TYPE 2 DIABETES MANAGEMENT: A FOCUS ON METABOLIC DEFECTS

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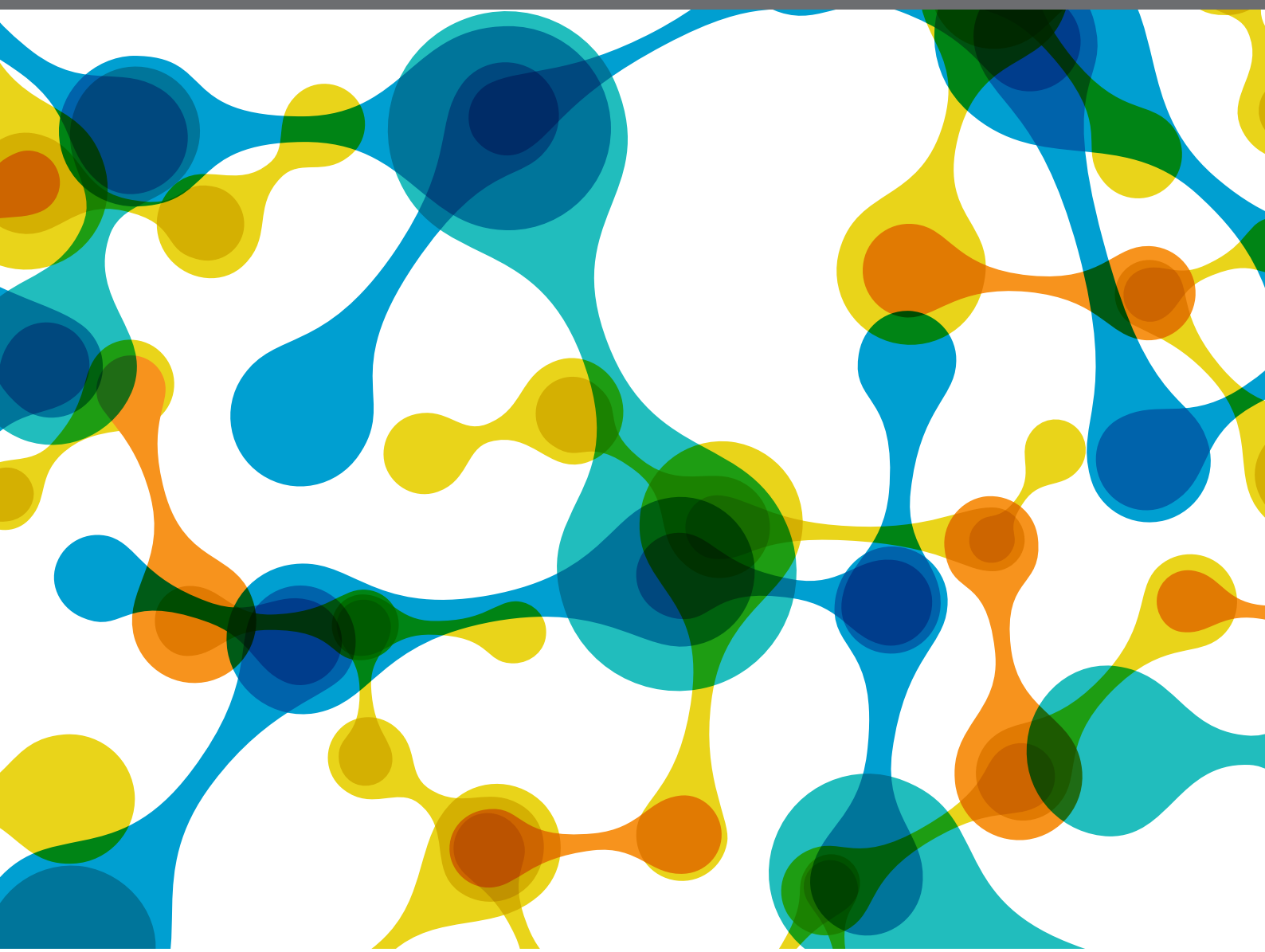

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TYPE 2 DIABETES MANAGEMENT: A FOCUS ON METABOLIC DEFECTS

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Associations of Insulin Levels and Insulin Resistance With Urine Glucose Excretion Independent of Blood Glucose in Chinese Adults With Prediabetes and Newly Diagnosed Diabetes

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Several studies have demonstrated that renal glucose reabsorption is increased in patients with type 2 diabetes. However, the increased renal glucose reabsorption may contribute to the progression of hyperglycemia. Therefore, promoting urine glucose excretion (UGE) by suppression of renal glucose reabsorption is an attractive approach for the treatment of diabetes. Insulin resistance is identified as a major characteristic in the pathogenesis of type 2 diabetes. Thus, our aim was to evaluate the association of UGE with serum insulin levels and insulin resistance in subjects with glucose abnormalities, including prediabetes and newly diagnosed diabetes (NDD). The present study included 1129 subjects, 826 individuals with prediabetes and 303 individuals with NDD. Urine samples were collected within 2 h of oral glucose loading for the measurement of glucose. Fasting serum insulin was measured. Homeostatic model assessment of insulin resistance (HOMA-IR) was assessed. Multiple linear regression analysis and multivariate logistic regression analysis were performed to determine the association of UGE with insulin levels and HOMA-IR. A negative association between serum insulin levels and UGE was observed. The relationship remained significant after adjustment for potential confounders, including age, gender, blood pressure and glucose ($\beta = -5.271$, 95% CI: -9.775 to -0.767, $p = 0.022$). Furthermore, multivariable logistic regression model showed that increased insulin levels were associated with a decreased risk for high UGE after multivariable adjustment. In addition, similar correlation was also observed between HOMA-IR and UGE. HOMA-IR was negatively correlated with UGE after controlling for potential confounders. Moreover, an independent inverse relationship between HOMA-IR and the risk of high UGE was found (OR = 0.85, 95% CI: 0.78–0.93, $p < 0.001$). In conclusion, insulin levels and HOMA-IR were negatively correlated with UGE after adjusting for potential confounders. Subjects with increased insulin levels or IR were at a decreased risk of high UGE independent of blood glucose. The study suggests that insulin might affect UGE through other ways, in addition to the direct blood glucose-lowering effect, thereby resulting in reduced UGE.

Keywords: diabetes mellitus, glycosuria, urine glucose excretion, insulin, insulin resistance

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INTRODUCTION

The kidney plays a central role in glucose homeostasis, largely through glucose reabsorption (Gerich, 2010; DeFronzo et al., 2012). Sodium-glucose cotransporters 2 (SGLT2), an important mediator of glucose reabsorption on the luminal surface of proximal tubules, is responsible for more than 90% of glucose reabsorption (Wilding, 2014; Mondick et al., 2016). Accumulating evidences have demonstrated that renal glucose reabsorption is increased in patients with type 2 diabetes mellitus since enhanced SGLT2 expression (DeFronzo et al., 2013; Osaki et al., 2016). However, increased glucose reabsorption may contribute to the progression of hyperglycemia. Therefore, promoting urine glucose excretion (UGE) by inhibition of renal glucose reabsorption has been recognized as an effective strategy for the treatment of diabetes (Ferrannini, 2017). In addition, assessing the significance of UGE in clinical practice, such as glycemic control and diabetes screening, has become a noteworthy field (Lu et al., 2011; Yang et al., 2015; Chen et al., 2018a).

Insulin resistance (IR), a major characteristic in the pathogenesis of type 2 diabetes (Defronzo, 2009), is manifested by increased hepatic glucose production and impaired glucose uptake. Moreover, IR is accompanied by chronic kidney disease (Thomas et al., 2015). Insulin receptor has been found in renal tubular cells, and insulin signaling plays an important role in tubular function (Artunc et al., 2016). Furthermore, it has been reported that insulin can stimulate sodium reabsorption in renal proximal tubules (Baum, 1987; Horita et al., 2016). As is well known, the transport of glucose from the tubular lumen into tubular cells is sodium dependent. Accordingly, insulin may play an important role in glucose reabsorption in the renal proximal tubular cells. To date, few studies have focused on the association between insulin levels and renal glucose reabsorption. Renal threshold for glucose reabsorption can reflect the capacity of renal glucose reabsorption. However, gold-standard stepwise hyperglycemic clamp procedure (SHCP) method cannot be widely used in the clinical practice to estimate renal threshold for glucose reabsorption, because of specialized laboratory demand. However, it is easy to obtain data on UGE.

Therefore, the aim of the present study was to investigate the association of serum insulin levels and IR with UGE in subjects with glucose abnormalities, including prediabetes and diabetes.

MATERIALS AND METHODS

Study Design and Participants

Data were obtained from a cross-sectional study undertaken to evaluate the efficacy of UGE in diabetes screening in Chinese population, aged 18–65 years and without previously diagnosed diabetes or taking anti-diabetic medication (Chen et al., 2018b). This study was approved by Ethics Review Committee of Jiangsu Provincial Center for Disease Control and Prevention and followed the tenets the Declaration of Helsinki. Written informed consent was obtained from each participant. All participants were given a standard 75 g glucose solution. All the urine samples were collected over a 2 h period after oral glucose loading for quantitative measurement of urine glucose. Finally, after confirming their oral glucose tolerance test and excluding those who had no data on fasting serum insulin level, 1129 subjects with glucose abnormalities including prediabetes and newly diagnosed diabetes (NDD) were included in the present study.

Anthropometric and Laboratory Measurements

Demographic characteristics and medical histories were obtained using a structured questionnaire. Weight, height, heart rate (HR), and blood pressure (BP) were measured according to standardized protocols. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Fasting plasma glucose (FPG) and 2 h plasma glucose (2h-PG) were measured with the glucose oxidase method using an automatic chemistry analyzer (Synchron LX-20, Beckman Coulter Inc., CA, United States). The concentration of urinary glucose was measured with a quantitative urine meter (UG-201-H, Tanita Corporation, Tokyo, Japan). UGE was calculated as the urinary glucose concentration (mg/dl) × the urine volume (dl). Fasting serum insulin level was evaluated by electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, United States). Insulin resistance was estimated by homeostasis model assessment for insulin resistance (HOMA-IR): FPG (mmol/L) × fasting insulin (mIU/L)/22.5.

Definitions

Prediabetes and newly diagnosed diabetes were defined according to the 1999 World Health Organization (WHO) criteria. In our previous study, UGE displayed an excellent sensitivity of 82.9% and a high specificity of 84.7% in detecting NDD at the corresponding optimal cutoff of 130 mg (Chen et al., 2018a). Therefore, in the present study, UGE exceeding 130 mg was considered as high UGE, while UGE less than 130 mg was considered as low UGE.

Statistical Analysis

The continuous variables in this study were presented as the means ± SD or median (25th–75th percentiles) as appropriate. The categorical variables were presented as numbers (%). The differences between the groups were analyzed using independent Student's *t*-tests for normally distributed variables, non-parametric Mann–Whitney *U*-tests for non-normally distributed variables and chi-square test for categorical data. The relationships between UGE and other clinical indicators was examined using Spearman's correlation. Multiple linear regression analysis with adjustment for potential confounders was conducted to access the association of insulin levels and IR with UGE. Potential confounders included in the multivariate analysis were age, gender, HR, BP, FPG, 2h-PG, total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), creatinine, blood urea nitrogen (BUN), and BMI, which were chosen depend on reaching statistical significance in the Spearman's correlation analysis and based on clinical judgment.

TABLE 1 | Baseline characteristics of the study participants according to UGE.

	Low UGE[a]	High UGE[b]	*P*
Number	586 (51.9%)	543 (48.1%)	
Age (years)	48.60 ± 11.08	48.62 ± 9.75	0.977
Male (%)	243 (41.5%)	318 (58.6%)	<0.001
HR (beats/min)	78.62 ± 12.15	79.47 ± 12.12	0.241
BLOOD PRESSURE (mmHg)			
Systolic	135.15 ± 19.83	138.66 ± 19.30	0.003
Diastolic	82.46 ± 11.67	85.33 ± 11.51	<0.001
PLASMA GLUCOSE (mmol/L)			
FPG	5.94 ± 0.70	7.00 ± 1.86	<0.001
2h-PG	7.95 ± 1.78	10.90 ± 4.19	<0.001
Insulin (mIU /L)	6.90 (4.80–10.30)	6.60 (4.20–9.50)	0.053
HOMA-IR	1.79 (1.25–2.66)	1.98 (1.23–3.01)	0.066
TC (mmol/ L)	4.91 ± 0.99	5.06 ± 0.97	0.010
TG (mmol/ L)	1.41 (0.96–2.05)	1.67 (1.11–2.36)	<0.001
HDL-c (mmol /L)	1.38 ± 0.39	1.35 ± 0.36	0.102
LDL-c (mmol /L)	2.79 ± 0.78	2.88 ± 0.77	0.054
Creatinine (umol/L)	71.45 ± 15.76	72.68 ± 16.73	0.205
BUN (mmol/L)	5.21 ± 1.51	5.36 ± 1.45	0.086
BMI (kg/m^2)	26.10 ± 3.79	26.80 ± 3.74	0.002

Data are presented as n (%), mean ± SD, or median (25th–75th percentiles) as appropriate.[a]UGE < 130 mg. [b]UGE ≥ 130 mg. HR, heart rate; FPG, fasting plasma glucose; 2h-PG, 2 h plasma glucose; TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein–cholesterol; LDL-c, low-density lipoprotein–cholesterol; BUN, blood urea nitrogen; LUGE, low urine glucose excretion; HUGE, high urine glucose excretion; BMI, body mass index.

A binary logistic regression analysis was performed to determine the odds ratios of high nUGE associated with insulin levels and IR. A P-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

General Characteristics of the Study Participants

A total of 1129 subjects, including 826 individuals with prediabetes and 303 individuals with NDD, were included in the present study. The general characteristics of the study population, according to UGE, were summarized in **Table 1**. Subjects with high UGE exhibited significant higher BP, FPG, 2h-PG, TC, TG, and BMI compared with those with low UGE. In addition, no significant differences in age, HR, insulin, HOMA-IR, HDL-c, LDL-c, creatinine, and BUN were found between the two groups.

Correlations of UGE With Other Clinical Indicators

Spearman's correlation showed that UGE was positively related to FPG and 2h-PG, whereas negatively correlated with serum insulin levels ($r = -0.063$, $p = 0.034$). Moreover, significant positive correlations of UGE with HR, BP, HOMA-IR, TG, TC, LDL-c, BUN, and BMI were observed (**Table 2**).

TABLE 2 | The correlations of UGE with other clinical indicators in subjects with glucose abnormalities.

	UGE	
	R	*P*-value
Age	0.010	0.736
HR	0.068	0.022*
Systolic BP	0.127	<0.001*
Diastolic BP	0.148	<0.001*
FPG	0.468	<0.001*
2h-PG	0.468	<0.001*
Insulin	−0.063	0.034*
HOMA-IR	0.071	0.017*
TC	0.077	0.009*
TG	0.146	<0.001*
HDL-c	−0.083	0.005*
LDL-c	0.063	0.035*
BUN	0.076	0.011*
Creatinine	0.030	0.308
BMI	0.110	<0.001*

Data are presented as r (P-value).
**Significance, p < 0.05. UGE, urine glucose excretion; HR, heart rate; BP, blood pressure; FPG, fasting plasma glucose; 2h–PG, 2–h plasma glucose; HbA1c, glycated hemoglobin; TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; BUN, blood urea nitrogen; BMI, body mass index.*

Multiple Linear Regression Analysis With UGE as the Dependent Variable

To identify the association of UGE with serum insulin levels and HOMA-IR, and eliminate the influence of confounders, multiple linear regression analysis with UGE as a dependent variable was presented in **Table 3**. HOMA-IR and insulin were analyzed in separate models due to collinearity. Insulin levels were negatively associated with UGE after adjustment for potential confounders, including age, gender, FPG, 2h-PG, systolic and diastolic blood pressure, and BMI (β = −5.271, 95% CI: −9.775 to −0.767, $p = 0.022$). The males were more likely to have higher UGE than females after multivariable adjustment. In addition, FPG and 2h-PG were still positively associated with UGE in this model. Moreover, a negative association between HOMA-IR and UGE was observed after controlling for other variables (β = –4.767, 95% CI: −28.477 to −1.057, $p = 0.035$).

Logistic Regression Analysis of Odds Ratio for High UGE

Furthermore, a binary logistic regression analysis was performed to identify the factors associated with odds ratios of high UGE. Increasing FPG and 2h-PG were significantly associated with an increased odds ratio of HUGE in the multi-adjusted model (**Table 4**). However, increased serum insulin levels were significantly associated with a decreased odds ratio of high UGE (OR = 0.96, 95% CI: 0.93–0.98, $p < 0.001$). In addition, the data also showed an independent inverse relationship between the HOMA-IR and the risk of high UGE (OR = 0.85, 95% CI: 0.78–0.93, $p < 0.001$).

TABLE 3 | Multiple linear regression analyses with UGE as the dependent variable.

Independent variable	b Coefficient	95% CI	Standardized coefficient	*P*
MODEL 1				
Age (years)	−1.663	−4.858 to 1.532	−0.026	0.307
Gender[a]	−292.210	−363.542 to −218.877	−0.217	<0.001
FPG (mmol/L)	95.187	68.392 to 121.982	0.210	<0.001
2h-PG (mmol/L)	87.996	76.713 to 99.279	0.459	<0.001
Insulin (mIU/L)	−5.271	−9.775 to −0.767	−0.056	0.022
Systolic (mmHg)	0.939	−1.271 to 3.150	0.027	0.405
Diastolic (mmHg)	2.576	−1.066 to 6.219	0.045	0.165
TC (mmol/L)	2.746	−45.363 to 50.855	0.004	0.911
TG (mmol/L)	10.585	−5.982 to 27.152	0.034	0.210
BMI (kg/m^2)	5.367	−3.261 to 13.996	0.030	0.223
MODEL 2				
Age (years)	−1.521	−4.703 to 1.662	−0.024	0.349
Gender[a]	−291.520	−363.876 to −219.164	−0.217	<0.001
FPG (mmol/L)	99.721	72.044 to 127.397	0.220	<0.001
2h-PG (mmol/L)	88.229	76.952 to 99.505	0.460	<0.001
HOMA-IR	−14.767	−28.477 to −1.057	−0.052	0.035
Systolic (mmHg)	0.892	−1.318 to 3.102	0.026	0.428
Diastolic (mmHg)	2.671	−0.969 to 6.312	0.046	0.150
TC (mmol/L)	2.724	−45.403 to 50.851	0.004	0.912
TG (mmol/L)	10.421	−6.152 to 26.994	0.034	0.218
BMI (kg/m^2)	4.881	−3.687 to 13.449	0.028	0.264

Both models were adjusted for age, gender, heart rate, blood pressure, FPG, 2h-PG, TG, TC, high-density lipoprotein–cholesterol, low-density lipoprotein–cholesterol, creatinine, blood urea nitrogen, and body mass index. [a] 1 = male, 2 = female. UGE, urine glucose excretion; FPG, fasting plasma glucose; 2h-PG, 2 h plasma glucose; TC, total cholesterol; TG, triglycerides; BMI, body mass index; HOMA-IR, homeostasis model assessment for insulin resistance.

DISCUSSION

Studies on the associations of serum insulin levels and HOMA-IR with UGE are relatively scarce. Previous studies have demonstrated that insulin stimulates sodium reabsorption and the transport of glucose is sodium dependent in renal proximal tubules (Baum, 1987; Ferrannini, 2017). Thus, we hypothesized that insulin may participate in renal glucose reabsorption, which may have influence on UGE. In the present study, we found that serum insulin levels were negatively associated with UGE. The relationship remained significant after adjustment for age, gender, heart rate, blood pressure, FPG, 2h-PG, TG, TC, HDL-c, LDL-c, creatinine, BUN, and BMI (**Table 3**). Furthermore, multivariable logistic regression model showed that insulin levels were associated with a decreased risk of high UGE. Similar correlation was also observed between HOMA-IR and UGE. The study established that increased insulin levels and HOMA-IR were strongly correlated with decreased risk of high UGE after controlling for other variables, indicating that insulin might reduce UGE independent of blood glucose.

Glycosuria is the result of glycemic excursions in excess of the renal glucose threshold (Osaki et al., 2016). Much more attention has been paid to the significance of UGE on health and disease, such as glycemic control and diabetes screening (Lu et al., 2011; Dallosso et al., 2015; Yang et al., 2015). However, factors associated with UGE have not been elucidated clearly in patients with diabetes. UGE increases in a proportional manner with increasing blood glucose (Rave et al., 2006). Consistent with previous studies (Rave et al., 2006; Yang et al., 2015), positive relationships of UGE with FPG and 2h-PG were observed in the present study. In addition, our data showed that both increased insulin levels and HOMA-IR were significantly associated with a decrease in the risk of high UGE. The study by Ono et al. (2017) also revealed an inverse association between the insulinogenic index and UGE. However, this study was conducted in subjects with prediabetes. Our study

TABLE 4 | Multiple logistic regression analyses of odds ratios for high UGE.

Category	β	SE of β	OR	95% CI	*P*-value
MODEL 1					
FPG (mmol/L)	0.920	0.112	2.51	2.02–3.12	<0.001
2h-PG (mmol/L)	0.393	0.037	1.48	1.38–1.60	<0.001
Insulin (mIU/L)	−0.046	0.013	0.96	0.93–0.98	<0.001
MODEL 2					
FPG (mmol/L)	0.989	0.117	2.69	2.14–3.38	<0.001
2h-PG (mmol/L)	0.396	0.037	1.49	1.38–1.60	<0.001
HOMA-IR	−0.160	0.042	0.85	0.78–0.93	<0.001

Both models were adjusted for age, gender, heart rate, blood pressure, FPG, 2h-PG, TG, TC, high-density lipoprotein–cholesterol, low-density lipoprotein–cholesterol, creatinine, blood urea nitrogen, and body mass index.
SE, standard error; OR, odds ratio; CI, confidence interval; FPG, fasting plasma glucose; 2h-PG, 2 h plasma glucose; UGE, urine glucose excretion; HOMA-IR, homeostasis model assessment for insulin resistance.

population was consisted of participants with prediabetes and NDD. Furthermore, a recent study reported that individuals with increased HOMA-IR were at an increased risk for high renal threshold for glucose reabsorption (Yue et al., 2017), suggesting that subjects with IR are more likely to have enhanced renal glucose reabsorption, which may cause a reduction in UGE. Taken together, we found insulin levels and IR were negatively associated with UGE independent of blood glucose, and further suggested that in addition to glucose-lowering action, as a major polypeptide hormone, insulin may affect UGE in other ways.

SGLT2, a highly specific and major glucose transporter in kidney tubules, is responsible for more than 90% of tubular glucose reabsorption (Nair and Wilding, 2010; Ferrannini, 2017). Overexpression of SGLT2 has been observed in both animal models and humans with diabetes (Rahmoune et al., 2005; Vallon et al., 2013). Obviously, increased glucose reabsorption may contribute to the progression of hyperglycemia (Wilding, 2014). Several studies have demonstrated the efficacy of SGLT2 inhibitors for the improvement of glucose control by inhibiting glucose reabsorption and increasing UGE (Devineni et al., 2012). As is well known, insulin resistance is one of the major characteristics in the pathogenesis of type 2 diabetes. Besides, insulin receptor has been found in renal tubular cells. A recent study found the deletion of insulin receptor significantly reduced SGLT2 expression and increased UGE, the study suggested that insulin, rather than glucose, may primarily regulate SGLT2 abundance and glucose transport (Nizar et al., 2018). In addition, another study demonstrated that insulin could stimulate SGLT-2-mediated glucose entry into proximal tubular cells (Nakamura et al., 2015). Taken together, elevated insulin levels may be a major factor to influence the expression of SGLT2 and UGE. Therefore, subjects with elevated insulin levels or HOMA-IR were more likely to have low UGE independent of blood glucose, may be attributed to enhanced glucose reabsorption via upregulation of SGLT2.

To date, few studies have focused on the association of insulin levels and IR with UGE. Our study population is consisted of subjects with no history of previous diabetes or taking any antidiabetic medication. Therefore, the correlation between insulin and UGE may be more accurate due to the elimination of the impacts of antidiabetic medication on UGE or insulin levels. However, some limitations should be noticed in this study. First, individuals with increased insulin levels and IR were at a decreased risk of high UGE independent of blood glucose, which might be attributed to increased renal glucose reabsorption. However, renal glucose reabsorption was not evaluated in this study. Future studies are needed to evaluate the renal threshold of glucose reabsorption in subjects with hyperinsulinemia or IR. In addition, further study is necessary to confirm whether hyperinsulinemia may induce SGLT2 overexpression in humans or animal models. Second, most subjects with normal glucose tolerance did not have obvious UGE. There is no significant correlation between insulin levels and UGE in subjects with normal glucose tolerance. Our study only involved subjects with prediabetes and NDD, so that the present findings may not be extrapolated to subjects with normal glucose tolerance or previous history of diabetes. Third, the causal relationships could not be deduced in the cross-sectional study. Finally, we did not measure insulin levels 30, 60, and 90 min post-glucose loading. Nevertheless, the present study could still provide valuable information to help us understand the association of UGE with insulin.

CONCLUSION

In conclusion, increased serum insulin levels and HOMA-IR were associated with a decreased risk of high UGE independent of blood glucose in subjects with glucose abnormalities, which might be attributed to the increased renal glucose reabsorption.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

Z-LS and JC were responsible for the study design. JC was responsible for data collection, data analysis, interpretation, and writing of the manuscript. S-HQ, H-JG, and WL were responsible for data collection and assisted with data interpretation and writing. All authors read the manuscript critically and approved the submitted version.

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Physical Exercise and Its Protective Effects on Diabetic Cardiomyopathy: What Is the Evidence?

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As one of the most serious complications of diabetes, diabetic cardiomyopathy (DCM) imposes a huge burden on individuals and society, and represents a major public health problem. It has long been recognized that physical exercise has important health benefits for patients with type 2 diabetes, and regular physical exercise can delay or prevent the complications of diabetes. Current studies show that physical exercise has been regarded as an importantly non-pharmacological treatment for diabetes and DCM, with high efficacy and low adverse events. It can inhibit the pathological processes of myocardial apoptosis, myocardial fibrosis, and myocardial microvascular diseases through improving myocardial metabolism, enhancing the regulation of Ca^{2+}, and protecting the function of mitochondria. Eventually, it can alleviate the occurrence and development of diabetic complications. Describing the mechanisms of physical exercise on DCM may provide a new theory for alleviating, or even reversing the development of DCM, and prevent it from developing to heart failure.

Keywords: exercise, diabetic cardiomyopathy, myocardial metabolism, oxidative stress, myocardial fibrosis, apoptosis

INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) continues to increase dramatically, and is now considered as an epidemic worldwide. It is estimated that the number of people with diabetes will exhibit a 45% increase in three decades (1). Uncontrolled diabetes can lead to a number of long-term health complications, including heart diseases, nerve damage, vision problems, and amputation. Among the various complications of diabetes, cardiovascular disease (CVD) represents the major cause of mortality and morbidity in diabetic patients, accounting for nearly 70% of related heart failure cases (2). Diabetic patients, when compared with non-disease subjects, are two to four times more likely to experience CVD events, due to micro- and macrovascular atherosclerosis, which is often exacerbated by the presence of concomitant CVD risk factors, including hypertension, dyslipidemia, and activation of neuro-hormonal and inflammatory mechanisms (3, 4).

THE PATHOGENESIS OF DCM

Diabetic cardiomyopathy (DCM), a distinct condition that develops in diabetic patients, is defined by the presence of myocardial dysfunction in the absence of coronary atherosclerosis, overt clinical coronary artery disease (CAD), and valvular heart disease (5, 6). It is characterized by cardiac structure and function disorders, including myocardial fibrosis, dysfunctional remodeling, and associated metabolic deregulation and left ventricular dysfunction (7–9). DCM is associated with diastolic dysfunction, with depressed myocardial contractility and relaxation, and eventually by clinical heart failure (10). Left ventricular diastolic dysfunction with increased wall stiffness is common in subjects with well-controlled T2DM who are free of clinically detectable heart diseases (11). The pathogenesis of DCM is a multifactorial process that includes altered myocardial metabolism (impaired energy metabolism, calcium regulation, and mitochondrial function) (12, 13), increased oxidative stress (14), altered myocardial structure with fibrosis (15), higher induction of apoptosis (16), and microvascular disease (9).

CURRENT STRATEGIES FOR DCM INTERVENTION

Despite prominent advances in the prevention and treatment of diabetes, diabetic complications, especially for DCM still remain rigorous in patients with T2DM (17, 18). Nowadays, treatments for DCM include glucose and lipid control, hypertension treatment, and CAD intervention. Currently, pharmacological treatment is accepted as the common strategy for CVD in diabetic patients, such as β-blockers, Ca^{2+} antagonist, β-blockers, renin–angiotensin–aldosterone system inhibitors (9). In recent years, it demonstrated that metformin and sodium-dependent glucose transporters 2 (SGLT2) inhibitors are also benefit for DCM (19). A systematic review of observational studies showed that metformin reduced all-cause mortality in patients with diabetes with congestive heart failure (20). Pan et al. found that empagliflozin ameliorated DCM in diabetic ALDH2*2 mutant patients (21). However, the incidence and mortality rate of DCM still remains high. Physical exercise has been shown to improve health and quality of life in patients with a variety of diseases, including obesity (22), T2DM (23), chronic kidney disease (24), and cancers (25). Currently, physical exercise has been regarded as an importantly non-pharmacological treatment for the prevention and treatment of diabetes and its complications. It has been long known that physical activity can decrease the occurrence of cardiac events, including heart attacks and strokes, and the need for a coronary revascularization intervention (26–28). Exercise induces adaptations to the heart itself, as well as the cardiovascular system. These adaptations include protection against ischemic damage, increase of cardiac growth, and modulation of cardiac metabolism, function, and vascular supply (26). Myocardial apoptosis, myocardial fibrosis, and hemodynamic disorders caused by high glucose can be improved or even reversed by physical exercise. Studies have shown that exercise can improve myocardial metabolism, lower blood glucose, increase insulin sensitivity, inhibit myocardial fibrosis, improve oxidative stress, and decrease the risks of CVDs. Ultimately, it can improve heart function and decrease the mortality of DCM (29, 30).

CLINICAL STUDIES ABOUT EXERCISE AND DIABETES-RELATED CARDIOVASCULAR DISEASES

Physical activity plays an essential role in the maintenance of human health. Chronic diseases, such as metabolic syndrome and diabetes, are a tremendous burden to our society. Regular physical activity is a primary recommendation for the prevention and treatment of these diseases (36). Physical exercise has a wide array of beneficial effects, including improving glucose and insulin metabolism, and reducing the risks of CVDs in diabetic patients (37). The intensive weight loss intervention was effective in increasing physical activity and improving cardiorespiratory fitness in overweight and obese individuals with T2DM (31). Physical activity was associated with reduced risks of CVD,

TABLE 1 | Clinical studies about exercise and diabetes-related cardiovascular diseases.

Subjects included	Exercise profile	Metabolic effects	References
4,376 overweight or obese adults with T2DM	At least 50 min/week, progressing to at least 175 min/week	Increased physical activity Improved cardiorespiratory fitness	Jakicic et al. (31)
3,058 patients with T2DM	Leisure-time physical activity	Reduced risk of CVD, cardiovascular death, and total mortality	Tanasescu et al. (32)
575 patients with T2DM	Low (<4 METs), moderate (≥4 to <7 METs) and high fitness (≥7 METs)	Lower hemoglobin A1c and C-reactive protein levels A decreased prevalence of left ventricular hypertrophy and increased aortic stiffness	Cardoso et al. (33)
3,708 patients with T2DM	Occupational, commuting, and leisure-time physical activity	Decreased total and cardiovascular mortality	Hu et al. (34)
539 patients with T2DM	Leisure-time physical activity	Reduced risks of short-term CVD outcome	Karjalainen et al. (35)

CVD, cardiovascular disease; METs, metabolic equivalents.

cardiovascular death, and total mortality in patients with T2DM (32, 38). In addition, the benefits of exercise may depend on the intensity of exercise. It showed that a moderate to high level of physical fitness was independently associated with several cardiovascular risk markers, which may contribute to decreasing the burden of morbidity and mortality in patients with T2DM (33). Hu et al. also showed that a moderate or high level of physical activity was associated with a reduced risk of total and cardiovascular mortality among patients with T2DM, which was regardless of the levels of body mass index, blood pressure, total cholesterol, and smoking (34). Furthermore, the duration of exercise is also an important factor for its benefits of CVD. Karjalainen et al. found that there was an inverse association between leisure-time physical activity and short-term CVD outcome. However, controlled, home-based exercise training had minor effects on the risk profile of CVD in CAD patients with T2DM. These findings highlight the significance of lifelong physical activity instead of a short-term exercise program in the prevention of future unfavorable outcomes in patients with CAD (35). However, clinical studies about exercise and its benefits in diabetes-related CVDs are limited. It is suggested to carry out long-term exercise program to ensure maximum exercise efficiency, with an appropriate amount of exercise. According to the evidence of the aforementioned studies and the 2018 guideline of American Diabetes Association (ADA) (39), we recommend a moderate to high level [$\geq$4 to $<$7 metabolic equivalents (METs)] of physical fitness for patients with T2DM, such as 150 min or more of moderate-to-vigorous intensity aerobic activity per week. The clinical studies about exercise and diabetes-related cardiovascular diseases are listed in **Table 1**.

PRE-CLINICAL EXPERIMENTS ABOUT PHYSICAL EXERCISE AND DCM

Physical Exercise Improves Cardiomyocyte Metabolism

Physical Exercise Increases Energy Metabolism

Disorders of myocardial glucose and lipid metabolism lead to changes in pathways related with myocardial energy metabolism. Abnormalities that produce cardiac structure and function are called "metabolic remodeling of the heart," which ultimately leads to the development of cardiomyopathy. Glucose transporter-4 (GLUT-4) is an intracellular protein that can be translocated to cell membrane induced by insulin, and then it can participate in glucose uptake and utilization. The expression of GLUT-4 was decreased and abnormally distributed in diabetic state, resulting in a significant decrease in glucose transport and impaired myocardium energy utilization (58). Studies indicate that moderate exercise can upregulate GLUT-4 expression, and also can increase glucose transport and activate pyruvate dehydrogenase complexes, even in the absence of insulin (59). It suggests that exercise can compensate for impaired energy metabolism in insulin-deficient state, which may be related to the increase of insulin-sensitive adenosine monophosphate activated protein kinase (AMPK) expression, thereby protecting pancreatic β cells. Exercise may also enhance insulin-mediated glucose transport by increasing the expression of protein kinase C-δ (60, 61). Exercise can also increase insulin and its downstream protein expressions in the myocardium of diet-induced obesity rats, as well as forkhead box protein o1 (Foxo1) and other key regulators of pancreatic β cells, and also activate insulin signaling pathway (40). Thus, exercise can protect pancreatic β cells, promote insulin secretion, activate insulin signaling pathway, increase GLUT4 expression, improve intracellular energy metabolism, and ultimately protect cardiomyocytes.

Physical Exercise Enhances Calcium Regulation

Calcium is a crucial mediator of cell signaling in skeletal muscles for cellular functions and specific functions, including contraction, fiber-type differentiation, and energy production. Intracellular Ca^{2+} dyshomeostasis is one of the main markers of DCM, which can affect myocardial contractile function, directly leading to the occurrence and development of DCM. It is even worse in altered sarcoplasmic reticulum Ca^{2+} uptake rate accompanied by decreased function of sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) (62). In T2DM patients, the Na^{+}-Ca^{2+} exchange of cardiomyocytes is inhibited, while the sarcoplasmic reticulum Ca^{2+} pump is normal, and Ca^{2+} is gradually concentrated in the sarcoplasmic reticulum. Thus, the amplitude and attenuation rate of Ca^{2+} concentration in the myocardium is decreased. Conversely, exercise can improve the expression and activity of SERCA2a, which can regulate Ca^{2+} release and recapture in the myocardium. Exercise can increase Ca^{2+}-calmodulin-dependent protein kinase phosphorylation, reduce Ca^{2+} efflux, facilitate Ca^{2+} regulation, and ultimately improves myocardial contraction and diastolic function (41). Stølen et al. found that high intensity intermittent exercise improved myocardial contractility by restoring L-type Ca^{2+} channels, increasing the density of T-transverse tubules, and increasing the synchrony of Ca^{2+} release and excitatory contraction coupling (42).

Physical Exercise Improves Mitochondrial Function

Mitochondrion is the center of energy metabolism, and recent evidence suggests that mitochondrial dysfunction may play a critical role in the pathogenesis of DCM. The imbalance of energy supply and demand directly leads to the decline of myocardial function and induction of DCM (63). The ultrastructure of mitochondria in DCM shows reduced density, mitochondrial swelling, and destruction of the intima and adventitia, and an increase in mitochondrial matrix, while exercise attenuates diabetes-induced ultrastructural changes in rat cardiac tissue (43). Moderate exercise intervention has a protective effect on mitochondrial function. Exercise can regulate the key regulator of mitochondrial metabolism, peroxisome proliferator-activated receptor gamma co-stimulatory factor-1α (PGC-1α), and activate its downstream transcription factors. Thus, it can enhance mitochondrial DNA replication and transcription, and increase mitochondria biosynthesis (44). Furthermore, the mechanisms by which exercise improves mitochondrial function may be related to the regulation of Ca^{2+} in mitochondria. Ca^{2+} is a key metabolic enzyme activator in mitochondria, and mitochondrial Ca^{2+} circulatory balance can be easily affected by intracellular Ca^{2+} homeostasis (41, 42). Resistance exercise improves cardiac function and

mitochondrial efficiency in hearts, of diabetic rat, which were accompanied by higher expressions of mitochondrial biogenesis proteins such as PGC-1α and mitochondrial transcription factor A (TFAM) (45). In addition, studies have shown that high intensity exercise can increase myocardial mitochondrial contents, but no change in moderate intensity exercise (46, 47). However, Veeranki et al. showed that moderate intensity exercise prevented DCM associated contractile dysfunction through restoration of mitochondrial function and connexin 43 levels in db/db mice (30). These indicate that myocardial mitochondrial biosynthesis may be associated with exercise intensity, and exercise intensity should be further investigated about its effects on DCM.

Physical Exercise Relieves Oxidative Stress Damage

Oxidative stress is considered to be a key link in the development of DCM. Under physiological conditions, there is a balance system of oxygen free radicals and free radicals in the body. Oxygen atoms play an important role in the redox signaling pathway. Moderate oxidation can increase protein activity, but excessive reactive oxygen species can cause pathological changes through interaction with lipids, proteins, and DNA (64). Hyperglycemia can directly promote the production of oxygen free radicals, induce oxidative stress, and promote cardiomyocyte apoptosis. The mechanisms by which exercise ameliorates oxidative stress is complex, including: (1) reducing the production of reactive oxygen species. Exercise can ameliorate the damage caused by excessive oxidative stress in the diabetic myocardium and pancreas, thereby improving glucose metabolism and reducing damage caused by reactive oxygen species (48). Long-term exercise can also directly reduce the level of reactive oxygen species in the body by reducing the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in diabetic rats (49). (2) Enhancing the ability of anti-oxidative stress. Exercise can increase the expression of nitric oxide synthase and nitric oxide, and ultimately enhance the antioxidant function in endothelial cells (49). Nuclear factor E2-related factor 2 (Nrf2) can regulate the expressions of antioxidants mediated by antioxidant response elements. It is an important transcription factor for intracellular defense of reactive oxygen species (50, 65). Studies have shown that acute exercise can promote the function of Nrf2, activate downstream antioxidant response elements, and ultimately enhance the activity of anti-oxidative stress. In addition, knocking out the Nrf2 gene can increase the sensitivity of cardiomyocytes to oxidative stress, leading to increased oxidative damage in cells (50). Kanter et al. showed that low intensity exercise decreased the elevated tissue malondialdehyde (MDA) levels and increased the reduced activities of the enzymatic antioxidants superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in cardiac tissue (51). It indicates that exercise improves the biological mechanisms of DCM by affecting the levels of plasminogen activator inhibitor 1 (PAI-1) and endothelial nitric oxide synthase (eNOS), and it is dependent on the intensity of exercise (52).

Physical Exercise Improves Myocardial Fibrosis

Myocardial fibrosis is the most prominent histopathological change in DCM, characterized by myocardial cell collagen deposition, interstitial fibrosis, and perivascular fibrosis, and ultimately induce the reconstruction of cardiac structure and function (53). Numerous studies indicated that moderate exercise can decrease blood glucose, reduce myocardial fibrosis, promote myocardial reverse remodeling in diabetic rats, and improve cardiac function (30, 53). The mechanisms may be that exercise reduces pressure overload by improving blood pressure, thereby alleviating myocardial fibrosis (53). Exercise can increase the content of matrix metalloproteinase-2 (Mmp-2) in obese rats, increase the degradation of collagen and inhibit the formation of myocardial fibrosis (47). The interaction of collagen with glucose can further cause chemical modification of glycated collagen to form advanced glycation end products (AGEs) that promote arterial and cardiac cirrhosis, as well as endothelial dysfunction (66). Other mechanism by which exercise improves myocardial fibrosis may be related to improving energy metabolism, decreasing blood glucose, and myocardial glycogen deposition (54). Novoa et al. showed that high intensity chronic exercise had a positive impact on cardiac remodeling, evidenced as reduction in myocyte hypertrophy, reduced collagen deposition, and amelioration of myocardial fibrosis (55).

Physical Exercise Inhibits Cardiomyocyte Apoptosis

Diabetes-induced cardiomyocyte apoptosis is a typical feature of DCM. Hyperglycemia can directly promote cytochrome C release to the cytoplasm by activating cytochrome C in mitochondria, triggering cascade activation of caspase-3, leading to endogenous apoptosis of cardiomyocytes. This change plays an important role in the development of diabetic cardiac hypertrophy, myocardial remodeling, and heart failure. C-Jun N-terminal kinase is a member of the mitogen-activated protein kinase (MAPK) family, which can activate caspase-8 and the apoptotic protein Bax, and release cytochromes to promote apoptosis (67). Veeranki et al. found that exercise can also reduce cytochrome C leakage into cytoplasm by increasing mitochondrial transmembrane potential, thus prevent cardiomyocyte apoptosis (30). A number of studies have shown that exercise can reduce the phosphorylation of c-Jun N-terminal kinase in obese rats, block the transmission of downstream apoptotic signals. Exercise can also increase the expression of B-cell lymphokine 2 in the myocardium of diabetic mice, which can affect the activation of pro-apoptotic proteins by binding to pro-apoptotic proteins, and ultimately play an anti-apoptotic role on cardiomyocytes in diabetic mice (56). Kanter et al. showed that low intensity exercise had a therapeutic effect on diabetes-induced morphological, biochemical, and apoptotic changes in the cardiac tissue of rats (51). Khakdan et al. found that high intensity interval training effectively increased the expression of Sirtuin 1 (Sirt1) and B cell leukemia/lymphoma 2

TABLE 2 | Pre-clinical experiments about physical exercise and DCM.

Animals	Exercise intervention	Main findings	References
PHYSICAL EXERCISE IMPROVES CARDIOMYOCYTE METABOLISM			
Diet-induced obesity rats	Treadmill running (50-min/day, 5 days per week velocity of 1.0 km/h for 2 months)	- Increased protein levels of phospho-P38MAPK, REDD1 in the myocardium - Decreased 14-3-3 protein levels in the myocardium	Pieri et al. (40)
STZ-induced diabetic SD rats	accumulated about 3,554 m/day of voluntary wheel running for 12 weeks	- Prevented diastolic dysfunction in diabetic mice - Normalized sarcoplasmic reticulum protein content and expression in diabetic animals - Enhanced SERCA2a activity	Epp et al. (41)
Cardiomyocytes from mice with T2DM (db/db)	13 weeks of aerobic interval training (4 min at 85–90% of VO_{2max} and 2 min at 50% of VO_{2max} for 80 min /day, 5 days/week)	- Restored contractile function associated with restored SR Ca^{2+} release synchronicity, T-tubule density, twitch Ca^{2+} amplitude, SR Ca^{2+} ATPase and Na^{+}/Ca^{2+}-exchanger activities, and SR-Ca^{2+} leak - Reduced phosphorylation of cytosolic CaMKIIδ - Normalized enhanced fractional Ca^{2+} release	Stølen et al. (42)
STZ-induced diabetic SD rats	Run daily on a treadmill for 9 weeks (60 min/day, at a pace of 20 m/min)	- Attenuated diabetes-induced changes in collagen fibrils, cytoplasmic area, and level of mitochondrial disruption	Searls et al. (43)
C57BL/6 db/db mice	Run daily on a treadmill for 15 weeks (10 m/min for 1 h/day)	- Reversed reduction in EF and FS - Reversed reduction of mtDNA replication and transcription, together with reduced mtDNA content and impaired mitochondrial ultrastructure - Activated PGC-1α and Akt signaling	Wang et al. (44)
Otsuka Long-Evans Tokushima Fatty rats	20 repetitions of climbing a ladder 5 days per week for 12 weeks	- Increased EF and FS - Increased mitochondrial numbers - Higher expression of PGC-1α and TFAM	Ko et al. (45)
C57BL/6J mice	10 weeks of treadmill running (4 min at 85–90% of VO_{2max})	10% increase in heart weight-to-body weight ratio 36% increase in glucose oxidation and a concomitant reduction in fatty acid oxidation	Hafstad et al. (46)
Diet-induced obesity C57BL/6 mice	8–10 weeks of treadmill running (4 min at 85–90% of VO_{2max})	- Improved aerobic capacity, reduced obesity, improved glucose tolerance - Normalized left ventricular mechanical efficiency and mechanoenergetics - Improved mitochondrial capacity and efficiency, as well as reduced oxidative stress	Hafstad et al. (47)
db/db mice	300 m run on a treadmill for 5 days/week at the speeds of 10–11 m/min for 5 weeks	- Prevented diabetic cardiac functional deficiencies: EF and FS - Improvements in contraction velocity and contraction maximum, OCR, and tissue ATP levels - Attenuated transmembrane potential decline and cytochrome c leakage	Veeranki et al. (30)
PHYSICAL EXERCISE RELIEVES OXIDATIVE STRESS DAMAGE			
STZ-induced diabetic Wistar rats	9 weeks of treadmill running (11 m/min, 18 min/day)	- Lower left atrium diameter - Higher catalase and superoxide dismutase activities - Higher glutathione peroxidase activity	Gimenes et al. (48)
Diabetic Goto-Kakizaki (GK) rats	9 weeks of treadmill running (60 min/day and 5 days/week)	- Increased plantaris muscle cytochrome oxidase, improved glycosylated hemoglobin and insulin sensitivity - Increased both total eNOS expression and the dimer:monomer ratio in the left ventricle - Increased nitric oxide (+28%) production and decreased eNOS-dependent superoxide (−12%) production Decreased NADPH-dependent O_2-activity	Grijalva et al. (49)
$Nrf2^{-/-}$ mice	Exercise on a treadmill for 2 consecutive days (60 min/day; 14 m/min; 10% grade)	- Activated Nrf2/ARE signaling and promoted antioxidant - Activation of Nrf2/ARE (antioxidant response element) signaling - Enhancement of antioxidant defense pathways - Increased trans-activation of ARE-containing genes	Muthusamy et al. (50)

(Continued)

TABLE 2 | Continued

Animals	Exercise intervention	Main findings	References
STZ-induced diabetic SD rats	Exercise on a treadmill for 30 min daily for 4 weeks at a speed of 10 m/min	- Decreased the elevated tissue MDA levels - Increased the reduced activities of the enzymatic antioxidants SOD, GSH-Px, and CAT in cardiac tissue	Kanter et al. (51)
STZ-induced diabetic SD rats	Exercise on a treadmill for 60 min/day on 5 days for 6 weeks (10–20 m/min)	- Higher serum level of NO and eNOS - Reduced PAI-1 and vWF - Reduced PKC levels	Chengji et al. (52)
PHYSICAL EXERCISE ATTENUATES MYOCARDIAL FIBROSIS			
db/db mice	300 m run on a treadmill for 5 days/week at the speeds of 10–11 m/min for 5 weeks	- Normalized overall collagen accumulation at both the perivascular regions and in interstitial regions of heart tissue - Prevented the tendency for decline in the fast twitch cardiac MHC isoform, α-MHC	Veeranki et al. (30)
STZ-induced diabetic Wistar rats	Swimming training for 8 weeks (5 days/week, 90 min/day, with a load of 5% body weight)	- Decreased interstitial collagen and reticular fibers on the extracellular matrix - Attenuated glycogen accumulation	Silva et al. (53)
Diet-induced obesity C57BL/6 mice	8–10 weeks of treadmill running (4 min at 85–90% of VO_{2max})	- Increased the content of Mmp-2 in obese rats, increase the degradation of collagen and inhibited the formation of myocardial fibrosis	Hafstad et al. (47)
High-fat diet fed C57BL/6J mice	5 weekly HIT (10 × 4 min at 85–90% of maximum oxygen uptake)	- Normalized diastolic function, attenuated diet-induced changes in myocardial substrate utilization - Inhibited cardiac reactive oxygen species content and fibrosis	Lund et al. (54)
Alloxan-induced diabetic SD rats	Exercise on a treadmill for 4 weeks at 80% of maximal performance	- Inhibited cardiomyocyte hypertrophy - Inhibited collagen deposition in the heart and interstitial fibrosis	Novoa et al. (55)
PHYSICAL EXERCISE INHIBITS CARDIOMYOCYTE APOPTOSIS			
db/db mice	300 m run on a treadmill for 5 days/week at the speeds of 10–11 m/min for 5 weeks	- Attenuated transmembrane potential decline and cytochrome c leakage	Veeranki etal. (30)
STZ-induced diabetic Wistar rats	Exercise on a treadmill for 60 min/day, 5 days/week, for 10 weeks	- Increased cardiac survival pathway (IGF1, IGF1-R, PI3K, and Akt) and the pro-survival Bcl-2 family proteins (Bcl-2, Bcl-xL, and p-BAD) - Reduced cardiac TUNEL-positive apoptotic cells - Decreased the apoptotic key component caspase-3	Cheng et al. (56)
STZ-induced diabetic SD rats	Exercise on a treadmill for 30 min daily for 4 weeks at a speed of 10 m/min	- Reduced cardiac TUNEL-positive apoptotic cells	Kanter et al. (51)
High-fat high-fructose diet-induced Wistar diabetic rats	Exercise on a treadmill for 5-min at 30–40% of VO_2max, 2-min intervals at 85–90% VO_2max with recovery cycles at 30–40% VO_2max and finished by 3-min cooling down by running at 30–40% of VO_2max for 10 weeks	- Increased the expression of Sirt1 and BCL-2 - Increases LVEF% and FS%	Khakdan et al. (57)
STZ-induced diabetic SD rats	Exercise on a treadmill for 60 min/day on 5 days for 12 weeks (20 m/min for LIT and 34 m/min for HIT)	- Reduced serum cTn-I levels - Reduced GRP78, CHOP, and cleaved caspase-12 protein expression	Chengjier et al. (52)

DCM, diabetic cardiomyopathy; STZ, Streptozotocin; SD, Sprague-Dawley; MAPK, mitogen-activated protein kinase; REDD1, regulated in development and DNA damage response 1; SR, sarcoplasmic reticulum; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; mtDNA, mitochondrial DNA; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM, mitochondrial transcription factor A; Akt, protein kinase B; VO2max, maximal oxygen consumption; LVEF, left ventricular ejection fraction; EF, ejection fraction; FS, fractional shortening; OCR, oxygen consumption rate; ATP, adenosine triphosphate; PAI-1, plasminogen activator inhibitor 1; vWF, Von Willebrand factor; PKC, protein kinase C; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant responsive element; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MHC, myosin heavy chain; Mmp-2, matrix metalloproteinase-2; HIT, high intensity interval training; IGF1, insulin-like growth factor 1; IGF1-R, IGF1-receptor; PI3K, phosphatidylinositol 3′-kinase; TUNEL, TdT-mediated dUTP nick-end labeling; LIT, low intensity exercise training; cTn-I, cardiac troponin I; Sirt1, Sirtuin 1; BCL-2, B cell leukemia/lymphoma 2; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

(BCL-2) in diabetic rats, with improved left ventricular ejection fraction (LVEF%) and fractional shortening (FS%) (57). A recent study suggested that exercise appeared to ameliorate DCM by inhibiting endoplasmic reticulum stress-induced apoptosis in diabetic rats, which was in an intensity-dependent manner (52).

Physical Exercise Improves Microvascular Disorders

Microvascular disease is also one of the pathological changes of DCM. Under the influence of hyperglycemia, the function and structure of microvessels will undergo pathological changes, which are characterized by vascular

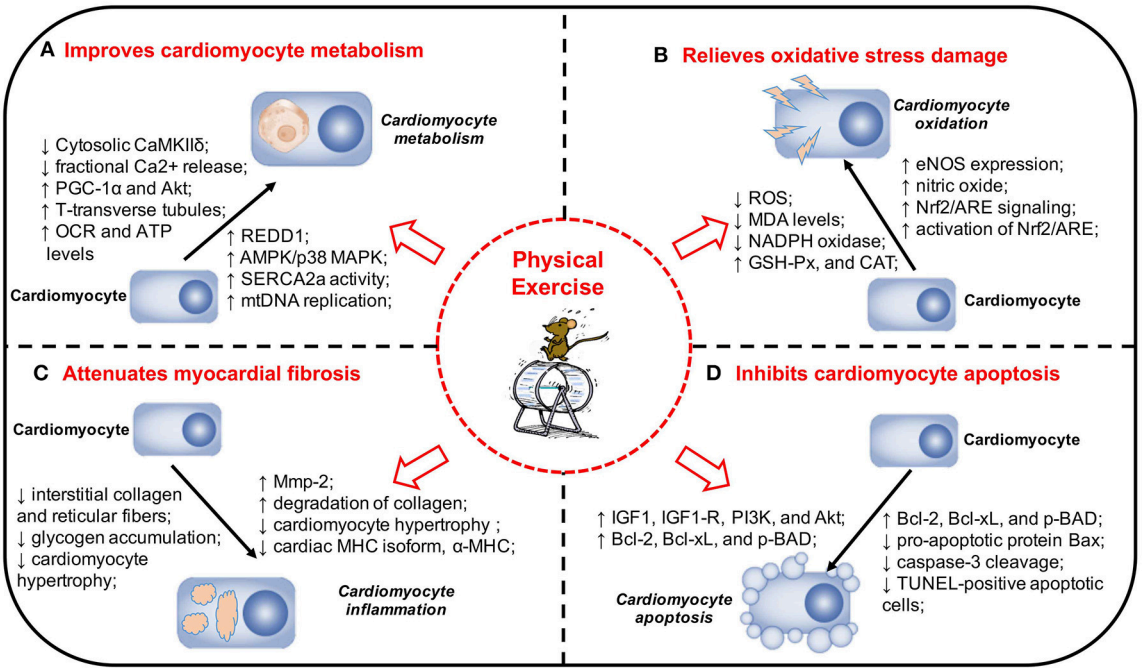

FIGURE 1 | Physical exercise and its protective effects and potential mechanisms in DCM. **(A)** Physical exercise Improves cardiomyocyte metabolism in DCM. **(B)** Physical exercise relieves oxidative stress damage in DCM. **(C)** Physical exercise attenuates myocardial fibrosis in DCM. **(D)** Physical exercise inhibits cardiomyocyte apoptosis in DCM. The pathogenesis of DCM is a multifactorial process and a range of mechanisms play a significant role in the onset and development of DCM. Physical exercise can exert a variety of protective effects on DCM, including improving cardiomyocyte metabolism, relieving oxidative stress damage, attenuating myocardial fibrosis, and inhibiting cardiomyocyte apoptosis. DCM, diabetic cardiomyopathy; MAPK, mitogen-activated protein kinase; REDD1, regulated in development and DNA damage response 1; SERCA2a, sarcoplasmic reticulum Ca2+-ATPase; mtDNA, mitochondrial DNA; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Akt, protein kinase B; VO2max, maximal oxygen consumption; EF, ejection fraction; FS, fractional shortening; OCR, oxygen consumption rate; ATP, adenosine triphosphate; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant responsive element; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MHC, myosin heavy chain; Mmp-2, matrix metalloproteinase-2; IGF1, insulin-like growth factor 1; IGF1-R, IGF1-receptor; PI3K, phosphatidylinositol 3′-kinase; TUNEL, TdT-mediated dUTP Nick-End Labeling.

endothelial defects, endothelial cell dysfunction, and aggravated inflammatory response of partial vascular endothelium. It can affect the transport of substances, such as glucose and insulin into other tissues of the body, which can lead to abnormal tissue function. The mechanisms underlying the protective effects of exercise on microvessels mainly include two aspects: (1) exercise can protect vascular endothelial cells, increase the expression of nitric oxide, enhance the diastolic function of microvessels, and increase blood perfusion (66). (2) Exercise can enhance microvascular response to insulin and improve insulin signaling. Increased insulin can activate both insulin receptor substrate-1/phosphatidylinositol-3-kinase/protein kinase B (IRS-1/PI3K/AKT) and MAPK pathways, leading to equilibrium between the vasopressor substance nitric oxide and endothelin-1, ensuring normal vasomotor function (68). The pre-clinical experiments and the potential mechanisms about physical exercise and DCM is shown in **Table 2** and **Figure 1**.

CONCLUSIONS

In summary, as a fundamental component of the human condition, physical exercise plays a critical role in human health. Exercise training is considered as a cornerstone in the management of T2DM, possessing a potency to decrease the risks of CVD in patients with diabetes. Exercise can protect the myocardium by improving myocardial cell metabolism, alleviating oxidative stress damage, improving myocardial fibrosis, inhibiting apoptosis, and ameliorating microvascular disorders, and ultimately it is proposed to have the potential impacts to protect against DCM. Exercise is an importantly non-pharmacological strategy in reducing the risk factors of diabetes and its complications. It can be considered as a promising agent for alternative therapies for the prevention and treatment of diabetes and its cardiovascular complications. However, more clinical trials and pre-clinical studies are required to promote the translation of molecular findings to therapeutics of physical exercise.

AUTHOR CONTRIBUTIONS

JZ and JC collected data, synthesized data, and wrote the manuscript. SZ, LZ, and XHG reviewed and edited the manuscript. JQZ and XHX contributed to the design of this review.

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REVIEW
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Heterogeneity of Metabolic Defects in Type 2 Diabetes and Its Relation to Reactive Oxygen Species and Alterations in Beta-Cell Mass

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Type 2 diabetes (T2D) is a complex and heterogeneous disease which affects millions of people worldwide. The classification of diabetes is at an interesting turning point and there have been several recent reports on sub-classification of T2D based on phenotypical and metabolic characteristics. An important, and perhaps so far underestimated, factor in the pathophysiology of T2D is the role of oxidative stress and reactive oxygen species (ROS). There are multiple pathways for excessive ROS formation in T2D and in addition, beta-cells have an inherent deficit in the capacity to cope with oxidative stress. ROS formation could be causal, but also contribute to a large number of the metabolic defects in T2D, including beta-cell dysfunction and loss. Currently, our knowledge on beta-cell mass is limited to autopsy studies and based on comparisons with healthy controls. The combined evidence suggests that beta-cell mass is unaltered at onset of T2D but that it declines progressively. In order to better understand the pathophysiology of T2D, to identify and evaluate novel treatments, there is a need for *in vivo* techniques able to quantify beta-cell mass. Positron emission tomography holds great potential for this purpose and can in addition map metabolic defects, including ROS activity, in specific tissue compartments. In this review, we highlight the different phenotypical features of T2D and how metabolic defects impact oxidative stress and ROS formation. In addition, we review the literature on alterations of beta-cell mass in T2D and discuss potential techniques to assess beta-cell mass and metabolic defects *in vivo*.

Keywords: type 2 diabetes, diabetes classification, oxygen stress, reactive oxygen species, beta-cell, beta-cell mass, imaging, positron emission tomography

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INTRODUCTION

In the year 2030, it is estimated that 439 million people will be affected by diabetes (American Diabetes Association, 2009) and that the number will rise to 642 million by 2040 (Zimmet et al., 2016). Type 2 diabetes (T2D) accounts for 90–95% of all diabetes cases and is a global disease with major health- and financial implications for both the affected and the society. Already, in the 19th century, it was recognized by Lancereaux that there were at least two forms of diabetes which he

divided into *diabetes maigre* and *diabetes gras* meaning diabetes of the "thin" and "fat" (National Diabetes Data Group, 1979). With increasing knowledge, the classifications of diabetes have become more detailed and complex, but these early observations still play an important role since they reflect different aspects of pathophysiology. Indeed, diet and body weight have a major impact on the risk of developing T2D which at least in part can explain the dramatic increase in prevalence. Over the last 10 years, there has also been a substantial addition of drugs approved for the treatment of T2D. Despite that, a large number of those affected by T2D fail to reach an acceptable metabolic control (Safai et al., 2018). This can be explained by a number of factors including physical inactivity, diet, adherence to medications but also the underlying pathophysiological process and stage of disease is of importance for the effect of glucose lowering drugs. Over the last years, it has become increasingly recognized that T2D is a heterogeneous disease which requires an individualized treatment with adaptive changes over time as the disease progresses. In addition, hyperglycemia and coupled metabolic defects in diabetes increase the production of oxidative stress and reactive oxygen species (ROS) which can have vast deleterious effects and contribute to beta-cell dysfunction, failure, and loss. As T2D progresses, the initial hyperinsulinemia declines and a large number of patients are rendered insulin deficient due to the loss of beta-cells. In this review, we will highlight the different phenotypical features of T2D and how metabolic defects impact oxidative stress and ROS formation in different tissues. In addition, we review the literature on alterations of beta-cell mass in T2D and discuss potential imaging techniques in order to assess beta-cell mass and metabolic defects *in vivo*.

THE RATIONALE OF DIABETES CLASSIFICATION AND PHENOTYPICAL PRESENTATIONS OF TYPE 2 DIABETES

The diagnostic criteria for diabetes mellitus (DM) should preferably be based on parameters and laboratory tests which can be assessed in primary care facilities and broad enough to encompass all afflicted individuals. The more common the disease, the more phenotypically heterogeneous is the affected population. Classification criteria are used to divide the heterogeneous population to more homogeneous subpopulations for research and treatment guidelines (Aggarwal et al., 2015). The current diabetes classification into type 1 diabetes (T1D) and T2D is based on the ability to secrete insulin and the presence or absence of autoantibodies. Patients with T1D must be treated with insulin already at diagnosis whereas patients with T2D initially should be treated with dietary regimes, biguanides, or sulfonylureas. In clinical practice, 90–95% of people that fulfill the DM diagnostic criteria are classified as T2D.

In 1993, Tuomi et al. (1993) identified diabetes patients with a phenotype of both T1D and T2D. Typically, these patients are indistinguishable from T2D at diagnosis but over time they develop a more T1D like phenotype. The subgroup, coined latent autoimmune diabetes in the adult (Brophy et al., 2008), is defined as patients older than 35 years, with glutamate decarboxylase antibodies (GADA) 65 reactive against pancreatic beta-cells and remaining endogenous insulin secretion at least 6 months after diagnosis (Tuomi et al., 1993). The prevalence of LADA ranges from 4–6% in Eastern Asia (Takeda et al., 2002; Zhou et al., 2013) to 10–12% in Northern Europe (Turner et al., 1997; Laugesen et al., 2015), leaving 90% of the heterogeneous T2D population undifferentiated. LADA patients also share genetic characteristics of both T1D and T2D and the rate of beta-cell loss and thereby time to exogenous insulin dependence correlates to the levels of GADA 65 (Niskanen et al., 1995; Fourlanos et al., 2005; Brophy et al., 2008; Cervin et al., 2008). In addition, patients with a classical T1D can develop insulin-resistance despite the lack of endogenous insulin production, often referred to as double-diabetes (Cleland et al., 2013). Thus, the categorization of DM into T1D and T2D is not clear-cut but rather a mix of etiology resulting in a phenotypic continuum (Niskanen et al., 1995; Fourlanos et al., 2005; Buzzetti et al., 2007; Laugesen et al., 2015).

Before 1995, there were only two classes of anti-diabetic agents apart from insulin, sulfonylurea (1946), and biguanide (1959). The identification of LADA implied the need for a tighter glucose monitoring compared to T2D and avoidance of sulfonylurea, but apart from this the classification contributed little to improve the clinical management of diabetes. Since then six pharmacodynamically different drug classes have been approved for clinical use, α-glucosidase inhibitors (1995), thiazolidinediones (1996), metglitinides (1997), glucagon-like peptide-1 (GLP-1) analogs (2005), dipepidylpepidase-4 (DPP-4) inhibitors (2006), and sodium glucose co-transporter 2 (SGLT2) inhibitors (2013) (White, 2014). Aside from providing new treatment possibilities, they elucidate the need for a revised diabetes classification.

From being a rational DM classification in 1979, the classification into T1D and T2D is now obsolete both for research and for clinical guidance. This realization has spurred efforts to find new classifications of diabetes. With a data-driven topologic analysis on electronic medical records data using 73 clinical features associated to variations in single nucleotide polymorphisms, Li et al. (2015) identified three subgroups of T2D that differ both in phenotype and genotype. Subtype 1 (31%) was characterized by obesity, kidney disease, and hyperglycemia whereas subtype 2 (25%) and 3 (44%) were associated with cancer and neurological disease, respectively. The study did not include disease duration and did not reveal if individuals switched subtype over time. However, this study is of interest since it links real-life data to genome-wide association studies.

By using latent class trajectory analysis on a five-time point oral glucose tolerance test (OGTT), Hulman et al. (2018) identified five sub-classes of metabolic control even among non-diabetic individuals that differed in regard to insulin sensitivity and acute insulin response, obesity, lipid levels, and inflammatory markers. The classes were also correlated to different pathophysiological processes. The strongest determinant of time to glucose peak during the OGTT was insulin sensitivity and those patients who shifted sub-class over time could mainly be explained by life-style changes that affect insulin sensitivity.

Ahlqvist et al. (2018) presented a novel diabetes classification by cluster analysis of five phenotypically diabetogenic risk factors. Cluster 1 (severe autoimmune diabetes, SAID) includes all patients with positive GADA 65 antibody titer. Cluster 2 (severe insulin deficient diabetes, SIDD) with low fasting (f) C-Peptide and high HbA1c at diagnosis. Cluster 3 (severe insulin resistant diabetes, SIRD) with high fC-Peptide and high HbA1c. Cluster 4 (mild obesity-related diabetes, MOD) with high BMI and relatively low HbA1c at diagnosis. Cluster 5 (mild age-related diabetes, MARD) was the largest with 39% of the population and characterized by higher age at diagnosis and relatively low HbA1c. During follow-up (median 3.9 years), cluster 2 (SAID) and 3 (SIRD) were more prone to complications than cluster 4 (MOD) and 5 (Abdo et al., 2010).

Udler et al. (2018) aimed to find pathophysiological clusters by takeoff from publically available genome-wide assay study (GWAS). Ninety-four genetic variants and 47 diabetes-related metabolic traits were included to a Bayesian non-negative factorization clustering, which yielded five clusters. The clinical impact of the clusters was then assessed in four separate cohorts (N = 17 874). Cluster 1 (beta-cell) and 2 (proinsulin) were associated with beta cell dysfunction, cluster 1 had increased proinsulin levels whereas cluster 2 had decreased proinsulin levels. Clusters 3 (obesity), 4 (lipodystrophy), and 5 (liver/lipid) were associated with mechanisms of insulin resistance. The obesity-liked loci FTO and MC4R were more common in cluster 3, concordantly also waist and hip circumference. Individuals in cluster had decreased adiponectin, low insulin sensitivity index and HDL levels, and increased triglycerides. Cluster 5 was associated with loci related to non-alcoholic liver disease (NAFLD) and these individuals had increased levels of urate and fatty acids related to NAFLD (serum triglycerides, palmitoleic acid, and linolenic acid).

These ambitious attempts to reform diabetes classification, summarized in **Figure 1** and **Supplementary Table S1**, take on the long time insight that diabetes is not a single disease of hyperglycemia, but rather a syndrome of multiple metabolic disturbances. If the addition of genetic and phenotypic parameters actually identifies novel diabetes subgroups, we may well stand in front of a shift of paradigm in both treatment and monitoring diabetes.

METABOLIC DEFECTS AND REACTIVE OXYGEN SPECIES IN TYPE 2 DIABETES

Type 2 diabetes, though primarily a disease characterized by decreased insulin sensitivity, also involves the destruction of insulin producing beta-cells during the later stages of the disease (Sakuraba et al., 2002; Butler et al., 2003). An ever-increasing demand for insulin production to overcome progressing insulin resistance becomes harmful to the beta-cells and hyperglycemia and increased free fatty acids, cause oxidative stress (Donath et al., 2005). Albeit the assessment of beta-cell mass in humans has been rather challenging, most current research suggests that beta-cell mass declines with the progression of T2D. While the mechanisms behind beta-cell failure and death in T2D are not fully understood, increasing interest has been directed toward the role of oxidative stress. Increased production of ROS, driven by chronic hyperglycemia and hyperlipidemia, is thought to be a major cause of the beta-cell dysfunction in diabetes (Robertson et al., 2007; Graciano et al., 2013). ROS are known to damage components of the cellular machinery, including DNA, proteins, and lipids which leads to a vast array of deleterious effects (Schieber and Chandel, 2014). In fact, signs of increased ROS activity have been observed in pancreatic islets of deceased T2D patients (Sakuraba et al., 2002). Moreover, oxidative stress likely contributes to the development of peripheral insulin resistance and many of the long-term micro- and macrovascular complications of diabetes (Styskal et al., 2012).

Reactive oxygen species are inevitable byproducts of aerobic metabolism, produced primarily from "leakage" of electrons in the mitochondrial electron transport chain. Under basal conditions, ROS serve in various pathways regulating biological and physiological processes involving mainly stress response signaling (Schieber and Chandel, 2014). Antioxidant enzymes and low molecular ROS-scavengers balance ROS activity in the physiological redox biology. An overexpression of ROS, or overwhelming of the antioxidant responses, results in oxidative stress. Accumulating evidence suggests that oxidative stress is involved both in the early events surrounding the development of T2D, as well as the later hyperglycemia induced tissue damage (Nowotny et al., 2015). Moreover, pancreatic islets and particularly beta-cells express less antioxidant enzymes compared to other tissues, making them more susceptible to the damaging effects of oxidative stress and ROS (Lenzen et al., 1996; Miki et al., 2018).

While excessive ROS levels have long been viewed as responsible for many undesirable effects (Stadtman, 1992, 2001), it is becoming more evident that moderate levels of ROS are often not only inevitable but also necessary and beneficial for many normal cellular functions (Cai and Yan, 2013), this is the case also in beta-cells. In the production of insulin, ROS are unavoidable byproducts of enzyme driven (Sevier and Kaiser, 2002; Tu and Weissman, 2004) folding of proinsulin in the endoplasmic reticulum (ER). With each formation of a disulphide bond, one molecule of ROS is produced. With the insulin molecule having three disulphide bonds important for its function (Chang et al., 2003), production of one molecule of insulin would be associated with the production of three molecules of ROS. Hyperglycemic conditions can cause a 50-fold increase in insulin biosynthesis (Goodge and Hutton, 2000). Under these conditions, beta-cells each produce up to 1 million molecules of insulin per minute (Scheuner and Kaufman, 2008), this could possibly signify production of 3 million molecules of ROS per minute in every beta-cell (Gross et al., 2006; Shimizu and Hendershot, 2009). Regulation of insulin translation and the unfolded protein response (UPR) play an important role, but the exact mechanisms by which beta-cells cope with this amount of ROS production are still not fully understood, particularly as they express relatively low levels of antioxidant enzymes. Autophagy also protects against oxidative stress and ER-stress (Kroemer et al., 2010), and failure of this system may worsen beta-cell function in diabetic conditions (Watada and Fujitani, 2015).

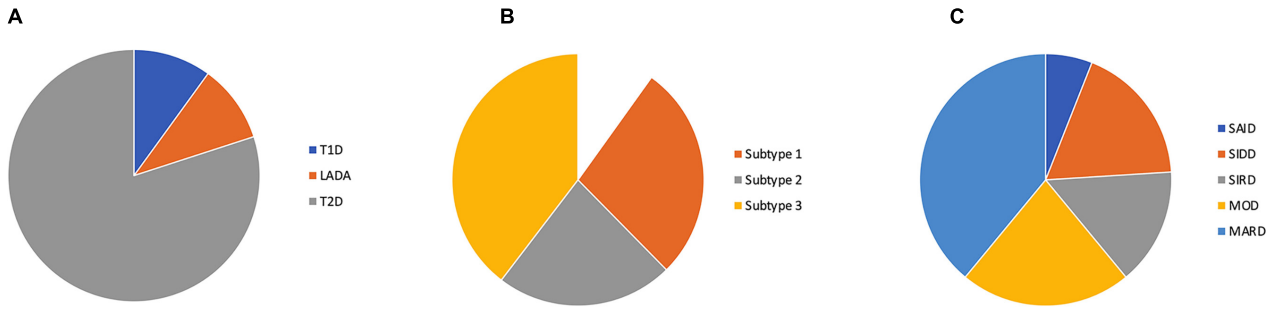

FIGURE 1 | Proportions of diabetes subtypes by **(A)** the current classification, **(B)** subtyping of type 2 diabetes by Li et al. (2015) and **(C)** cluster classification by Ahlqvist et al. (2018) SAID (severe auto-immune diabetes), SIDD (severe insulin deficient diabetes), SIRD (severe insulin resistant diabetes), MOD (mild obestity-related diabetes) and MARD (mild age-related diabetes).

Shimizu and Hendershot (2009) elegantly summarize possible molecular mechanisms coping with oxidative stress in secretory tissues. Common for the protective mechanisms, such as the UPR, is that prolonged activation often results in diversion to cytodestructive effects, giving us a possible explanation of how ROS might take part in the beta cell loss observed particularly in T1D. If conditions of ER-stress are not resolved, Ca2+ leaks from the ER, further increase ROS production by causing mitochondrial dysfunction (Deniaud et al., 2008), thus leading to an increased oxidative stress load in the already struggling cells, and subsequently apoptosis. This ties the role of ER-stress and oxidative stress in with the notion that mitochondrial apoptotic pathway plays a central role in cytokine induced beta-cell failure in T1D (Grunnet et al., 2009). Furthermore, ER-stress in beta-cells has also been suggested to be in part responsible for sustaining the autoimmune response observed in T1D (Marré et al., 2015).

It is also conceivable that during diabetic conditions, when demand for insulin production is increased, and beta-cell ROS production remains high for prolonged time periods, further insulin production is inhibited as a cytoprotective measure. Indeed, Ire1, which is one of the effector branches of the UPR, when continuously activated has been shown to cause suppression of insulin gene expression (Lipson et al., 2006), possibly explaining why prolonged hyperglycemia in T2D patients leads to diminished insulin production also in the absence of apoptosis (Shimizu and Hendershot, 2009). Thus, the subject of ROS is both complex and rather paradoxical, being both an integral part of the islets basic functioning, and perhaps even necessary for their proliferation, but left uncontrolled part of their demise.

In addition to ROS production from increased metabolism and insulin production, hyperactivity in the NADPH oxidases (NOX) also leads to excessive ROS production. Seven membrane bound isoforms of the NOX enzymes (NOX1-5 and DUOX1-2) have been identified. These perform normal cellular functions at physiological conditions, but excessive activation produces harmful levels of ROS. Increased activity of some NOX isoforms has been shown to play an important role in metabolic defects and diabetes through mitochondrial dysregulation in the beta-cells (Guichard et al., 2008; Syed et al., 2011). Increased NOX activity has also been linked to lipid induced ROS production and fatty acid promoted amplification of glucose-stimulated insulin secretion (Graciano et al., 2013). Some NOX isoforms seem to be activated by glucose stimulation, and in the short term potentiate insulin release (Morgan et al., 2009); however, excessive long-term activation is detrimental to beta-cell function (Syed et al., 2011). The NOX4 isoform has been suggested to function as a mitochondrial energy sensor, being negatively regulated by ATP (Shanmugasundaram et al., 2017), and might thus be a source of ROS in both islets and other tissues experiencing metabolic stress in diabetes. Of great interest, we have recently demonstrated that selective NOX4 inhibitors protect human islets and reduce beta-cell death under *in vitro* conditions mimicking the T2D environment (Wang et al., 2018a), making it a potential drug target.

Advanced glycation end products (AGEs) are modified proteins and lipids formed under conditions of oxidative stress. AGEs can, however, also sustain oxidative stress by increasing ROS formation and negatively impact antioxidant systems (Nowotny et al., 2015). Moreover, AGEs such as methylglyoxal are highly abundant in the standard western diet (Uribarri et al., 2007), rendering them as both endogenous and exogenous contributors to oxidative stress. While the full role of AGEs in T2D is not yet completely understood, it is generally accepted that they play an important role by contributing to the oxidative stress, causing both beta-cell damage and peripheral insulin resistance (Vlassara and Uribarri, 2014).

Besides oxidative stress related to glucolipotoxicity, AGEs, and dietary factors, there are many other environmental factors associated with deterioration of beta-cell function that are less well understood. One such factor is disruption of the islets circadian rhythm, which has also recently been suggested to cause increased ROS production and a decreased production of anti-oxidant genes in beta-cells, leading to beta-cell dysfunction and diabetes (Lee et al., 2018).

Numerous studies have attempted to delineate the potential antioxidant effects of current oral antidiabetic treatments as well as for exogenous insulin substitution, with biguanides being the most studied substance. The Biguanide Metformin has been evaluated as a potential treatment for many diseases apart from T2D with promising results. Its potential use in various

forms of cancer (Cheng and Lanza-Jacoby, 2015; Vancura et al., 2018), infectious diseases (Kajiwara et al., 2018), cardiovascular disease (Diaz-Morales et al., 2017; Nesti and Natali, 2017), skin disorders (Wang et al., 2018b), and much more is continuously being investigated. In many cases, alterations in redox status are suggested as a main mechanism of action. While Metformin is suggested to ameliorate many disorders by decreasing oxidative stress (Cheng and Lanza-Jacoby, 2015; Diaz-Morales et al., 2017), others suggest the opposite, that Metformin acts by increasing ROS production (Kajiwara et al., 2018; Wang et al., 2018b). While at first glance these conflicting reports seem discerning, it is not unexpected that cells and tissues with vastly differing physiological processes respond differently to Metformin and oxidative stress. In general, research concerning the effects of Metformin on oxidative stress in diabetes suggests that it decreases peripheral ROS production and thereby protects against diabetic atherosclerosis and other complications (Esteghamati et al., 2013; Singh et al., 2016; Diaz-Morales et al., 2017). A recent study implies that aberrant complex I activation in the pancreas of diabetic patients causes an overflow of NADH that is diverted into ROS production leading to beta-cell dysfunction and death (Wu et al., 2017), and Metformin being a mitochondrial complex I inhibitor effectively counteracts this. So, while Metformin's main mechanism of action in reducing hepatic gluconeogenesis and increasing peripheral insulin sensitivity has been known for years, many of its functions are yet to be fully understood (Rena et al., 2017), including its effect on oxidative stress.

Multiple studies also suggest that GLP-1 analogs may positively influence redox homeostasis, summarized in Petersen et al. (2016). When it comes to other oral antidiabetics, some research suggests effects on ROS production in various tissues by sulfonylureas (Sawada et al., 2008), α-glucosidase inhibitors (Aoki et al., 2012), thiazolidinediones (Singh et al., 2016), and DPP4 inhibitors (Rizzo et al., 2012), but it must be noted that research on these matters are limited.

In a study where exogenous insulin analogs were administered to T2D patients who failed to achieve satisfactory glycemic control on Metformin and sulfonylurea alone, showed a significant decrease in oxidative stress markers (Tuzcu et al., 2013). Interestingly, this was not related to changes in mean glucose levels, suggesting instead some direct inhibitory effects on ROS formation. While insulin in high levels may promote oxidative stress (Rains and Jain, 2011), this study and others (Monnier et al., 2011) suggest that insulin has a rather complex relationship with oxidative stress in T2D.

The relationship between glucose variability and oxidative stress in T2D has also been examined and has yielded somewhat conflicting results. Monnier et al. (2011) reported a significant correlation between glucose variability and oxidative stress whereas a repeating study failed to find a relationship (Siegelaar et al., 2011). A possible explanation for this, as the authors mention in their discussion, is that the latter study mainly examined patients with significantly better glycemic control. This hypothesis is strengthened by results from another study by Monnier et al. (2010) where the relationship between glucose variability and oxidative stress was also associated with HbA1c.

A major limitation for studies of oxidative stress *in vivo* is the difficulty to measure ROS in a reliable way, summarized by Halliwell and Whiteman (2004). In general, the short half-life of reactive species limits our possibilities to measure them directly. Instead, we are limited to measuring the levels of markers for oxidative damage or trapping the reactive species and measuring levels of the trapped molecules. This entails a number of problems, as the marker or trap preferably has to be stable, specific, quantifiable, present in the studied tissue and in addition not confounded by diet or alternative activation pathways. As of now, there are no biomarkers of ROS that are considered to be ideal, but some are better than others. For instance, isoprostanes are considered a rather reliable biomarker for lipid peroxidation, which is a common way to measure the effects of oxidative stress *in vivo* (Halliwell and Whiteman, 2004; Kaviarasan et al., 2009). Indeed, the levels of isoprostanes have been found to be increased in T2D (Kaviarasan et al., 2009).

In conclusion, there are multiple pathways for excessive ROS formation in T2D, and a deficit in the beta-cells capacity to cope with oxidative stress, summarized in **Figure 2**. Oxidative stress may not only be caused by a number of metabolic defects in T2D but can also in itself contribute to aggravating the defects and the different phenotypes of diabetes. While there exist some difficulties in studying oxidative stress *in vivo* in humans, our current understanding is that it appears to have a central role in many processes involving the development and progression T2D and its long-term complications. Aside from assessing redox properties of currently available medication, novel treatments targeting ROS production such as specific NOX inhibitors are also being researched (Wang et al., 2018a).

ADAPTIVE CHANGES OF BETA-CELL MASS IN OBESITY AND TYPE 2 DIABETES

Due to the lack of established *in vivo* techniques, our current knowledge on beta-cell mass in humans fully relies on autopsy studies. Although a very valuable source, autopsy material has a number of drawbacks including technical difficulties but of outmost importance is the inherent lack of repeated measurements. The number and volume of islets increase substantially from fetal life to adulthood and is estimated to increase fivefold from birth to adulthood, in parallel the exocrine pancreas increase 15-fold in size (Witte et al., 1984). Therefore, islets compose 20% of the total pancreas volume in newborns, 7.5% in children but only 1–2% in adults (Witte et al., 1984). In addition, the proportion of beta-cells within the islets varies in the different anatomical regions of the pancreas with more beta-cells in corpus and cauda. In adults, the pancreas weighs in average around 100 g but can range from 50 to approximately 170 g. Combined with the potential twofold difference in islet percentage, this gives a ≥fivefold theoretical difference in islet mass even among healthy individuals. Well in line with this, a fivefold difference in beta-cell mass is often observed among healthy individuals in reports based on autopsy material (Rahier

et al., 2008). This is important to keep in mind since our knowledge and view on beta-cell mass in T2D is based on comparisons with healthy controls.

In adulthood there are in particular two physiological conditions which lead to an increase in beta-cell mass; pregnancy and obesity (Van Assche et al., 1978; Kloppel et al., 1985; Butler et al., 2010; Hanley et al., 2010; Saisho et al., 2013). The alterations of beta-cell mass during pregnancy are elegantly reviewed by Nielsen (2016). Of importance, beta-cell mass has been found to be increased by 50% in obese individuals when compared to lean individuals (Rahier et al., 1983; Kloppel et al., 1985; Hanley et al., 2010; Saisho et al., 2013). In fact, Saisho et al. (2013) found that beta-cell mass correlates with BMI. In patients with T2D, there have been conflicting results on beta-cell mass which can be explained by the above-mentioned difficulties regarding comparisons with non-diabetic individuals and by the disease duration and heterogeneity of T2D. In patients with recent onset T2D, the beta-cell mass has been found to be unaltered (Rahier et al., 1983; Hanley et al., 2010); however, in the report by Hanley et al. (2010) obese patients with T2D displayed a decreased beta-cell mass. The unaltered beta-cell mass in recent onset T2D in combination with the normal or increased C-peptide levels make a strong argument for that T2D is not primarily developed due to a loss of beta-cells but rather due to insulin resistance and beta-cell dysfunction. Others have reported on a moderate (≈25%) decrease in beta-cell mass (Sakuraba et al., 2002) but there are also reports on a more pronounced reduction (≈50%) of beta-cell mass in patients with long-standing T2D (Maclean and Ogilvie, 1955; Butler et al., 2003; Rahier et al., 2008). The degree of beta-cell loss in T2D has been found to correlate to disease duration, with a distinct reduction after 20 years of disease (Rahier et al., 2008). In addition, there are a number of reports supporting the deposition of amyloid in islets of patients with T2D which could contribute to beta-cell dysfunction and apoptosis (Bell, 1952; Ehrlich and Ratner, 1961; Clark et al., 1988; Sakuraba et al., 2002; Huang et al., 2007). However, the role of islet amyloid deposits in the pathogenesis of T2D is beyond the scope of this review, but for the interested the comprehensive review on islet amyloid by Westermark et al. (2011) is warmly recommended.

In non-diabetic individuals, the beta-cell mass is tightly regulated during adulthood by a balance between beta-cell replication and apoptosis (Bonner-Weir, 2000). Interestingly, ROS is known to increase apoptosis and in high concentrations induce cell cycle arrest. However, in low to moderate concentrations, ROS have been found to stimulate cell proliferation (Boonstra and Post, 2004; Ahmed Alfar et al., 2017). In addition, experimental studies have shown that mitochondrial ROS play an important role in beta-cell proliferation (Ahmed Alfar et al., 2017) and in the establishment of beta-cell mass during development (Zeng et al., 2017). The loss of beta-cell mass in T2D can be explained by the increased apoptosis rate observed in islets from T2D patients (Butler et al., 2003; Rahier et al., 2008; Hanley et al., 2010) and the lack of increased beta-cell proliferation (Butler et al., 2003). Of great interest, pancreatic beta-cells inherently express low levels of antioxidant enzyme superoxide dismutase (Falk-Delgado et al., 2015) but there is also support of further decreased levels of SOD in beta-cells of T2D patients (Sakuraba et al., 2002). This further supports the role of beta-cell failure and loss due to ROS activity in T2D.

The combined evidence suggests that beta-cell mass is relatively unaltered at the onset of T2D, but declines progressively with the disease. It seems as if the loss of beta-cell mass is more pronounced in obese T2D individuals which could be due to a loss of stimulatory signals or a lack of beta-cell proliferatory response to obesity in combination with an increased metabolic stress under diabetic conditions. In addition, the local milieu and metabolic challenges of beta-cells in T2D obesity may also have deleterious effects (Prentki et al., 2002) and increase the production of ROS (Graciano et al., 2013).

IN VIVO IMAGING OF BETA-CELL MASS

Given the pandemic increase in T2D and the heterogeneous nature of the disease in combination with large individual variations in beta-cell mass, it would be of great importance to establish a technique allowing *in vivo* monitoring of beta-cell mass. With such a technique, we would gain valuable insight on the pathophysiology of the disease and the decline of beta-cell mass over time in different phenotypical presentations of T2D. Due to the size and distribution of pancreatic islets, imaging beta-cell mass is a tough challenge since there are no non-invasive imaging modalities with a high enough resolution to delineate single islets in human. However, by using positron emission tomography (PET) in combination with a beta-cell specific PET-tracer, it would be possible to monitor the combined signal from all islets within the pancreas and thereby beta-cell mass. There have been several attempts to find a beta-cell specific PET-tracer over the last decade, summarized in a recent review by Eriksson et al. (2016).

We have focused our attempts on the clinically available PET-tracer [^{11}C]5-hydroxy-tryptophan ([^{11}C]5-HTP), a serotonin precursor, which, however, is not completely specific for beta-cells but is also retained in remaining endocrine cells within the islets of Langerhans. Using PET in combination with computed tomography (CT), we have found that the pancreatic uptake of [^{11}C]5-HTP is reduced by 66% in patients with long-standing T1D when compared with healthy controls, which is well in line with a complete beta-cell loss (Eriksson et al., 2014). We have also evaluated the use of [^{11}C]5-HTP in combination with magnetic resonance tomography (MRT) in patients with T2D. Patients were first categorized into four groups based on BMI (lean or obese) and treatment regime, either oral antidiabetic drugs (OADs) alone or in combination with insulin (OAD+insulin). The functional beta-cell mass was determined based on acute C-peptide response to a bolus of arginine and C-peptide response to a glucose potentiated arginine test. We found that the patients, both lean and obese, treated with OAD had a normal C-peptide response to arginine but a marked reduction of C-peptide secretion in response to the glucose potentiated arginine test. Patients treated with OAD+insulin displayed a marked reduction in C-peptide secretion in both

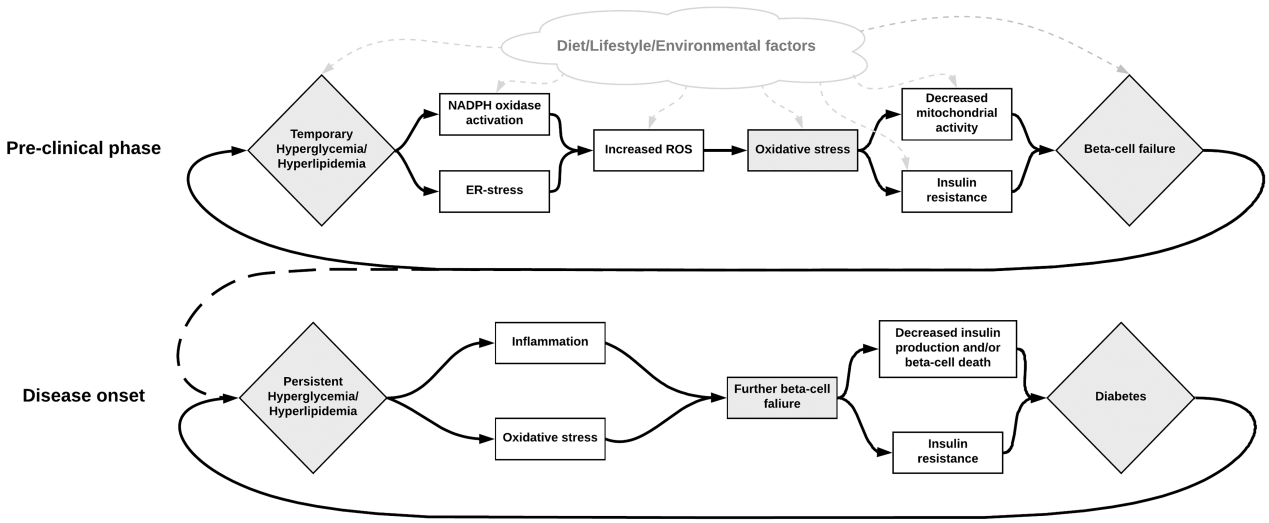

FIGURE 2 | Illustration of the possible role of NOX activation in the development of beta-cell failure, hyperglycemia, and diabetes. Metabolic dysregulation leading to hyperactivity in the NOX-enzymes results in excessive ROS production and oxidative stress. This increased oxidative stress may subsequently be responsible for beta-cell failure, which in turn contributes to increased metabolic dysregulation. Various other factors may also influence these steps in different ways. For instance, diet can contribute to increased oxidative stress directly by containing excessive AGEs, or indirectly by contributing to the metabolic dysregulation. Inhibition of the NOX enzymes seems to be a promising solution for breaking this deleterious cycle.

the acute- and glucose potentiated arginine test. However, the pancreatic uptake of [^{11}C]5-HTP did not differ between the groups (Carlbom et al., 2017). Of interest, we observed a twofold difference of [^{11}C]5-HTP pancreatic uptake in healthy controls but among the T2D group the difference was close to fivefold. In fact, among lean T2D patients with OAD+Insulin two thirds of the patients displayed a pancreatic uptake well below the absolute levels of healthy controls. In line with this, the pancreatic volume was quite homogenous in healthy controls but varied in T2D groups with a significantly increased volume in obese T2D patients with OAD+insulin.

There could be several contributors to the observed discrepancy between functional beta-cell mass and [^{11}C]5-HTP pancreatic uptake. In fact, isolated islets from patients with T2D contain and secret less insulin when compared to non-diabetic donors (Deng et al., 2004; Marchetti et al., 2004). Since [^{11}C]5-HTP is not a beta-cell specific tracer, the result could still reflect a true finding of islet mass in T2D since the alpha-cell mass has been found to be unaltered (Henquin and Rahier, 2011) and there have been reports supporting a de-differentiation of beta-cells rather than a beta-cell destruction in T2D (Talchai et al., 2012; Spijker et al., 2015). However, the data should be interpreted with caution given the small number of individuals in each group. Given the large variability even in normal physiology, cross-sectional comparisons will be difficult in order to discern more discrete alterations of beta-cell mass. However, this could be overcome by the use of repeated paired measurements. Indeed, by using a retrospective design of repeated examinations, we have found that the pancreatic uptake of [^{11}C]5-HTP in T2D decrease over time as the disease progresses (Eriksson et al., 2014). In addition, new PET-traces targeting GPR44 that are specific for beta-cells are currently being developed which could potentiate future studies of adaptive changes of beta-cell mass during different stages of T2D (Eriksson et al., 2018).

IN VIVO IMAGING OF GLUCOSE- AND LIPID METABOLISM IN TYPE 2 DIABETES

Apart from the possibilities of imaging and quantifying beta-cell mass with PET, the technique also opens up for possibilities of mapping metabolic alterations in specific tissues *in vivo*. As discussed above, hyperglycemia and hyperlipidemia increase ROS activity which contributes to tissue damage and diabetic complications. Currently, we base most of our clinical decisions and classifications on the circulating levels of glucose, free fatty acids, and hormones. We can also detect indirect measurements of ROS but it has been difficult to establish reliable biomarkers, in addition the effects and activity of ROS can vary in different tissue. However, by using PET, we could actually image and map how these metabolic defects occur in different tissues which could relate to the risk of complications in T2D. In addition, these techniques would also provide important insights on the pathophysiological processes and thereby guide us in which treatments to use. An important advantage with PET is the relatively low radiation burden associated with tracers and the possibility to use MRT for anatomical mapping. In combination with the short radioactivity half-life of most tracers (<120 min), it is therefore possible to perform repeated examinations and to use a multi-tracer approach which makes it possible to examine several metabolic pathways at the same time.

The most widely used PET-tracer is in fact a glucose analog, 2-deoxy-2-(^{18}F)fluoro-D-glucose ([^{18}F]FDG), which is used in

different fields of medicine to identify everything from tumors to inflammation. [^{18}F]FDG is a general biomarker for any tissue relying on glycolysis, for example, the brain and the myocardium. Briefly, by relating the uptake rate of [^{18}F]FDG in different tissues to the glucose levels in plasma, a metabolic rate of glucose (MRGlu, μmol/g/min) in specific tissue can be determined (Phelps et al., 1979). By using [^{18}F]FDG PET, it has, for instance, been demonstrated that the brain glucose utilization is not affected by insulin-infusion in healthy individuals, i.e., the glucose uptake is maximized already at fasting conditions. However, in individuals with impaired fasting glucose (IGF), the brain glucose utilization increases in response to insulin infusion suggesting that the brain glucose metabolism is disturbed even in IFG (Hirvonen et al., 2011). Using whole-body [^{18}F]FDG PET and hyperinsulinemic euglycemic clamp, it was recently demonstrated that the brain glucose utilization is increased in patients with T2D (Boersma et al., 2018). In contrast, the authors found that the glucose utilization was decreased in skeletal muscle, visceral- and adipose tissue, and the liver in patients with T2D (Boersma et al., 2018).

In addition, several fatty acids have been labeled with positron emitting nuclides in order to track their fate in the human body (Mather and DeGrado, 2016). Fatty acid uptake and the rate of oxidation can be determined by relating the uptake and retention, respectively, of the labeled fatty acid with the circulating amounts of non-esterified fatty acids in plasma (p-NEFA). Some of the clinically more commonly used markers for fatty acid metabolism are [^{11}C]Palmitate (Weiss et al., 1976) and 14-^{18}F-fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA) (DeGrado et al., 1991). Depending on the design of the PET tracers and the trapping mechanisms in different cellular compartments, the tracers tend to reflect either fatty acid uptake or fatty acid oxidation. By using a multi-tracer approach, it has been demonstrated in patients with T2D that the myocardial metabolism is shifted toward fatty acid oxidation instead of glycolysis (Rijzewijk et al., 2009). In addition, it has been demonstrated by PET imaging that patients with T2D have an increased myocardial fatty-acid uptake and fatty-acid oxidation compared to healthy individuals (Mather et al., 2016). In both of these studies, the patients displayed a good metabolic control and had not yet established any micro- or macrovascular complications.

As discussed, metabolic defects increase production of ROS and by imaging metabolism in different tissue using PET this could give indirect evidence on ROS formation and activity. In fact, the uptake of [^{18}F]FDG has been linked to ROS concentration in tumor cell-lines and tumor-bearing mice (Jung et al., 2013). Also, increasing oxidative stress has been related to decreased brain [^{18}F]FDG uptake in neurodegenerative disorders (Mosconi et al., 2008). However, the volatile nature of ROS in tissue results in a less than exact assessment using indirect approaches for measurement. Therefore, it is potentially a major advancement that PET tracers specific for ROS are now being developed. In experimental studies, [^{18}F] and [^{11}C] labeled dihydrophenantridine derivatives have been used which bind to DNA in their oxidized forms and therefore becomes trapped within cells. The uptake was found to be ROS specific in both *in vitro* and *in vivo* experimental studies (Chu et al., 2014; Wilson et al., 2017). In addition, also other imaging techniques are being developed for measuring free radicals directly in living organisms by using electron spin resonance and special probes (Berliner et al., 2001; Elas et al., 2012). However, these probes are currently only available for preclinical use. Furthermore, genetically encoded ROS probes such as HyPer-3 (Bilan et al., 2013) and roGFP2-Orp1 (Gutscher et al., 2009) have been presented in recent years (Shimizu and Hendershot, 2009; Meyer and Dick, 2010), and while perhaps not suited for the clinic, allows visualization of specific ROS detection *in vivo* in disease models. PET imaging using tracers targeting ROS will likely soon be available in the clinical setting, which will be a valuable contribution in many fields of medicine, not the least in diabetes research.

DISCUSSION

Given the diverse nature of T2D, it is a challenging and costly disease to manage, both in perspective of the individual patient, as well as the society as a whole. Classification and monitoring of diabetes has until recently relied primarily the on quantification of circulating glucose and insulin levels in combination with the presence or absence of autoantibodies. While this is a massive step up from the characterizations of Lancereauxs classification based on body weight, it falls short for use in modern research and drug development. Common for the classifications by Li et al. (2015), Ahlqvist et al. (2018), and Udler et al. (2018) is that they see T2D as a result of different pathophysiological disturbances and that the heterogeneity can be explained by identifying which disturbance is the dominant. Li et al. (2015) and Udler et al. (2018) emphasize genetic variance as the underlying cause and originate their classification from there. They find expected concordance between genetic and phenotypic traits in several cohorts. Ahlqvist et al. (2018) and Hulman et al. (2018) take on a more pragmatic approach by focusing on phenotype characteristics. By using variables that can be easily measured in primary care, the classification by Ahlqvist et al. (2018) has the potential to be widely accepted. However, the use of phenotype for classification may not be robust over time. Successful treatment may lead to a switch in cluster belonging, requiring re-classification on a regular basis.

Type 2 diabetes is associated with a number of metabolic defects resulting from decreased insulin sensitivity, many of which likely take part in the development and progression of the disease. Accumulating evidence points toward oxidative stress as a culprit, responsible not only for the devastating consequences of peripheral hyperglycemia and hyperlipidemia, but also for the dysfunction and loss of beta-cells. Multiple sources for this oxidative stress have been suggested (Styskal et al., 2012; Graciano et al., 2013; Nowotny et al., 2015). Much points to insulin deficiency being correlated with increased oxidative stress, providing a possible explanation to why the SIDD and SIRD classes suggested by Ahlqvist et al. (2018) are more prone to complications than MOD and MARD during follow-up. As elevated insulin levels promote oxidative stress, the insulin resistance observed in SIRD may in part be caused by elevated

peripheral oxidative stress. SIDD in contrast, characterized by insulin deficiency, while having similar peripheral complications as SIRD, may initially have its oxidative stress primarily localized to the pancreatic islets, causing impairment of beta-cell function and survival. Improved understating of the metabolic defects that occur, the implications of oxidative stress, and delineation of mechanisms which are most important for the progression of the disease, will help us combat T2D as well as provide potential targets for novel treatment strategies. In order to succeed in this challenging task, both basic research and clinical studies are warranted to pinpoint the exact mechanisms. For the latter, effective and specific monitoring tools are needed. With the pancreas being a quite inaccessible organ for invasive *in vivo* studies, the assessment of inflammation, beta-cell mass, and metabolism have so far been mostly been limited to autopsy studies. Furthermore, adversities in studying the mechanisms behind and processes surrounding beta-cell failure and death in T2D, especially *in vivo*, are likely present not solely due to limitations in methodology, but also because of de facto differences in mechanisms between phenotypically different subgroups. The ongoing development of non-invasive PET imaging techniques targeting beta-cell mass, as well as glucose and lipid metabolism and ROS, will hopefully provide us with tools to perform more extensive prospective studies in order to delineate the pathophysiological changes in the progression of diabetes. In addition, this will serve as a valuable tool for evaluating the effects of novel drug interventions and may also aid in further sub-classification attempts in order to tailor specific treatment regimes. Much of our current knowledge support the view that the loss of beta-cell mass is not the cause of but rather the effect of T2D. However, a number of important questions regarding the role of beta-cell loss in T2D remain unanswered. With the development of *in vivo* techniques based on PET for the assessment of beta-cell mass, a number of important questions can be addressed.

CONCLUSION

Type 2 diabetes is a complex and heterogeneous disease which affects millions of people in increasing numbers worldwide. T2D increases the risk of cardiovascular disease and causes a number of long-term complications with dramatic effects for both the affected individual and the society. The classification of diabetes is at an interesting turning point and we will likely have a number of sub-classifications of T2D within the next few years. Some of the metabolic defects of T2D are causal for the disease but many are secondary and can further contribute to an aggravated metabolic control, beta-cell dysfunction, and even beta-cell loss. An important, and perhaps so far underestimated, factor is the role of oxidative stress and ROS in the pathophysiology of T2D. ROS could be causal but also contribute to a large number of the metabolic defects observed in T2D including beta-cell dysfunction and beta-cell loss. Beta-cell mass is unaltered at the onset of T2D but progressively declines over time. Currently, our knowledge on beta-cell mass is limited to autopsy studies and based on comparisons with healthy controls. PET in combination with novel PET-tracers holds great potential for quantifying beta-cell mass *in vivo*. In addition, PET can be used to quantify and image metabolic defects as well as ROS activity in different tissues. With the use of these novel techniques, we anticipate that our understanding on the pathophysiology of T2D will dramatically increase over the coming years which hopefully will result in the development of new potent drugs to combat metabolic defects, ROS activity and beta-cell failure in T2D.

DATA AVAILABILITY STATEMENT

The datasets for this manuscript are not publicly available because historical published data will be provided by the corresponding author upon request. Requests to access the datasets should be directed to daniel.espes@mcb.uu.se.

AUTHOR CONTRIBUTIONS

DE and AE are responsible for the design of the manuscript. All authors have contributed in the writing and critical review of the manuscript.

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SUPPLEMENTARY MATERIAL

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Increased Ratio of Global *O*-GlcNAcylation to Tau Phosphorylation at Thr212 Site Is Associated With Better Memory Function in Patients With Type 2 Diabetes

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Objective: Aberrant *O*-GlcNAc modification has been implicated in type 2 diabetes mellitus (T2DM) and the pathogenesis of neurodegenerative diseases via competition with tau phosphorylation. We aimed to investigate the association between global *O*-GlcNAcylation, tau phosphorylation levels and mild cognitive impairment (MCI) in the whole blood of patients with T2DM.

Methods: Sociodemographic, clinical characteristics and cognitive performances of the enrolled T2DM subjects were extensively assessed. Global *O*-GlcNAcylation and tau phosphorylation levels in the whole blood were also determined using Western blot.

Results: Forty-eight T2DM subjects, including 24 with MCI and 24 with normal cognition, were enrolled in this study. Compared with cognitively normal controls, T2DM with MCI subjects displayed decreased global *O*-GlcNAcylation level, but increased tau phosphorylation levels (all $p < 0.05$). To reflect the combined effect, the ratios of global *O*-GlcNAcylation to tau phosphorylation levels, including specific sites, such as Ser396, Ser404, Thr212, and Thr231, were all significantly decreased in MCI subjects (all $p < 0.05$). Further multivariable logistic regression analysis revealed that high glycated hemoglobin A1c was an independent risk factor, whereas increased *O*-GlcNAc/p-T212 was an independent protective factor for MCI in patients with T2DM (odds ratio [OR] = 2.452, 95% confidence interval [CI] 1.061–5.668, $p = 0.036$; OR = 0.028, 95%CI 0.002–0.388, $p = 0.008$, respectively). With regard to each cognitive domain, *O*-GlcNAc/p-T212 was positively correlated with the score of Auditory Verbal Learning Test-delayed recall ($r = 0.377$, $p = 0.010$).

Conclusion: Our study suggests that increased ratio of global *O*-GlcNAcylation to tau phosphorylation at Thr212 site in the whole blood is associated with decreased risk of MCI, especially with better memory function in T2DM subjects.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier ChiCTR-OCC-15006060.

Keywords: tau protein, *O*-GlcNAcylation, phosphorylation, mild cognitive impairment, type 2 diabetes mellitus

INTRODUCTION

Current estimates indicated that 415 million adults are diagnosed with diabetes worldwide in 2015 (Ogurtsova et al., 2017). Among these adults, 90% manifested type 2 diabetes mellitus (T2DM). T2DM is a multifactorial metabolic disorder that can cause several acute and chronic complications, mainly including diabetic ketoacidosis, diabetic hyperosmotic coma, cardiovascular and cerebrovascular diseases, and microvascular diseases (Zheng et al., 2018). Previous studies suggested that T2DM is an independent risk factor for mild cognitive impairment (MCI) and dementia (Cheng et al., 2012; Gao et al., 2016), and also progression from MCI to dementia (Li et al., 2016; Ciudin et al., 2017). Moreover, the annual conversion rate of MCI to Alzheimer's disease (AD) is 10–15%, and 80% of MCI patients are converted to AD within 6 years, which will cause huge family and social burdens (Tiermey et al., 1996). Therefore, it is urgent to provide early prevention and intervention of MCI in patients of T2DM. The underlying mechanisms of T2DM-related cognitive dysfunction are complex and include chronic hyperglycemia (Kim D. J. et al., 2016), recurrent hypoglycemia (Sheen and Sheu, 2016; Mehta et al., 2017), insulin resistance (IR) (Ma et al., 2015), β-amyloid aggregation, and tau protein hyperphosphorylation (Baglietto-Vargas et al., 2016; Mittal and Katare, 2016). However, the exact mechanisms require further studies.

O-*N*-acetylglucosaminylation (*O*-GlcNAcylation) is a ubiquitous and dynamic posttranslational modification regulated by only two known enzymes, namely, *O*-linked β-*N*-acetylglucosamine transferase (OGT) and *O*-linked β-*N*-acetylglucosaminidase (*O*-GlcNAcase, OGA) (Yang and Qian, 2017). OGT transfers *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) to the hydroxyl group of serine (Ser) or threonine (Thr) residues of certain cytoplasmic, nuclear and mitochondrial proteins, whereas OGA removes it from proteins. To date, thousands of *O*-GlcNAc-modified proteins reportedly regulate many cellular pathways, such as epigenetics, gene expression, translation, protein degradation, signal transduction, mitochondrial bioenergetics, cell cycle, and protein localization (Zachara et al., 2015). *O*-GlcNAc pathway utilizes uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) from the hexosamine biosynthetic pathway. Thus, previous studies linked it to circulating glucose levels. For example, aberrant protein *O*-GlcNAc modification has been associated with hyperglycemia and IR (Park et al., 2010; Myslicki et al., 2014). Moreover, increased expression of *O*-GlcNAcylation in erythrocyte or leukocyte proteins (particularly granulocyte) is considered as a unique marker for early and efficient detection of T2DM (Wang et al., 2009; Springhorn et al., 2012). In addition, *O*-GlcNAcylation has been also implicated in the pathogenesis of diabetic complications, such as but not limited to retinopathy, nephropathy, and vascular disease (Peterson and Hart, 2016).

O-GlcNAcylation and phosphorylation modify Ser and/or Thr side chains of substrate proteins. Consequently, *O*-GlcNAcylation can compete with phosphorylation at certain sites of various proteins, including microtubule-associated protein tau (Hart et al., 2011). Studies demonstrated that the formation of neurofibrillary tangles, in which the major components are abnormally hyperphosphorylation tau proteins, is one of the defining pathological features of AD (Grundke-Iqbal et al., 1986). As a consequence, aberrant tau *O*-GlcNAcylation has been implicated in the pathogenesis of Alzheimer and neurodegenerative diseases. In human postmortem brain tissues, tau protein was demonstrated to be modified by *O*-GlcNAcylation, and protein *O*-GlcNAcylation level in AD brain was lower than that in healthy controls (Liu F. et al., 2004). Evidence from an animal study indicated an imbalance between tau phosphorylation and *O*-GlcNAcylation in the hippocampus of 3xTg-AD mice (Gatta et al., 2016). Intervention studies also suggested that increased *O*-GlcNAcylation may prevent (β-amyloid plaque formation and pathological tau aggregation, thereby rescuing cognitive impairment in transgenic mouse models (Kim et al., 2013; Graham et al., 2014; Yuzwa et al., 2014).

However, knowledge is lacking on the relationships among O-GlcNAcylation, tau phosphorylation level and cognitive functions in peripheral blood samples from patients with T2DM. Given the researches that suggested a link of O-GlcNAcylation with phosphorylation modification and cognition, as well as IR and diabetes, we hypothesized that the imbalance between O-GlcNAcylation and tau phosphorylation may be involved in diabetes-associated cognitive decline. Herein, we aimed to investigate the association among global O-GlcNAcylation, tau phosphorylation levels, MCI, and the different cognitive domain performances in the whole blood of patients with T2DM.

MATERIALS AND METHODS

Ethics Statement

This study was conducted in the Department of Endocrinology, Affiliated Zhongda Hospital of Southeast University. The study protocol and informed consent documents were approved by the Research Ethics Committee of the Affiliated Zhongda Hospital of Southeast University. All enrolled subjects were informed about the study and gave written consent.

Subject Recruitment

Subjects were recruited from the Department of Endocrinology, Affiliated Zhongda Hospital of Southeast University from January 2017 to October 2017. All patients with T2DM were diagnosed according to the World Health Organization (1999) criteria (Alberti and Zimmet, 1998). They were aged between 45 and 75 years with a history of diabetes $>$ 3 years. Patients with any of the following criteria were excluded: (1) presence of acute diabetic complications, including diabetic ketoacidosis, hyperosmolar non-ketotic diabetic coma, diabetic lacatocidosis, and severe hypoglycemia; (2) presence of acute cardiovascular or cerebrovascular accident, such as acute myocardial infarction, and acute cerebral hemorrhage; (3) any known history of central nervous system diseases that could affect cognition, including stroke (Hachinski ischemic score [HIS] $\geq$ 4), head trauma, epilepsy, major anxiety and depression; (4) history of alcoholism or drug abuse within 2 months; (5) the presence

of thyroid dysfunction or other severe medical illness, such as cancer, anemia and serious infection; and (6) history of severe visual or hearing loss. MCI was diagnosed based on criteria established by the National Institute on Aging-Alzheimer's Association workgroups (Albert et al., 2011). The criteria included the following: (1) a reported decline in cognitive function (self/informant/clinician report); (2) objective evidence of impairment in one or more cognitive domains, which were assessed by the Montreal Cognitive Assessment (MoCA) in this study; (3) preservation of normal activities of daily living (ADL), measured by ADL questionnaire; and (4) the absence of dementia (based on the Diagnostic and Statistical Manual of Mental Disorders-IV criteria).

Sociodemographic and Clinical Characteristic Collection

Standard sociodemographic and clinical characteristics of the enrolled subjects were collected at study entry, including age, gender, educational level, weight, height, blood pressure (BP), and diabetes duration. Body mass index (BMI) was calculated as body weight (kg) divided by the square of body height (m^2). Hypertension was defined as BP $\geq$ 140/90 mmHg and/or use of antihypertensive medication. Lifestyle risk factors such as smoking and alcohol history were also included. The laboratory data comprised fasting blood glucose (FBG), fasting C-peptide (FCP), glycated hemoglobin A1c (HbA1c), triglycerides, total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, serum creatinine (SCr), blood urea nitrogen (BUN), and uric acid (UA). IR was evaluated according to the modified homeostatic model assessment of insulin resistance (HOMA-IR) formula based on FCP: FBG (mmol/L) $\times$ FCP (ng/mL)/22.5 (Kim J. D. et al., 2016). Data on antidiabetic drug usage, including metformin and insulin, were also collected in this study.

Cognitive Performance Assessment

Cognitive performances were assessed through a battery of neuropsychological scales designed to evaluate the performance in approximately 60 min across an array of cognitive domains including memory, executive function, visual spatial processing, attention, and information processing speed. The MoCA test was used to assess overall cognitive function ranging from 0 to 30 score and also regarded as a highly sensitive screening tool of MCI in patients with T2DM (Alagiakrishnan et al., 2013). The normal MoCA score was $\geq$26, with one point added if the subject had less than 12 years of formal education (Nasreddine et al., 2005). Other tests included Digit Span Test, Verbal Fluency Test, Clock Drawing Test (CDT), Trail Making Test-A and B, and Auditory Verbal Learning Test (AVLT). HIS, clinical dementia rating (CDR), and self-rating depression scale were also obtained. All neuropsychological tests were performed by a skilled neuropsychiatrist from the Department of Neurology, Affiliated Zhongda Hospital of Southeast University, and all the subjects were blinded to the study design.

Western Blot

Whole blood samples were collected from the enrolled subjects by venipuncture in anticoagulant-free tubes, and then immediately stored at −80°C. In preparation for Western blot, blood samples were homogenized in a lysis buffer, which consists of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and various protease and phosphatase inhibitors, including phenylmethanesulfonyl fluoride, sodium pyrophosphate, and β-glycerophosphate. The samples were then centrifuged at 12,000 *g* at 4°C for 10 min, and the lysate was recovered and diluted 20 times. Protein concentrations were determined by the bicinchoninic acid assay according to the manufacturer's protocol (NanJing KeyGen Biotech Co., Ltd.). 20 μg total protein was resolved on SDS-polyacrylamide gel electrophoresis gels and blotted to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were then blocked by 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h, and incubated overnight at 4°C with appropriate primary antibodies. Primary antibodies included anti-*O*-linked *N*-acetylglucosamine [RL2] (1:1000; Abcam, ab2739), anti-OGT/*O*-linked *N*-acetylglucosamine transferase (1:5000; Abcam, ab177941), anti-MGEA5 (1:5000; Abcam, ab124807), anti-Tau [Tau-5] (1:800; Abcam, ab80579), anti-Tau (phosphor S396) (1:10000; Abcam, ab109390), anti-Tau (phosphor S404) (1:1000; Abcam, ab92676), anti-Tau (phosphor T212) (1:1000; Abcam, ab4842), and anti-Tau (phosphor T231) (1:5000; Abcam, ab151559) with glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (1:3000; CMCTAG, AT0002) serving as the control. Secondary antibodies were as follows: goat anti-mouse IgG horseradish peroxidase (HRP) (1:50000; MyBioScience, MKA001A) and goat anti-rabbit IgG HRP (1:25000; MyBioScience, MKA003A). Signals were detected with chemiluminescence (Millipore, WBKLS0500) and then exposed using MiniChemi^{TM}610 Imaging and Analysis System (Beijing Sage Creation Science Co., Ltd.).

Statistical Analyses

Densitometry data of global *O*-GlcNAc, OGT, OGA, and tau phosphorylation levels were analyzed by the Image J program. Data are presented as mean ± standard deviation/standard error of mean (SEM), median (interquartile range) or *n* (percentage). Student's *t*-test, non-parametric Mann–Whitney *U* test or Chi-square test was used to determine differences of clinical parameters, global *O*-GlcNAcylation level, its key enzyme levels, and tau phosphorylation levels, as well as ratios of global *O*-GlcNAcylation to tau phosphorylation levels between T2DM with MCI patients and those with normal cognition subjects. A simple logistic regression model was first used to explore so-called independent risk or protective factors for MCI in patients with MCI. Then, forward stepwise multivariable regression analysis was used to explore the "strongest" factors affecting the MCI presence. Spearman correlation analyses were performed to explore the relationships of the ratio of global *O*-GlcNAcylation to tau phosphorylation levels with other sociodemographic and clinical

characteristics. Furthermore, we conducted partial correlation analyses to assess the relationships between the ratio of global *O*-GlcNAcylation to tau phosphorylation levels and different cognitive domain performances. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, United States), and p-value < 0.05 (two-tailed) was considered statistically significant.

RESULTS

Sociodemographic, Clinical Characteristics and Cognitive Performances

This study enrolled a total of 48 T2DM subjects, including 24 persons with MCI and 24 cognitively normal controls. The detailed sociodemographic, clinical characteristics and cognitive performances of the participants are summarized in **Table 1**. The MCI and control group were well matched in age, gender and education levels, as well as the prevalence of smoking, alcohol, hypertension and metformin and insulin usage (all $p > 0.050$). Compared with cognitively normal controls, T2DM with MCI subjects displayed elevated HbA1c and FBG, while decreased FCP and UA (all $p < 0.05$). No significant differences were observed including BMI, diabetes duration, HOMA-IR (FCP), blood lipids, SCr and BUN (all $p > 0.05$). Moreover, T2DM with MCI patients showed significantly poorer overall and different domains of cognitive performances than control subjects (all $p < 0.05$, except the p-value for CDT).

TABLE 1 | Sociodemographic, clinical characteristics and cognitive performances.

Characteristic	MCI group (n = 24)	Control group (n = 24)	p-value
Age (years)	60.67 ± 6.92	61.25 ± 6.77	0.769[a]
Female, *n* (%)	14 (58.33)	9 (37.50)	0.149[c]
Education Levels (years)	9.50 (9.00–12.00)	11.50 (8.25–12.00)	0.800[b]
Smoking, *n* (%)	11 (45.83)	9 (37.50)	0.558[c]
Alcohol, *n* (%)	8 (33.33)	5 (20.83)	0.330[c]
BMI (kg/m^2)	25.19 ± 3.45	26.01 ± 3.36	0.409[a]
Hypertension, *n* (%)	17 (70.83)	12 (50.00)	0.140[c]
Diabetes duration (years)	10.00 (8.25–15.75)	10.00 (7.00–13.00)	0.367[b]
Metformin usage, *n* (%)	21 (87.50)	16 (66.67)	0.086[c]
Insulin usage, *n* (%)	17 (70.83)	13 (54.17)	0.233[c]
HbA1c (%)	9.25 ± 1.27	7.95 ± 0.90	<0.001[a]
FBG (mmol/L)	9.68 ± 2.11	7.83 ± 1.62	0.001[a]
FCP (ng/mL)	0.46 (0.33–0.55)	0.58 (0.43–0.83)	0.021[b]
HOMA-IR (FCP)	0.20 (0.13–0.27)	0.21 (0.15–0.28)	0.353[b]
TG (mmol/L)	1.62 (0.89–2.33)	1.34 (0.97–1.98)	0.688[b]
TC (mmol/L)	4.87 ± 1.03	4.38 ± 1.24	0.149[a]
HDL (mmol/L)	1.22 ± 0.27	1.09 ± 0.27	0.102[a]
LDL (mmol/L)	3.04 ± 0.93	2.70 ± 0.88	0.206[a]
SCr (μmol/L)	68.96 ± 19.60	70.28 ± 20.09	0.819[a]
BUN (mmol/L)	5.70 ± 1.15	5.95 ± 1.26	0.469[a]
UA (μmol/L)	282.33 ± 65.91	336.92 ± 98.24	0.029[a]
Neuropsychological tests			
MoCA	24.00 (22.00–25.00)	28.00 (27.00–29.00)	<0.001[b]
DST	11.00 (10.00–11.75)	12.00 (11.00–13.00)	0.023[b]
VFT	14.50 (13.00–18.00)	18.00 (15.25–20.00)	0.012[b]
CDT	4.00 (3.00–4.00)	4.00 (3.00–4.00)	0.596[b]
TMT-A	68.50 (56.50–87.50)	55.00 (45.50–71.00)	0.020[b]
TMT-B	171.50 (116.50–215.00)	123.00 (90.25–159.75)	0.042[b]
AVLT-immediate recall	16.00 (14.00–18.75)	19.00 (15.25–23.50)	0.041[b]
AVLT-delayed recall	5.00 (3.00–6.00)	6.00 (5.00–8.75)	0.016[b]

Data are presented as n (%), mean ± standard deviation, or median (interquartile range) as appropriate.

[a]Student's t-test for comparison of normally distributed quantitative variables between MCI group and control group.

[b]Mann–Whitney U test for comparison of asymmetrically distributed quantitative variables between MCI group and control group.

[c]χ^2 test for comparison of qualitative variables between MCI group and control group.

MCI, mild cognitive impairment; BMI, body mass index; HbA1c, glycosylated hemoglobin; FBG, fasting blood glucose; FCP, fasting C-peptide; HOMA-IR(FCP): replacing fasting insulin with FCP in the homeostasis model assessment of insulin resistance formula; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SCr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid; MoCA, Montreal Cognitive Assessment; DST, Digit Span Test; VFT, Verbal Fluency Test; CDT, Clock Drawing Test; TMT, Trail Making Test; AVLT, Auditory Verbal Learning Test.

Global *O*-GlcNAcylation and Tau Phosphorylation Levels Between the MCI and Control Subjects

Global *O*-GlcNAcylation and tau phosphorylation levels were determined by western blot and analyzed by densitometry (**Figure 1**). Compared to T2DM with cognitively normal controls, global *O*-GlcNAcylation level was lower in MCI subjects ($p = 0.012$) (**Figures 1A,B**). Moreover, there was a significant decrease in OGT expression, but an increase in OGA expression in the MCI group (both $p < 0.05$) (**Figures 1A,B**). Results also showed that the decrease in global *O*-GlcNAcylation level was accompanied by an increase in total tau level, as well as hyperphosphorylation of tau protein at specific sites including Ser396, Ser404, Thr212 and Thr231 (all $p < 0.05$) (**Figures 1C,D**). In order to reflect the combined effect and to magnify the effect, we performed relative ratios of global *O*-GlcNAcylation to tau phosphorylation levels, and found that *O*-GlcNAc/Tau-5, *O*-GlcNAc/p-S396, *O*-GlcNAc/p-S404, *O*-GlcNAc/p-T212, and *O*-GlcNAc/p-T231 were all decreased in T2DM with MCI subjects in comparison to control subjects (all $p < 0.05$) (**Figure 2**).

Exploration of Risk Factors for MCI in T2DM Patients

To explore risk factors for MCI in T2DM patients, we first conducted a simple logistic regression analysis via entering all sociodemographic and clinical characteristics. The results showed that T2DM subjects with higher HbA1c and FBG were associated with increased risk of MCI, while increased FCP, UA, *O*-GlcNAc/Tau-5, *O*-GlcNAc/p-S396, *O*-GlcNAc/p-S404, *O*-GlcNAc/p-T212, and *O*-GlcNAc/p-T231 were associated with decreased risk of MCI (all $p < 0.05$) (**Table 2**). Further forward stepwise multivariable logistic regression analysis revealed that high HbA1c was an independent risk factor for MCI, while increased *O*-GlcNAc/p-T212 was an independent protective factor for MCI in T2DM patients (OR = 2.452, 95%CI 1.061–5.668, $p = 0.036$; OR = 0.028, 95%CI 0.002–0.388, $p = 0.008$, respectively).

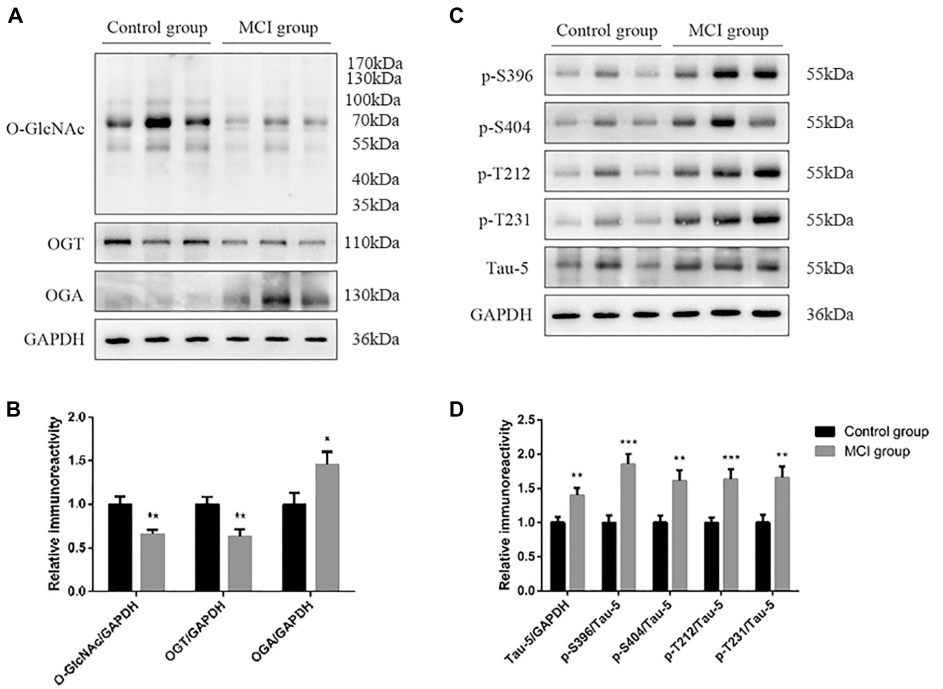

FIGURE 1 | Global *O*-GlcNAcylation and tau phosphorylation levels in the whole blood of T2DM subjects. **(A)** Representative Western blot analysis showing that global *O*-GlcNAcylation and OGT expression are decreased, while OGA expression are increased in T2DM with MCI compared to those with normal cognition. **(B)** Quantitation of Western blot analysis **(A)**. **(C)** Representative Western blot analysis showing that total tau level, as well as phosphorylation of tau protein at specific sites including Ser396, Ser404, Thr212, and Thr231 are increased in T2DM with MCI compared to those with normal cognition. **(D)** Quantitation of Western blot analysis **(C)**. All data represents $n = 48$, and are means ± standard error of mean. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Relationships of *O*-GlcNAc/p-T212 With Different Cognitive Domain Performances in T2DM Patients

The Spearman correlation analyses revealed that *O*-GlcNAc/p-T212 was negatively associated with HbA1c and FBG ($r = -0.346$, $p = 0.016$; $r = -0.329$, $p = 0.023$, respectively). No significant associations were found between *O*-GlcNAc/p-T212 and other sociodemographic and clinical characteristics (all $p > 0.05$). Further partial correlation analyses showed that *O*-GlcNAc/p-T212 was positively associated with MoCA after adjustment for HbA1c and FBG ($r = 0.397$, $p = 0.006$). With regard to each cognitive domain, *O*-GlcNAc/p-T212 was positively correlated with the score of AVLT-delayed recall, which represent delayed verbal learning and memory functions ($r = 0.377$, $p = 0.010$) (**Table 3**).

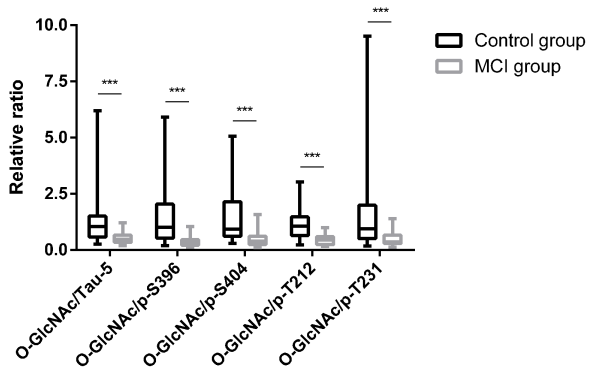

FIGURE 2 | Relative ratio of global *O*-GlcNAcylation to tau phosphorylation levels in T2DM subjects. *O*-GlcNAc/Tau-5, *O*-GlcNAc/p-S396, *O*-GlcNAc/p-S404, *O*-GlcNAc/p-T212, and *O*-GlcNAc/p-T231 were all decreased in T2DM with MCI subjects in comparison to control subjects. All data represents $n = 48$, and are median (interquartile range). $^{***}p < 0.001$.

TABLE 2 | Exploration of risk factors for MCI in T2DM patients.

	β	SE of β	*p*-value	OR	95%CI
HbA1c (%)	1.106	0.358	0.002	3.023	1.500–6.092
FBG (mmol/L)	0.568	0.205	0.006	1.765	1.181–2.637
FCP (ng/mL)	−2.714	1.280	0.034	0.066	0.005–0.814
UA (umol/L)	−0.010	0.005	0.042	0.990	0.981–1.000
O-GlcNAc/Tau-5	−3.325	1.100	0.003	0.036	0.004–0.311
O-GlcNAc/p-S396	−3.752	1.260	0.003	0.023	0.002–0.277
O-GlcNAc/p-S404	−2.173	0.877	0.013	0.114	0.020–0.635
O-GlcNAc/p-T212	−4.209	1.301	0.001	0.015	0.001–0.190
O-GlcNAc/p-T231	−2.103	0.813	0.010	0.122	0.025–0.601

MCI, mild cognitive impairment; β, regression coefficient; SE, standard error; OR, odds ratio; CI, confidence interval for odds ratio; HbA1c, glycosylated hemoglobin; FBG, fasting blood glucose; FCP, fasting C-peptide; UA, uric acid.

TABLE 3 | Relationships of *O*-GlcNAc/p-T212 with other clinical characteristics and different cognitive domains performances in T2DM patients.

	***O*-GlcNAc/p-T212**	
	r	***p*-value**
Age (years)	0.077	0.604[a]
Education levels (years)	−0.019	0.897[a]
BMI (kg/m^2)	0.239	0.102[a]
Diabetes duration (years)	−0.065	0.659[a]
HbA1c (%)	−0.346	0.016[a]
FBG (mmol/L)	−0.329	0.023[a]
FCP (ng/mL)	0.127	0.389[a]
HOMA-IR (FCP)	−0.050	0.734[a]
TG (mmol/L)	0.112	0.450[a]
TC (mmol/L)	−0.143	0.334[a]
HDL (mmol/L)	−0.245	0.094[a]
LDL (mmol/L)	−0.194	0.187[a]
SCr (μmol/L)	0.265	0.069[a]
BUN (mmol/L)	−0.062	0.674[a]
UA (μmol/L)	0.185	0.207[a]
MoCA	0.397	0.006[b]
DST	0.038	0.803[b]
VFT	0.197	0.189[b]
CDT	0.104	0.491[b]
TMT-A	0.053	0.725[b]
TMT-B	0.053	0.729[b]
AVLT-immediate recall	0.215	0.152[b]
AVLT-delayed recall	0.377	0.010[b]

[a] *Spearman correlation.*
[b] *Partial correlation after adjustment for HbA1c and FBG.*
MCI, mild cognitive impairment; BMI, body mass index; HbA1c, glycosylated hemoglobin; FBG, fasting blood glucose; FCP, fasting C-peptide; HOMA-IR(FCP): replacing fasting insulin with FCP in the homeostasis model assessment of insulin resistance formula; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SCr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid; MoCA, Montreal Cognitive Assessment; DST, Digit Span Test; VFT, Verbal Fluency Test; CDT, Clock Drawing Test; TMT, Trail Making Test; AVLT, Auditory Verbal Learning Test.

DISCUSSION

Several key findings were obtained from this case-control study which assessed the relationships among global *O*-GlcNAcylation, tau phosphorylation levels and MCI in T2DM subjects. (1) Global *O*-GlcNAcylation level was significantly decreased, whereas tau phosphorylation levels were increased in T2DM with MCI subjects compared with those with normal cognition. (2) High HbA1c was an independent risk factor for MCI, whereas increased *O*-GlcNAc/p-T212 was an independent protective factor for MCI in patients with T2DM; (3) *O*-GlcNAc/p-T212 was positively associated with overall cognitive function, especially with delayed learning and memory functions.

In the current study, we first performed a correlation study between global *O*-GlcNAcylation level, tau phosphorylation levels and cognitive functions in the whole blood of patients of T2DM and observed a decreased global *O*-GlcNAcylation level but increased tau phosphorylation levels in T2DM with MCI subjects. These findings were consistent with those obtained in human postmortem brain tissues, which demonstrated that protein *O*-GlcNAcylation level in AD brain was lower than that in controls (Liu F. et al., 2004). Previous study also revealed an imbalance between tau *O*-GlcNAcylation and phosphorylation in the hippocampus of a mouse model of AD (Gatta et al., 2016). In addition, *O*-GlcNAcylation elevation via the OGA inhibitor can lead to a significant reduction of pathological tau and then provides protection against neuron loss in animal studies (Yuzwa et al., 2012; Graham et al., 2014; Hastings et al., 2017). Furthermore, in accordance with our study, plasma tau levels were found higher in MCI subjects compared with cognitively normal controls in the population-based Mayo Clinic Study of Aging (Dage et al., 2016). However, our data were contradictory with the results of several studies performed on patients with AD and MCI. The plasma levels of total tau decreased among subjects with MCI and AD compared with cognitively normal controls (Sparks et al., 2012). In another study exploring the utility of plasma tau as diagnostic markers for MCI and AD, Zetterberg et al. reported that plasma tau levels were significantly elevated in AD but not in MCI compared with CN subjects (Zetterberg et al., 2013). The inconsistency in these findings may be attributed to differences in disease populations (T2DM with MCI or MCI), sample source (whole blood or plasma) and detection method of tau protein (Western blot or enzyme-linked immunosorbent assay).

High HbA1c was an independent risk factor for MCI in our T2DM subjects, which was consistent with the result reported in the action to control cardiovascular risk in diabetes-memory in diabetes (ACCORD-MIND) trial. The study indicated that higher HbA1c levels were associated with lower cognitive function in individuals with diabetes (Tali et al., 2009). When adjusted for age, sex and education, a 1% higher HbA1c level was associated with a 0.09-point lower MoCA score in non-demented elderly patients with type 2 diabetes (Huang et al., 2015). In patients with newly diagnosed T2DM, higher HbA1c was also associated with worse cognitive performances assessed by the modified 13-item version of the telephone interview for cognitive status (TICS-M) (Moulton et al., 2016). On the contrary, increased *O*-GlcNAc/p-T212 was an independent protective factor for MCI in patients with T2DM. Furthermore, *O*-GlcNAc/p-T212 was positively associated with overall cognitive performances, especially with learning and memory functions. The exact mechanisms for these relationships are not fully understood. One possible reason is that *O*-GlcNAcylation level was shown to correlate negatively with tau phosphorylation levels, which are a primary regulator of neuronal functions, including regulating long-term synaptic plasticity and learning and memory (Anggono and Huganir, 2012; Luscher and Malenka, 2012). With regard to specific phosphorylation sites of tau protein, Alonso et al. (2010) suggested that a single phosphorylation site alone had little influence on the biological activity of tau protein, while phosphorylation at Thr212 along with a modification on the C-terminal of the protein could facilitate tau aggregation. In addition, *O*-GlcNAc modification is highly abundant in the mammalian brain, especially with markedly

dense expression in the hippocampus (Liu K. et al., 2004), whereas the hippocampus is mainly responsible for learning and memory function in rodents (Lazarov and Hollands, 2016). Moreover, *O*-GlcNAcylation has been linked to regulate protein homeostasis, which is essential to maintain synaptic contacts and memory (Akan et al., 2018). Xie et al. (2016) also reported that *O*-GlcNAcylation downregulation suppresses protein kinase A (PKA)-cAMP-response element binding protein signaling and consequently causes learning and memory deficits in AD. By contrast, increasing the levels of *O*-GlcNAcylation by caloric restriction can lessen learning impairment associated with diabetes (Jeon et al., 2016). Therefore, all the above explanations supported our finding that increased *O*-GlcNAc/p-T212 was a protective factor and associated with lower MCI incidence in T2DM subjects.

Our study was the first to investigate the association among *O*-GlcNAcylation level, tau phosphorylation levels, and cognitive performances in T2DM patients with MCI. Moreover, the samples used in our study were peripheral blood cells other than cerebrospinal fluid (CSF) or brain tissues, which were easier to obtain and generalize to the clinic. However, certain limitations should be noted in this study. First, due to the lack of specific *O*-GlcNAcylation antibody for tau protein, we couldn't obtain tau *O*-GlcNAcylation level. We are also unable to obtain the level of UDP-GlcNAc because of the difficulties of detection methods (High-performance liquid chromatography/Quadrupole-Time of flight-Mass spectrometry) (Oikari et al., 2018), which could directly reflect the associations among blood glucose, key enzymes and tau *O*-GlcNAcylation level. Moreover, the relatively small sample size and sample composition of this study limited the interpretation of our results to a certain degree. In addition, this is a case-control study, and findings derived from this work cannot elucidate the direction of the relationship and it is not possible to determine the causality.

CONCLUSION

Our study suggests that increased ratio of global *O*-GlcNAcylation to tau phosphorylation at Thr212 site in the whole blood is associated with decreased risk of MCI, especially with better learning and memory function in T2DM subjects. Further prospective studies with a substantial sample size should be conducted to validate these observations.

AUTHOR CONTRIBUTIONS

SW and RH contributed to study conception and design. RH, ST, RC, HL, DG, and JW acquired the data. RH and ST performed the analyses. RH wrote the first draft. SW, JH, and ST revised it critically for important intellectual content. All authors approved the final version to be published.

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Efficacy of Co-administration of Liuwei Dihuang Pills and Ginkgo Biloba Tablets on Albuminuria in Type 2 Diabetes: A 24-Month, Multicenter, Double-Blind, Placebo-Controlled, Randomized Clinical Trial

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Purpose: We investigated the effects of Traditional Chinese Medicine (TCM) on the occurrence and progression of albuminuria in patients with type 2 diabetes.

Methods: In this randomized, double-blind, multicenter, controlled trial, we enrolled 600 type 2 diabetes without diabetic nephropathy (DN) or with early-stage DN. Patients were randomly assigned (1:1) to receive Liuwei Dihuang Pills (LWDH) (1.5 g daily) and Ginkgo biloba Tablets (24 mg daily) orally or matching placebos for 24 months. The primary endpoint was the change in urinary albumin/creatinine ratio (UACR) from baseline to 24 months.

Results: There were 431 patients having UACR data at baseline and 24 months following-up in both groups. Changes of UACR from baseline to follow-up were not affected in both groups: −1.61(−10.24, 7.17) mg/g in the TCM group and −0.73(−7.47, 6.75) mg/g in the control group. For patients with UACR ≥30 mg/g at baseline, LWDH and Ginkgo biloba significantly reduced the UACR value at 24 months [46.21(34.96, 58.96) vs. 20.78(9.62, 38.85), $P < 0.05$]. Moreover, the change of UACR from baseline to follow-up in the TCM group was significant higher than that in the control group [−25.50(−42.30, −9.56] vs. −20.61(−36.79, 4.31), $P < 0.05$].

Conclusion: LWDH and Ginkgo biloba may attenuate deterioration of albuminuria in type 2 diabetes patients. These results suggest that TCM is a promising option of renoprotective agents for early stage of DN.

Trial registration: The study was registered in the Chinese Clinical Trial Registry. (no. ChiCTR-TRC-07000037, chictr.org)

Keywords: diabetic nephropathy, urinary albumin, creatinine ratio, Liuwei Dihuang, Ginkgo biloba

INTRODUCTION

Diabetic nephropathy (DN) is one of the most common causes of end-stage renal disease (ESRD) world widely, accounting for considerable morbidity and mortality in patients with both type 1 and type 2 diabetes mellitus (DM) (1, 2). Estimated GFR and albuminuria are independent risk factors which associate with progression to ESRD strongly (3). The common pathologic feature of early DN is the presence of glomerular hypertrophy, with mesangial expansion and glomerular basement membrane thickening, which cause excessive glomerular filtration and increased urinary albumin excretion. Emerging evidence shows that both renal impairment and cardiovascular events occur in patients with developed micro-albuminuria earlier for diabetes, suggesting fundamental importance to the management of microalbuminuria.

The progression of albuminuria is associated with glomerular hypertension, inflammation, and oxidative stress. The present management for microalbuminuria includes strict control of blood glucose and blood pressure, with the ACE inhibitors (ACEI) or angiotensin receptor blockers (ARB). These therapies, however, are frequently unsatisfactory, probably reflecting inadequate targeting on the pathophysiology (4). For example, the use of ACEI/ARB failed to stop the progression of nephropathy to ESRD in type 2 patients (5). The albuminuria was demonstrated to have positive correlation with renal risk in patients with type 2 diabetic nephropathy (DN) (6). Albuminuria also has been demonstrated a direct toxic effect on renal tissue, leading to progressive renal damage (7). Previous clinical trial shows that initial reduction of albuminuria can reduce the risk of ESRD (8).

In China, Traditional Chinese Medicine (TCM) has been widely used as complementary therapy for DN, including Lumbrokinase (9), Xue Shuan Tong (10), Berberine (11), huangkui (12), Salvia miltiorrhiza (13), Liuwei Dihuang (LWDH), and Ginkgo biloba. The latter two have been proposed for the prevention and treatment of DN in China and other Asian regions recently (14–17). LWDH is a classic TCM, which is reported to ameliorate oxidation and improve insulin sensitivity as well as glucose tolerance (18, 19). Ginkgo biloba contains ∼24% flavone glycosides (primarily quercetin, kaempferol, and isorhamnetin) and 6% terpene lactones in weight, with a long history in the use for a variety range of conditions in China. Administration of Ginkgo biloba is associated with blood vessel dilation, reduction in blood viscosity and the density of oxygen free radicals, inhibition of platelet activating factor (20). Therefore, Ginkgo biloba may have renal protective effects in DN. In both animal models and patients with DN, Ginkgo biloba was shown to inhibit NF-κB, oxidation and accumulation of extracellular matrix (ECM) (21–23). However, whether combination of LWDH and Ginkgo biloba can prevent the progression of renal injury is unclear. This study was designed to evaluate co-administration of LWDH Pills and Ginkgo biloba Tablets on renoprotection for type 2 diabetes with normal urinary albumin excretion or microalbuminuria.

Abbreviations: TCM, Traditional Chinese Medicine.

MATERIALS AND METHODS

Subjects

This is a multicenter, randomized, double-blind, placebo-controlled trial. Briefly, patients were included if they had type 2 diabetes, urine albumin excretion (UAE) < 300 mg/d, were aged 30–70 years, provided written informed consent. Patients were excluded from participation for any of the following: (1) poor control of hypertension(>160/110 mmHg), (2) diagnosed macrovascular complications (such as myocardial infarction, stroke, transient ischemic attack, peripheral vascular disease), polyneuropathy, or proliferative retinopathy, (3) using ACEI, ARB, or statins within 6 months prior to the enrollment (in order to avoid potential effects on albumin excretion). All subjects gave written informed consent. The protocol was approved by the Human Research Ethics Committee of each study center, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

This clinical trial was compliant with the Consolidated Standards of Reporting Trials (CONSORT). Subjects were recruited and screened at 10 clinical diabetes centers according to the criteria above. After enrollment, subjects were randomized at 1:1 to receive 1.5 g LWDH Pills (8 pills/time; batch number Z41022128; Wanxi Pharmaceutical Co., Ltd.) and 24 mg Ginkgo biloba Tablets (2 tablets/time; batch number Z20027949; Yangtze River Pharmaceutical Co., Ltd.) (TCM group, $n = 300$) orally three times per day, or matching placebos (placebo group, $n = 300$) (**Figure 1**). Randomization was performed by an independent doctor in each clinical center with block randomization method. Patients, investigators, and the sponsor's clinical team were all blinded to treatment allocation. Subjects were followed up with clinic consultation for 2 years.

Primary End Point

The primary outcome variable was the change in urinary albumin/creatinine ratio (UACR) before and after treatment. On the first visit, each subject was fasted overnight (at least 8 h), and attended the clinical center at 08:00. An overnight first-void urine sample was collected from each patient to measure the UACR. Normal albuminuria was defined as an UACR < 30 mg/g. Patients were considered to have microalbuminuria if their UACR ranged in 30–299 mg/g. Macro albuminuria was defined as UACR ≥300 mg/g.

Other Outcomes

All blood samples were immediately obtained at 08:00 after overnight. Enzyme-linked immunosorbent assay was used to detect the high-sensitivity C-reactive protein (HS-CRP) (Lot 78034031, Bender Med Systems GmbH, Austria; minimum detection limit: 3 pg/ml; intra-assay CVs: 6.9%; inter-assay CVs: 13.1%), matrix metalloproteinase 2 (MMP2) (Lot 303216, R&D, USA; minimum detection limit: 0.047 ng/ml; intra-assay CVs: 5.6%; inter-assay CVs: 7.4%), soluble advanced glycation end products (sRAGE) (Lot 303510, R&D, USA; minimum detection limit: 4.12 pg/ml; intra-assay CVs: 5.7%; inter-assay CVs: 7.7%), and fractalkine (Lot 301156, R&D, USA; minimum detection limit: 0.018 ng/ml; intra-assay CVs: 2.6%; inter-assay

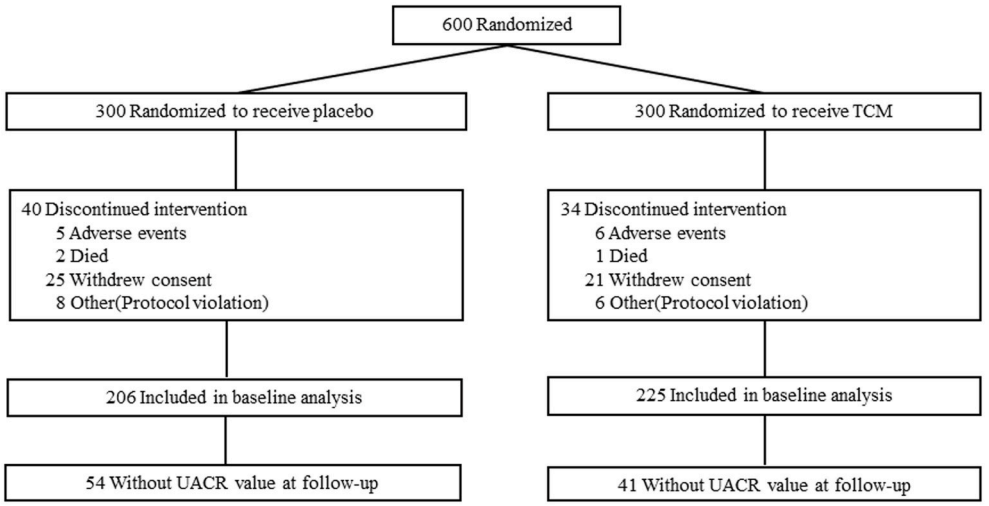

FIGURE 1 | Flow of participants in the trial.

CVs: 6.6%). The concentrations of serum AGE-peptides (AGE-P) were measured by flow injection assay (FIA) (24).

The subject was consumed a standardized breakfast (100 g steamed bread). Venous blood were sampled before and after breakfast, and fasting blood glucose (FBG), HbA1c, total cholesterol (TC), total triglyceride (TG), HDL, LDL, and postprandial blood glucose (PBG) were measured. The glomerular filtration rate (GFR) was estimated using the equation recommended by the National Kidney Foundation in the Modified Diet in Renal Disease (25).

Adverse Events

Adverse events included cancer, stroke, coronary artery disease, bleeding, and many transient minor complaints, such as dizziness, nausea, hypoglycemia, skin itching or headache. Participants were count only for once.

Statistical Analysis

Paired sample *T*-test is used for intragroup comparisons for clinical characteristics between the baseline and the end of follow-up. Independent samples *T*-test is used between the placebo and TCM group if the normal distribution is satisfied, otherwise, the non-parametric test is used. Family history of type 2 diabetes, history of retinopathy and the therapies between the two groups were compared using chi-squared test. Subgroup analysis was performed according to the baseline UACR ($\geq$30 or $<$30 mg/g). A probability of $p < 0.05$ was considered to be statistically significant. All analyses were performed using SPSS software (version 17.0; SPSS Inc). Data were presented as means $\pm$ SD or Median (lower quartile, upper quartile).

RESULTS

Basic Characteristics

Six hundred type 2 patients were enrolled, 74 of which were lost during the follow-up, i.e., 34 patients in the TCM group and 40 in the placebo group, and the reasons for these dropouts were reported in **Figure 1**. There was no difference in age, gender, duration of known diabetes, BMI, blood glucose, HbA1c, SBP, DBP, HDL, LDL, TC, TG, GFR, or the presence of microalbuminuria between the two groups at the baseline. Baseline clinical characteristics were well-balanced between the two groups during the 24 months treatment (**Table 1**).

Primary End Point

For all these subjects, 54 in the control group and 41 in the TCM group did not have UACR value at 24 months follow-up. There were no significant differences in UACR between the placebo ($n = 206$) and TCM group ($n = 225$) before and after treatment. The change of UACR in both groups was not affected. Patients were divided into subgroups according to the baseline values of UACR: UACR $<$ 30 mg/g, UACR $\geq$30 mg/g. For patients with UACR $\geq$30 mg/g at baseline, the reduction of UACR value between baseline and follow-up was much more obvious in the TCM group compared with that in the placebo group [$-$25.50($-$42.30, $-$9.56) vs. $-$20.61($-$36.79, 4.31), $P < 0.05$]. Moreover, UACR decreased significantly at 24 months compared with baseline in both placebo [44.79 (35.12, 65.11) vs. 32.19(12.32, 64.99), $P < 0.05$] and TCM groups [46.21(34.96, 58.96) vs. 20.78(9.62, 38.85), $P < 0.05$] (**Table 2**).

Onset and Remission Rate of Microalbuminuria and Macroalbuminuria

There was no significant difference in the occurrence of microalbuminuria at follow-up between the two groups for those with baseline normoalbuminuria ($P > 0.05$). Despite of the control of blood glucose, lipids and pressure, 2 patients progressed into macroalbuminuria in the placebo group (1.14%), without any occurrence among the patients treated with TCM. There was no significant reduction in the remission rate of microalbuminuria after 24 months in TCM treatment compared

TABLE 1 | The characteristics at baseline and 24 months treatment of placebo and TCM.

	Placebo group			TCMgroup		
	baseline	**end of follow-up**	***d***	**baseline**	**end of follow-up**	***d***
FBG (mmol/L)	7.45 ± 2.21	6.93 ± 1.58*	−0.51 ± 2.46	7.49 ± 2.31	6.85 ± 1.47*	−0.58 ± 2.31
PBG (mmol/L)	10.76 ± 3.83	9.73 ± 2.70*	−1.03 ± 4.37	11.00 ± 3.74	9.80 ± 2.98*	−1.13 ± 3.88
HbA1c (%)	7.28 ± 1.90	6.53 ± 1.21*	−0.66 ± 1.89	7.26 ± 1.81	6.66 ± 1.18*	−0.50 ± 1.79
SBP (mmHg)	125.62 ± 13.68	123.75 ± 11.28	−0.89 ± 14.38	125.87 ± 13.42	123.54 ± 11.67*	−2.21 ± 14.30
DBP (mmHg)	77.38 ± 8.99	75.48 ± 7.22*	−1.09 ± 8.31	77.81 ± 9.30	74.76 ± 7.37*	−2.03 ± 9.07
TC (mmol/L)	4.74 ± 0.97	4.73 ± 0.94	−0.11 ± 0.92	4.84 ± 0.90	4.94 ± 0.94*	−0.21 ± 0.91
TG (mmol/L)	1.54 ± 1.03	1.37 ± 0.83	−0.15 ± 1.29	1.56 ± 1.00	1.45 ± 1.19	0.05 ± 1.45
HDL (mmol/L)	1.26 ± 0.32	1.19 ± 0.28	−0.04 ± 0.34	1.29 ± 0.34	1.27 ± 0.37	−0.01 ± 0.26
LDL (mmol/L)	2.78 ± 0.71	2.74 ± 0.70	−0.00 ± 0.71	2.87 ± 0.78	2.76 ± 0.80	−0.08 ± 0.84
BMI (kg/m^2)	23.99 ± 2.73	24.33 ± 2.80	−1.51 ± 6.16	23.82 ± 2.76	23.98 ± 3.09*	−0.38 ± 4.03†
Age (years)	60.45 ± 6.19			60.81 ± 6.36		
Male sex, *n* (%)	151 (50.33)			146 (48.67)		
Diabetes duration (years)	5.30 ± 4.51			5.65 ± 5.15		
GFR (mL/min/1.73m^2)	86.52 ± 19.57			88.21 ± 19.98		
ACR>30 mg/g, *n* (%)	45 (17.31)			32 (12.03)		
Family history of T2DM, *n* (%)	105 (35)			109 (36.30)		
History of retinopathy disease, *n* (%)	61 (20.33)			68 (22.67)		
GLUCOSE-LOWERING THERAPIES, *N* (%)						
Diet only	40 (13.33)			38 (12.67)		
Sulfonylurea	120 (40)			102 (34)		
Alpha-glucosid ase inhibitor	46 (15.33)			41 (13.67)		
Glinides	18 (6)			26 (8.67)		
Insulin, insulin analog	80 (26.67)			83 (27.67)		
Metformin	115 (38.33)			104 (34.67)		
Thiazolidinedione	15 (5)			17 (5.67)		
ANTIHYPERTENSIVE THERAPIES, *N* (%)						
Calcium channel blockers	73 (24.33)			64 (21.33)		
Beta-blocker	15 (5)			10 (3.33)		
Diuretics	12 (4)			24 (8)		
Alpha-blocker	1 (0.33)			0		
Hydrochlorothiazide and irbesartan	0			1 (0.33)		
Vasodilator	3 (1)			2 (0.67)		

FBG, fasting blood glucose; PBG, postprandialblood glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; ACR, albumin/creatinine ratio.

d-value: difference between baseline and 24 months follow-up.

*Paired sample T-test was used for intragroup comparisons between the baseline and the end of follow-up, *$p < 0.05$.*

Independent samples T-test was used to determine the d-value between the placebo and TCM group, †$p < 0.05$.

Independent samples T-test was used to determine the statistical significance between the placebo and TCM group at baseline and follow-up, all the p values > 0.05.

Data are presented as means ± SD.

with control group in patients with existing microalbuminuria at baseline (65.9% vs. 48.4%, $p > 0.05$).

UACR and Markers of Inflammation in One Center

The characteristic at the baseline between the two groups were balanced (**Supplemental Table 1**). No significant difference of UACR was observed between placebo and TCM groups at baseline or after intervention. Serum fractalkine (0.56 ± 0.25 vs. 0.78 ± 0.43 ng/mL, $p = 0.01$) and AGE-P (14.92 ± 2.43 vs. 19.29 ± 4.96 μg/mL, $p = 0.03$) concentrations were lower, whereas sRAGE concentrations (2,242.75 ± 359.67 vs. 1,832.63 ± 324.83 pg/mL, $p = 0.04$) in the TCM group were higher than those in placebo at 24 months. However, there was no significant difference in CRP (0.73 ± 0.59 vs. 0.96 ± 0.79 mg/L, $p > 0.05$) or MMP2 (186.66 ± 72.68 vs. 173.43 ± 59.91 ng/mL, $p > 0.05$).

Adverse Events

The overall occurrence of adverse events was similar between the two groups. A total of 53 adverse events were reported (26 in the placebo group and 27 in the TCM group). Serious adverse events were: coronary artery disease (2 patients in both groups), cerebrovascular accident (4 patients in TCM group and 5 patients in control group), bleeding (1 patient in control

TABLE 2 | The effect of 24 months treatment on ACR in placebo and TCM group.

	Placebo ($n = 206$)			TCM ($n = 225$)		
(mg/g)	**Baseline**	**24 months**	***d*-value**	**Baseline**	**24 months**	***d*-value**
Total	14.32 (6.68, 23.73)	11.97 (7.49, 23.01)	−0.73(−7.47, 6.75)	15.31 (7.46, 25.37)	12.55 (7.57, 22.26)	−1.61 (−10.24, 7.17)
UACR ≥30	44.79 (35.12, 65.11)	32.19 (12.32, 64.99)*	−20.61(−36.79,4.31)	46.21 (34.96, 58.96)	20.78 (9.62,38.85)*	−25.50 (−42.30, −9.56)†
UACR < 30	12.63 (6.18, 18.40)	10.91 (6.69, 18.52)	−0.28 (−5.25,6.76)	13.10 (6.77, 19.23)	12.16 (7.48, 19.81)	0.96 (−6.50, 8.85)

Total: all the subjects. Subjects were divided into tertiles: UACR <30 mg/g, UACR ≥ 30 mg/g. d-value: difference between baseline and end of follow-up.
*Two-related-samples nonparametric tests was used for intragroup comparisons between the baseline and the end of follow-up, *$p < 0.05$.*
Analysis covariance was used for d-value of samples between the placebo and TCM group, †$p < 0.05$.
Two-independent-samples nonparametric tests was used to determine the statistical significance between the placebo and TCM group in the baseline and end of follow-up, all the $p > 0.05$.
Data are presented as median (lower quartile, upper quartile).

group) and cancer (4 patients in TCM group and 2 patients in control group).

DISCUSSION

In this multicenter, randomized, double-blind, and placebo-controlled prospective trial, we find that co-administration of 1.5 g LWDH and 24 mg Ginkgo biloba three times per day for 24 months is benefit to type 2 diabetes for microalbuminuria (as reflected by measures in UACR), associated with downregulation of inflammatory markers (i.e., fractalkine, AGE-P). The combined therapy may ameliorate the microalbuminuria in the patients with early DN. These observations provide evidence that Ginkgo biloba and LWDH are attractive alternatives for the management of DN.

Both Ginkgo biloba and LWDH are commonly prescribed for a variety of clinical conditions, without significant side effects. Increasing interest has been placed on their efficacy and safety for the management of early DN (16, 26–28) in China. However, the strength of these studies was limited by small sample size, short-duration, and lack of appropriate control. The present study represents the first large-scale, multicenter, randomized, double-blind, placebo-controlled trial, which evaluates the effects of combined treatment with LWDH and Ginkgo biloba on the progression of DN among relatively well-controlled type 2 diabetes for 24 months. We did not observe a significant difference in the change of UACR before and after intervention, but the change of UACR was alleviated significantly for a subgroup of patient with microalbuminuria. We failed to reveal statistical difference in UACR between the two groups probably due to the fact that patients with microalbuminuria were only a small portion of all these subjects (18.22% in TCM group and 15.05% in placebo group). It seems that this treatment have an effect on patients with microalbuminuria but not on those with normal albuminuria. TCM treatment didn't change the UACR for patients without albuminuria while they accounted for a large part of the study. Therefore, addition of TCM to standard clinical intervention appears to be promising for the DN with microalbuminuria. A larger clinical trial for this population is needed to confirm this conclusion.

As previously reported, diabetes mellitus is associated with multiple inflammatory responses, including AGEs accumulation, leukocyte infiltration, ECM depositing, cytokines, and adhesion molecule expression, which may contribute to the development of renal impairment (29, 30). In the present study, we demonstrated positive relationships of AGE-P and fractalkine, and a reverse relationship with sRAGE after 24 months intervention. In the circulation, small-sized AGE-P may act as a reactive intermediate to cause tissue injury via binding to susceptible target proteins both within and outside the vasculature and hence forming "second-generation" AGEs. We have reported that AGE-P represents a valuable marker for predicting the severity DN, and AGEs upregulates fractalkine expression in human renal mesangial cells (HRMC) (31). Fractalkine, an important chemoattractant with adhesiveness functions, acts to induce macrophage recruitment that may cause abnormality of the ECM metabolism and consequent deposition of excessive ECM (32). In contrast, sRAGE has been described as a "sponge" for AGEs which counteracts the detrimental effects of cellular RAGE by binding with serum AGEs and AGE-P (33). It has been shown that the lower level of sRAGE is associated with the higher risks of diabetes, coronary heart disease, and all-cause mortality (34).

Some studies contribute the protection of TCM to their anti-oxidative and anti-inflammatory properties. For example, LWDH was demonstrated to decrease cytokinesin mice with experimental autoimmune encephalomyelitis (35). Ginkgo biloba was reported to improve platelet function, to alter platelet-vessel wall interactions, and to reduce malondialdehyde levels in platelets in type 2 patients (36). In addition, Gingko biloba was also shown to reduce relative total superoxide dismutase activity in patients with DN (37). In the present study, TCM treatment leads to a lower fractalkine level and a higher sRAGE level.

Because of reducing the ability of the blood to clot, Gingko biloba was reported to increase the risk of bleeding with high dose varying from 120 to 240 mg daily (38). No one reported bleeding with lower dose (between 35 and 70 mg daily). The safety of Gingko biloba extract for early DN still needs further research to estimate if there is any side effect. Previous study and our trial did not observe the related side effect. So, patients with bleeding related disease should be treated with cautions.

Our study has several limitations. Firstly, a 24 months follow-up was relatively short to evaluate the natural progression of DN, and the prevention of TCM on the DN. Secondly, there was a

relatively high rate of premature withdrawals due to the longer follow-up period. Thirdly, this is a phase II trial and there was no formal sample size calculation due to the absence of available research on this topic at that time. As a result, the negative result in the primary endpoint may be subject to false-negative error. Finally, we were unable to obtain blood samples from all centers to measure inflammatory markers.

In summary, this multicenter, randomized, double-blind, placebo-controlled trial suggests that LWDH and Ginkgo biloba may be effective for managing DN. Further research to investigate the renal effects of co-administration prospectively is underway.

AUTHOR CONTRIBUTIONS

RS analyzed part of the data and wrote the manuscript. YanW collected the data and analyzed part of the data. ZS and JY involved in the design/implementation of the overall study, designed the analysis plan, and supervised the analysis and manuscript. TW and SQ contributed to the discussion and reviewed the manuscript. XY performed the statistical analyses guidance. XA, JM, SL, LH, LW, JL, JG, HY, XW, YaoW, and BY involved in the implementation of the overall study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00100/full#supplementary-material

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MINI REVIEW
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Impact of Nutrient Type and Sequence on Glucose Tolerance: Physiological Insights and Therapeutic Implications

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Pharmacological and dietary interventions targeting postprandial glycemia have proved effective in reducing the risk for type 2 diabetes and its cardiovascular complications. Besides meal composition and size, the timing of macronutrient consumption during a meal has been recently recognized as a key regulator of postprandial glycemia. Emerging evidence suggests that premeal consumption of non-carbohydrate macronutrients (i.e., protein and fat "preloads") can markedly reduce postprandial glycemia by delaying gastric emptying, enhancing glucose-stimulated insulin release, and decreasing insulin clearance. The same improvement in glucose tolerance is achievable by optimal timing of carbohydrate ingestion during a meal (i.e., carbohydrate-last meal patterns), which minimizes the risk of body weight gain when compared with nutrient preloads. The magnitude of the glucose-lowering effect of preload-based nutritional strategies is greater in type 2 diabetes than healthy subjects, being comparable and additive to current glucose-lowering drugs, and appears sustained over time. This dietary approach has also shown promising results in pathological conditions characterized by postprandial hyperglycemia in which available pharmacological options are limited or not cost-effective, such as type 1 diabetes, gestational diabetes, and impaired glucose tolerance. Therefore, preload-based nutritional strategies, either alone or in combination with pharmacological treatments, may offer a simple, effective, safe, and inexpensive tool for the prevention and management of postprandial hyperglycemia. Here, we survey these novel physiological insights and their therapeutic implications for patients with diabetes mellitus and altered glucose tolerance.

Keywords: macronutrient preloads, food order, gastric emptying, glucose tolerance, insulin secretion, postprandial glycemia, medical nutrition therapy, type 2 diabetes

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CONCEPTUAL FRAMEWORK

Type 2 diabetes (T2D) affects more than 400 million people worldwide and its prevalence is constantly increasing (1). The first metabolic alteration detectable in the progression of the disease is typically a loss of postprandial glucose control (2, 3), which is an independent risk factor for T2D (4, 5) and its complications (5–11). Targeting postprandial glycemia has proved effective for reducing the incidence of T2D (12–14). However, pharmacological control of postprandial glucose

in the prediabetic stage rises ethical and economic concerns, and novel cost-effective treatments are required.

Medical nutrition therapy is recommended as first line treatment for prediabetes and T2D (15, 16) and may be a useful tool for improving glucose tolerance. In fact, meal composition and size have a profound impact on the physiological processes that regulate postprandial glycemia, such as gastric emptying and intestinal glucose absorption, pancreatic, and gut hormone release, hepatic insulin extraction, glucose uptake by insulin-sensitive tissues, and endogenous glucose production (17). Adherence to lifelong nutritional interventions involving energy restriction is often poor, and therefore alternative dietary strategies focusing on eating patterns are gaining growing interest.

One emerging approach is premeal ingestion of non-carbohydrate macronutrients (namely protein and fat), which has been shown to reduce postprandial hyperglycemia in both T2D and at-risk individuals (**Figure 1A**) (20). It has long been known that non-carbohydrate components of the meal can markedly influence postprandial glycemia (18, 21–25). More recently, the magnitude of the glucose-lowering effect of protein and fat was found to be even greater when these macronutrients are consumed before carbohydrates than mixed with them (22, 23). Based on these observations, "preloading" each meal with protein and fat or tailoring the sequence of macronutrient ingestion (i.e., consuming protein- and fat-rich food before carbohydrate) has been proposed as a novel strategy for the prevention and management of postprandial hyperglycemia.

The number of experimental studies in support to the clinical application of this promising nutritional approach is rapidly growing. However, gathering all available evidence is challenging given the different keywords used by different groups to define similar dietary strategies [e.g., protein/fat/nutrient premeal consumption (26, 27) or preload (19, 23, 28–30), food/meal/nutrient sequence (31, 32) or order (33)]. A further degree of complexity in the interpretation and comparison of different findings is produced by the heterogeneity of study designs. In fact, the effect of preload-based nutritional interventions on postprandial glycemia appears largely dependent upon different variables, such as preload composition, size, and timing of ingestion, test meal stimulus, and individual glucose tolerance status (20) (**Figure 1B**).

Herein, we review the available evidence on the acute and chronic effect of protein and fat preloads on postprandial glycemia throughout the whole spectrum of glucose tolerance, from diabetic patients to prediabetic and healthy individuals (**Table 1**), the underpinning physiological mechanisms, and the potential therapeutic relevance in different clinical settings.

IMPACT OF MACRONUTRIENT PRELOADS ON POSTPRANDIAL GLYCEMIA

Type 2 Diabetes

In subjects with type 2 diabetes (T2D), premeal consumption of protein and fat—either alone or in combination—has proved effective in decreasing or even normalizing postprandial hyperglycemia (**Table 1**). In 2006, Gentilcore et al. (22) demonstrated that 30 ml olive oil ingested 30 min before a carbohydrate meal was able to reduce and delay the postprandial glucose excursion in 6 diet-controlled T2D subjects. In 2009, the same group observed that a 55 g whey protein preload led to an even greater reduction in postprandial hyperglycemia in 8 diet-controlled T2D subjects (23). Thereafter, the ingestion of food rich in protein or fat before carbohydrate has been consistently associated with reduced postload glycemic excursions in T2D patients when compared with a carbohydrate-first meal pattern (18, 19, 27, 30, 31, 33–36, 39). On average, a ∼40% reduction in glucose peak and a ∼50–70% reduction in glucose excursion has been observed when protein and vegetables were consumed before carbohydrate, rather than mixed together or consumed after carbohydrate (33, 39). In our studies (18, 19), a small mixed protein and fat preload (50 g parmesan cheese and 50 g egg) was associated with a 30–50% reduction in glucose peak and overall excursion during an oral glucose tolerance test (OGTT) in well-controlled T2D patients. Similarly, Jakubowicz et al. (36) showed a ∼30% reduction in postload glucose levels when 50 g whey protein were consumed before a high-glycemic index meal. Of note, the effect of macronutrient preloads on postprandial hyperglycemia in T2D appears comparable or even greater than that of current pharmacological therapy. In fact, Wu et al. (30) demonstrated that the glucose-lowering effect of a 25 g whey protein preload is similar to that of a dipeptidyl peptidase-4 (DPP-4) inhibitor (50 mg vildagliptin). Interestingly, combining the protein preload with vildagliptin was more effective for reducing postprandial glycemia compared with either treatment alone, thereby suggesting an additive effect. Further studies are needed to examine the interaction between nutrient preloads and oral hypoglycemic agents. In fact, preloading with saturated fat may lead to a deterioration in the glucose-lowering effect of DDP-4 inhibitors over time (48).

Prediabetic Subjects

In individuals with impaired glucose tolerance (IGT), a mixed nutrient preload ingested 30 min before an OGTT was able to decrease postload glucose concentrations by 37% when compared with a water preload (18) (**Table 1**). In agreement with this finding, Shukla et al. (43) observed a similar reduction (−39%) in postprandial glycemia in IGT subjects who consumed protein and vegetables before carbohydrate, compared with the same foods consumed in the reverse order (i.e., carbohydrate before protein and vegetables). In 20 subjects with IGT and/or isolated 1-h glucose ≥160 mg/dl, a small (14 g) almond preload reduced postprandial glycemia by 15% (42). Interestingly, the effect was greater in individuals with higher 2-h glucose concentrations, suggesting an inverse correlation between the individual degree of glucose tolerance and the magnitude of the glucose-lowering effect achievable with nutrient preloads (42).

Healthy Subjects

Nutrient preloads have been shown to reduce postprandial glucose concentrations even in subjects with normal glucose tolerance (NGT) (**Table 1**). Premeal consumption of either single amino acids (49, 50), whey protein (26, 44), a protein-enriched

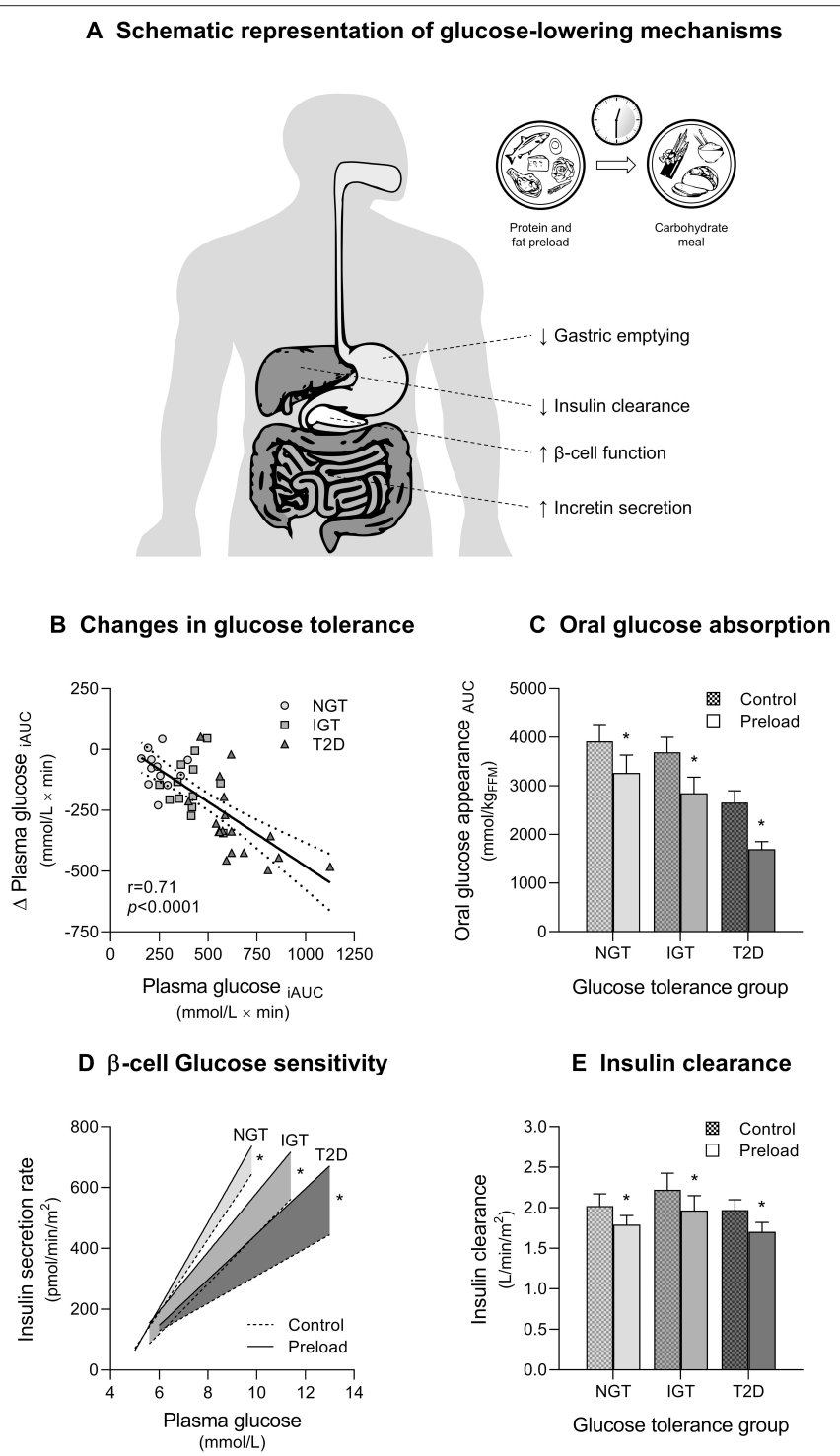

FIGURE 1 | Glucose-lowering effects of mixed nutrient preloads. **(A)** Schematic representation of postprandial glucose-lowering mechanisms activated by nutrient preloads. **(B)** Reduction of postload glucose excursions (Δ Plasma glucose $_{iAUC}$) during a 75 g oral glucose tolerance test (OGTT) after a mixed nutrient preload is proportional to the degree of glucose tolerance (Plasma glucose $_{iAUC}$) in subjects with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and type 2 diabetes (T2D). The physiological mechanisms responsible for the improvement in glucose tolerance during a 75 g OGTT preceded by a mixed nutrient preload (Preload) compared with a control OGTT (Control) are: **(C)** decreased rate of appearance of oral glucose for delayed gastric emptying; **(D)** enhanced glucose-stimulated insulin secretion (β-cell glucose sensitivity); and **(E)** reduced insulin clearance. Data are pooled from Trico et al. (18) and Trico et al. (19), for a total of 43 subjects examined (12 NGT, 13 IGT, and 18 T2D, except for B where T2D = 10). *p <0.05 using paired Wilcoxon signed-rank test for within-group difference between Preload and Control.

TABLE 1 | Available studies examining the glucose-lowering effects of preload-based nutritional interventions.

References	*n*	Preload	Timing	Control	Test meal	Effect on postload glycemia
TYPE 2 DIABETES						
Gentilcore et al. (22)	6	30 ml olive oil	−30′	30 ml water	65 g powdered potato + 20 g glucose + 250 ml water	Delayed glucose peak
Ma et al. (23)	8	55 g whey protein + 350 ml water	−30′	350 ml water	65 g powdered potato + 20 g glucose + 250 ml water	Glucose iAUC −51%
Chen et al. (34)	10	30 g soya beans + 75 g yogurt	−120′	None	51 g carbohydrate + 4.8 g fat +5.8 g protein	Glucose iAUC −36% 2 h glucose −9%
Clifton et al. (35)	24	17 g whey protein + 3 g lactose + 5 g guar + 150 ml water	−15′	150 ml water	2–3 slices of bread + jam and margarine, tea/coffee	Peak glucose −1.4 mM Mean glucose −0.8 mM
Jakubovicz et al. (36)	15	50 g whey protein + 250 ml water	−30′	250 ml water	High-glycemic index breakfast (353 kcal)	Glucose AUC −28%
Li et al. (28)	30	18 g Inzone Vitality (7.6 g protein + 1.8 g fat + 1.6 g fiber + 5.2 g carbs) + 150 ml water	−30′, each meal,12 weeks	None	Normal diet	HbA1c −0.3% 2 h glucose −14%
Ma et al. (37)	7	25 g whey protein + 100 ml water	−30′, 4 weeks	100 ml flavored water	Normal diet *Acute studies*: 65 g potato + 20 g glucose + 250 ml water	Fructosamine −9%* Peak glucose −5*-9%
Shukla et al. (33)	11	150 g chicken meat + 170 g vegetables	−15′	Reverse order	90 g ciabatta bread + 120 ml orange juice	Glucose iAUC −73% 2 h glucose −7%
Trico et al. (18)	10	50 g parmesan cheese + 50 g egg + 300 ml water	−30′	500 ml water	75 g oral glucose	Glucose iAUC−49%
Kuwata et al. (31)	12	100 g mackerel fish or 79 g beef meat	−15′	Reverse order	150 g rice	Glucose iAUC −30 to 40%
Trico et al. (19)	8	50 g parmesan cheese + 50 g egg + 300 ml water	−30′	500 ml water	75 g oral glucose	Glucose iAUC −28% Peak glucose −49%
Trico et al. (38)	17	Protein- and fat-rich food before carbohydrate	Before 2 meals, 8 weeks	Reverse order	Isocaloric diet	HbA1c −0.3% * Glucose CV −32% 2 h glucose rise −102%
Wu et al. (30)	22	25 g whey protein + 250 ml water	−30′	250 ml flavored water	400 g beef lasagna	Glucose AUC −1% * Peak glucose−5%
Shukla et al. (39)	16	150 g chicken meat + 170 g vegetables	−10′	Reverse order	90 g ciabatta bread + 120 ml orange juice	Glucose iAUC −53% Peak glucose −54%
Bae et al. (27)	15	30 g protein- and fiber-rich bar + 150 ml water	−30′	Reverse order	100 g bagel + 70 g cheese + 210 ml orange juice	Glucose iAUC−25%
Watson et al. (40)	79	17 g whey protein + 5 g guar gum + 150 ml water	15′ before 2 meals, 12 weeks	150 ml flavored water	65 g powdered potato + 1 egg yolk + 20 g glucose + 200 ml water	HbA1c −0.1% Peak glucose −15%
Watson et al. (41)	21	17 g whey protein ± 5 g guar gum + 60 mg sucralose + 150 ml water	−15′	60 mg sucralose + 150 ml water	65 g powdered potato + 1 egg yolk + 20 g glucose + 200 ml water	Glucose iAUC −15% (independent of guar gum consumption)

(Continued)

TABLE 1 | Continued

References	*n*	Preload	Timing	Control	Test meal	Effect on postload glycemia
IMPAIRED GLUCOSE TOLERANCE						
Trico et al. (18)	12	50 g parmesan cheese + 50 g egg + 300 ml water	−30′	500 ml water	75 g oral glucose	Glucose iAUC −37%
Crouch and Slater (42)	20	14.2 g almonds + 237 ml water	−30’	None	75 g oral glucose	Glucose AUC −16% 2 h glucose −14%
Shukla et al. (43)	15	100 g chicken meat + 285 vegetables + 15 ml olive oil	−20′	Reverse order	90 g ciabatta bread	Glucose iAUC −39%
NORMAL GLUCOSE TOLERANCE						
Cunningham and Read (21)	6	60 g margarine	−20′	300 ml beef consommé	300 g mashed potato + 230 ml water	Glucose AUC −39% Peak glucose −18% Delayed glucose peak
Akhavan et al. (26)	16	5–40 g whey protein + 400 ml water	−30′	300 ml water	12 kcal/kg pizza + 500 ml water	Dose-dependent reduction in glucose AUC (∼0–50%)
Akhavan et al. (44)	10	10 or 20 g whey protein + 400 ml water	−30′	300 ml water	12 kcal/kg pizza + 500 ml water	Mean glucose −4%
Trico et al. (18)	12	50 g parmesan cheese + 50 g egg + 300 ml water	−30’	500 ml water	75 g oral glucose	Glucose iAUC −32%
Kuwata et al. (31)	10	100 g mackerel fish or 79 g beef meat	15′	Reverse order	150 g rice	Glucose AUC −19 to 30% *
Sun et al. (45)	20	322 ml soy or dairy milk	−30′	None	91 g white bread + 322 ml water	Glucose iAUC −40 to 49%
Nishino et al. (46)	8	60 g pork meat + 150 g vegetables + 5 ml olive oil	Before carbs	Reverse order	150 g rice + 45 g pumpkin + 75 g orange + 150 ml water	Glucose AUC −48% *
Bae et al. (27)	15	30 g protein- and fiber-rich bar + 150 ml water	−30′	Reverse order	100 g bagel + 70 g cheese + 210 ml orange juice	Glucose iAUC −18%
TYPE 1 DIABETES						
Faber et al. (47)	20	22 g cheese + 30 g turkey meat	−15′	22 g cheese + 30 g turkey meat in test meal	2 slices bread + 15 g jam + 150 ml orange juice	Glucose AUC −19% * Mean glucose −9%
GESTATIONAL DIABETES						
Li et al. (29)	33 each group	18 g Inzone Vitality (7.6 g protein + 1.8 g fat + 1.6 g fiber + 5.2 g carbs) + 250 ml water	−30′, each meal, 7 weeks	18 g milk powder (3.5 protein + 1.1 g fat + 11.2 g carbs) + 250 ml water	Normal diet	Fasting glucose ∼-17% 2 h glucose ∼-5%

*$P < 0.05$ unless otherwise specified. *P = ns. AUC, area under the curve; CV, coefficient of variation; iAUC, incremental AUC.*

bar (27), dairy or soy milk (45), or margarine (21) before a carbohydrate-rich meal decreased postprandial glycemia in a dose-dependent manner in NGT subjects. Consistently, a mixed protein and fat preload reduced plasma glucose excursions after an OGTT by 32% in healthy young adults (18). Furthermore, the ingestion of either meat, fish, or vegetables before rice was able to decrease the postmeal glucose peak by ∼50% and to delay it by 30–60 min when compared with eating the same food in the reverse order (i.e., rice first) (31, 46).

Type 1 Diabetes

Despite recent improvements in insulin therapy, a tight control of postprandial hyperglycemia remains difficult to achieve in type 1 diabetes, and is frequently associated with an increased risk of insulin-induced hypoglycemia. In this setting, a recent study by Faber et al. (47) has shown that protein and fat consumed 15 min prior to carbohydrates reduced by ∼10% mean glucose levels in 20 type 1 diabetic children and adolescents. Remarkably, the nutrient preload was not associated with an increased risk of hypoglycemic episodes (47).

Gestational Diabetes

Glucose intolerance in pregnancy increases the risk of complications during delivery and the incidence of metabolic diseases later in life. In women with gestational diabetes (29), a treatment with low-carbohydrate preloads resulted in a significant reduction in both fasting and postprandial plasma glucose when compared with a dietary strategy implementing high-carbohydrate preloads. While low-carbohydrate preloads show promise, further studies are needed to determine efficacy and superiority of this approach.

LONG-TERM EFFICACY, SAFETY, AND FEASIBILITY

Despite numerous experimental studies demonstrated the acute beneficial effect of protein and fat consumption before carbohydrate on postprandial glycemia, only a few studies evaluated the long-term efficacy, feasibility, and safety of preload-based dietary strategies. In T2D subjects, a 25 g whey protein preload consumed 30 min before each meal for 4 weeks showed a sustained effect on postprandial glucose, with a nearly significant reduction in fructosamine levels ($p = 0.06$) (37). Furthermore, a 12-week intervention with mixed nutrient preloads was associated with decreased postprandial glucose and glycated hemoglobin levels in T2D subjects (28, 40). Finally, a recent study found a reduction in both fasting and postprandial plasma glucose in women with gestational diabetes consuming low-carbohydrate preloads for $\sim 9 \pm 1$ weeks (29).

Dietary strategies that require nutritional supplements (either food or artificial formulas) might be expensive and poorly accepted. Moreover, although previous studies did not show weight gain after chronic preload consumption (28, 37) possibly due to the compensatory satiating effect of protein (26, 28, 51–55), adding nutrient preloads to each meal may increase the total daily caloric intake, leading to an increase in body weight and diet-related metabolic alterations (56, 57).

To limit the risk of weight gain and to increase the feasibility and cost-effectiveness of dietary interventions exploiting the same glucose-lowering effects of nutrient preloads, other strategies have been proposed. Low-calorie fiber-rich preloads (e.g., guar gum, vegetables), alone or in combination with protein, have been shown to improve glucose tolerance in both healthy and diabetic subjects with negligible effects on body weight (40, 41, 58–61). Furthermore, our group (20, 38) and others (31–33, 39, 43, 46, 60) have recently proposed a nutritional approach that simply consists in manipulating the sequence of macronutrient ingestion during each meal. In a proof-of-concept study, postprandial glucose control significantly improved in T2D subjects instructed to consume protein- and fat-rich food before carbohydrate-rich food for 8 weeks under free-living conditions, with no differences in body weight, serum lipid profile and other metabolic markers (38). These data support carbohydrate-last meal patterns as an effective and safe behavioral strategy to reduce postprandial glucose excursions.

PHYSIOLOGICAL MECHANISMS

Gastric Emptying

The effect of non-carbohydrate nutrients on glucose tolerance is largely dependent on their ability to delay gastric emptying (18, 19, 22, 23, 31, 37, 45). Gastric emptying modulates the rate of oral glucose delivery and absorption in the small intestine, and it can account for about one third of the variance in the early glucose excursion during an OGTT (62–65). Fat is the most potent macronutrient in slowing gastric emptying (21, 62, 66–68). In 1989, Cunningham and Read (21) showed that the effect of fat on gastric emptying is greater when fat is consumed prior to carbohydrate rather than mixed with it, suggesting that this effect is dependent on the digestion of fat to fatty acids (22, 69, 70). In 2009, a protein preload was also found to be effective in slowing gastric emptying (23), as later confirmed by other groups (44). The effect of protein preloads on gastric emptying appears to be smaller compared to fat (23, 44, 71) and substantially unchanged after a 4-week consumption (37). Fat and protein may exhibit an additive effect on gastric emptying. In fact, a mixed protein and fat preload can markedly reduce oral glucose absorption across different classes of glucose tolerance (from −16% in NGT to−42% in T2D) (18) (**Figure 1C**). Consistently, Kuwata et al. (31) observed that both meat and fish consumed before rice are able to delay gastric emptying, particularly in T2D (31).

Insulin Secretion and β Cell Function

The effect of nutrient preloads on postprandial glycemia largely depends on their insulinotropic action (18, 22, 23, 31, 33, 36, 50, 72). Among non-carbohydrate macronutrients, protein is the most effective in enhancing glucose-stimulated insulin secretion. Ma et al. (23) showed that a 55 g whey protein preload increases glucose-stimulated insulin release by 2- to 3-fold in T2D, and these results have been confirmed in both non-diabetic and T2D subjects (27, 36, 71). The insulinotropic effect of protein is dose-dependent (26) and likely mediated by both direct and incretin-mediated interactions of protein and amino acids with β cells (72–76). Although fat can enhance

glucose-stimulated insulin secretion through direct and receptor-depended mechanisms (76–82), whether preloading with fat alone can affect insulin secretion is unclear. Indeed, the marked reduction in glucose responses due to the delay in gastric emptying after fat consumption usually leads to lower—rather than higher—absolute insulin levels in the early postprandial phase (21, 22). However, the insulin peak following a fat preload seems only delayed, but not reduced, even in the context of lower glucose levels (22). This observation suggests a positive—though small—effect of fat preloads on β cell function.

The effect of protein and fat on insulin secretion may be enforced by a synergistic interaction between the two classes of nutrients (18, 19, 83, 84). Our group showed that a mixed protein and fat preload increased plasma insulin levels during the first hour of an OGTT across the whole spectrum of glucose tolerance, despite lower glucose concentrations (18). The mixed preload increased the β cell responsiveness to plasma glucose (β cell glucose sensitivity) by 20% in NGT and prediabetic subjects, and almost doubled it in T2D subjects (**Figure 1D**). The greater enhancement of glucose-induced insulin secretion in T2D may be explained by a higher gradient of plasma amino acids after protein digestion and absorption in those subjects compared with healthy individuals (72, 85). Some studies with mixed preloads have reported different results (31, 39, 45, 46), likely due to a less rigorous estimation of β cell function (i.e., insulin and C-peptide levels were not adjusted for glucose concentrations) or to a stronger inhibition of gastric emptying by different preloads tested, which would minimize their impact on glucose-stimulated insulin secretion.

Insulin Clearance and Insulin Sensitivity

Besides a direct stimulation of insulin release by pancreatic β cells, macronutrient preloads may increase insulin bioavailability by reducing insulin degradation ("insulin clearance"), which mostly occurs in the liver. In fact, we reported an average ∼10% reduction in insulin clearance during a 2-h OGTT after a mixed nutrient preload, without significant differences between NGT, IGT and T2D subjects (18) (**Figure 1E**). A subsequent experiment showed a 52% increase in plasma insulin levels in T2D subjects during a 5-h OGTT, which was due to the combination of a 28% lower insulin clearance and a 22% higher insulin secretion after the nutrient preload (19).

Nutrient preloads may also impact on postprandial glucose homeostasis by affecting peripheral and hepatic insulin action ("insulin sensitivity"). However, no evidence so far has shown a significant influence of nutrient preloads on insulin sensitivity (18, 19).

Incretin Hormones

Macronutrient preloads may exert their glucose-lowering effects by stimulating the release of gut hormones, such as the glucagon like peptide-1 (GLP-1) and the glucose-dependent insulinotropic polypeptide (GIP) (23, 44, 71, 86–91). GLP-1 and GIP are usually referred to as "incretin hormones" to underscore their stimulatory effect on pancreatic β cells, which is glucose-dependent, dose-dependent, and—only for GLP-1—largely preserved in T2D (86, 92). Furthermore, incretin hormones exhibit pleiotropic actions that include the inhibition of gastric emptying and appetite [by GLP-1 (86, 92)] and the reduction of hepatic insulin clearance [by GIP (90, 91)]. Preloading with either protein or fat alone enhanced GLP-1 concentrations in both T2D and healthy subjects, while only protein increased GIP levels in T2D (22, 23, 27, 36, 44, 71). When protein and fat were consumed together as a mixed preload, we observed an almost doubled GIP response, alongside with a modest but significant increase in plasma GLP-1 (18). These effects were comparable in individuals with different glucose tolerance, with a tendency to be more pronounced in IGT and T2D subjects (18, 19). Similarly, consuming meat or fish before carbohydrate resulted in higher GLP-1 and GIP concentrations in both T2D and healthy individuals, and these effects were greater in T2D (31).

Additional Mechanisms

Several additional mechanisms have been proposed to explain the effect of non-carbohydrate nutrients on postprandial glucose control. Along with GLP-1 and GIP, protein (23, 44) and fat (93, 94) can stimulate the release of other gut hormones, such as cholecystokinin (CCK) and peptide YY (PYY), which inhibit gastric emptying and appetite (95–98) and stimulate insulin secretion (99–101).

The sight, smell and taste of nutrients may trigger neural signals leading to anticipatory insulin release, which may partly explain the insulinotropic effect of nutrient preloads (102). However, the contribution of the so-called "cephalic phase" of insulin secretion on glucose tolerance is little (∼1% of postprandial insulin release), transient (8–10 min from sensory stimulation) (102), and not supported by experimental evidence (18).

Furthermore, it should be noticed that the glucose-lowering effect of nutrient preloads occurs despite an increase in plasma glucagon levels (18, 19, 31, 71), which is expected to worsen glucose tolerance by promoting gluconeogenesis and glycogenolysis. However, endogenous glucose production was not affected by premeal consumption of protein and fat (18, 19), and the relevance of increased glucagon concentrations in this setting remains controversial.

Finally, the reduction of appetite and calorie intake following protein consumption, which is possibly mediated by the stimulation of GLP1 secretion, might contribute to weight loss after long-term consumption of protein preloads (51–55).

CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

The experimental evidence discussed above indicates that premeal consumption of protein and fat can markedly reduce postprandial glycemia across the whole spectrum of glucose tolerance. The mechanisms underlying this effect include a delay in gastric emptying as well as an enhancement of glucose-stimulated insulin release and a decrease in hepatic insulin clearance, resulting, respectively, in slower glucose absorption and hyperinsulinemia (22, 23, 72). From the

clinical perspective, the glucose-lowering effect of nutrient preloads is comparable in magnitude to that of current antihyperglycemic drugs (30), is proportionally greater in T2D than prediabetic and non-diabetic subjects (20), and appears to be sustained over time (37, 38). Preload-based dietary strategies can be useful in the management of T2D, either alone or in combination with pharmacological treatments, due to their additive effects (30). Furthermore, preload-based diets are of particular interest in clinical settings in which available pharmacological options are limited, including type 1 diabetes (47) and gestational diabetes (29), or not cost-effective, such as in the large number of individuals at high risk to develop T2D (18, 43). Remarkably, the same improvement in postprandial glycemia after nutrient preload consumption appears to be achievable by optimal timing of carbohydrate ingestion during a meal (i.e., carbohydrate-last meal pattern) (20, 31–33, 38, 39, 43, 46). This promising approach would avoid additional energy intake when compared with nutrient preloads, thereby minimizing the risk of body weight gain and diet-related metabolic alterations. Further refinement is required to determine the optimum timing and quantity of macronutrient consumption during a meal, as well as to standardize nutritional recommendations for targeting postprandial glycemia in different clinical settings. Larger studies are also needed to confirm the encouraging preliminary data on long-term efficacy, feasibility, and safety of these dietary approaches.

In summary, consistent experimental evidence suggests that preload-based nutritional strategies may offer a novel simple, effective, safe, and inexpensive therapeutic approach for the prevention and management of postprandial hyperglycemia and T2D.

AUTHOR CONTRIBUTIONS

LN and AM: data collection and analysis, interpretation of results, and manuscript writing; DT: funding, study design, data collection and analysis, interpretation of results, manuscript writing, and final editing. All authors read and approved the final submitted version of the manuscript.

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REVIEW
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Pancreatic Stellate Cells: A Rising Translational Physiology Star as a Potential Stem Cell Type for Beta Cell Neogenesis

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The progressive decline and eventual loss of islet β-cell function underlies the pathophysiological mechanism of the development of both type 1 and type 2 diabetes mellitus. The recovery of functional β-cells is an important strategy for the prevention and treatment of diabetes. Based on similarities in developmental biology and anatomy, *in vivo* induction of differentiation of other types of pancreatic cells into β-cells is a promising avenue for future diabetes treatment. Pancreatic stellate cells (PSCs), which have attracted intense research interest due to their effects on tissue fibrosis over the last decade, express multiple stem cell markers and can differentiate into various cell types. In particular, PSCs can successfully differentiate into insulin- secreting cells *in vitro* and can contribute to tissue regeneration. In this article, we will brings together the main concepts of the translational physiology potential of PSCs that have emerged from work in the field and discuss possible ways to develop the future renewable source for clinical treatment of pancreatic diseases.

Keywords: pancreatic stellate cell, quiescent, activation, stem/progenitor cell, physiological functions, β-cells neogenesis

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INTRODUCTION

Diabetes mellitus is one type of the most common chronic diseases over world, with an immense impact on public health. The number of individuals affected with this metabolic disorder is almost increased to 592 million by 2035 (Ogurtsova et al., 2017). Diabetes mellitus is caused by substantial deficits in functional β-cells in both type 1 and type 2 diabetes. Supplementation of deficient β-cells through stem cells transplantation provides ideal therapeutic effect, but this option is limited by donor sources and graft survival (Wang et al., 2015). Therefore, stem cell derived from adult pancreas that can be prompted to differentiate into insulin-secreting cells, would provide a self-healing source for β-cells neogenesis.

Adult tissue-specific stem cells are categorized as a cell type which has abilities to self-renewal and can differentiate into specialized functional cells. Based on their developmental potential, pancreatic stem cells, owing to their close relationship in term of developmental biology and

anatomy advantages compared with stem cells derived from other tissue sources, have attracted a great deal of research over the last decade (Bouwens et al., 2013; Gargett et al., 2016). However, the exact cell type(s) that function as pancreatic stem cells remain unclear.

Pancreatic stellate cells (PSCs) are a multifunctional cell type found in endocrine and exocrine pancreatic tissue and comprising about 7% of parenchymal cells in the pancreas (Apte et al., 2012). PSCs can be activated into myofibroblast-like phenotype along with expression of the activation marker protein α-smooth muscle actin (α-SMA) and a reduction in the number of retinoid-containing fat droplets, and they play a key pathological role in islet fibrosis, which contributes to the progression of β-cell dysfunction (Phillips, 2012). Recently, PSCs were proposed as a potential stem cell type for β-cell neogenesis (Jiang and Morahan, 2014). PSCs express multiple stem cell markers, are multipotent and can successfully differentiated into insulin-producing cells (Mato et al., 2009; Kordes et al., 2012; Zha et al., 2016; Pang et al., 2017). Thus, to summarize the physiological role of PSCs would not only to bring an improved understanding of the biological function of PSCs in pancreas homeostasis, but would likely yield new therapeutic strategies for β-cell regeneration.

NAMING AND CLASSIFICATION OF PSCs

Pancreatic stellate cells were first identified in the mouse pancreatic duct in 1982 as a cell type enriched in lipid droplets and appearing vitamin A (VA) -specific blue fluorescence (Watari et al., 1982). In 1990, These cells were identified in healthy sections from human and rat pancreas and named pancreatic stellate cells (Ikejiri, 1990). Later in 1998, two groundbreaking advancements were reported by Apte et al. (1998) and Bachem et al. (1998) groups; they developed a method to isolate, culture, and determine the characteristic expression of PSCs, which was a useful *in vitro* tool to study the biological characteristics of PSCs in their physiological state.

The existence of PSCs in islets was debated until 2016, when our group (Zha et al., 2014; Zha et al., 2016) isolated, identified, and named the fibrogenic cells obtained from mouse, rat, and human islets using collagenase digestion, islet stellate cells (ISCs). Furthermore, we compared the biological characteristics of ISCs with typical PSCs and found that ISCs had fewer lipid droplets than PSCs, appeared to be more easily activated by stimulators, and demonstrated reduced proliferation and migration abilities compared with PSCs (Wang et al., 2018). Using single-cell transcriptome technology, recent studies further confirmed that stellate cells are present in islets (Li J. et al., 2016; Lawlor et al., 2017). These results show that ISCs should be a sub-type of PSCs and appeared to be capable of exert direct effects on islet.

Pancreatic stellate cells can be divided into two biological phenotypes. In physiological conditions, PSCs are rich in intracellular lipid droplets and positive for glial fibrillary acidic protein (GFAP) and desmin expression. These are termed quiescent PSCs. When they are activated from the resting state to myofibroblast-like cells with a concurrent disappearance of lipid droplets, they are called activated PSCs. Activated PSCs specifically express α-SMA and secreted of collagen I, collagen III, fibronectin, and other ECM components to promote the formation of pancreatic fibrosis. The presence of lipid droplets, simultaneous expression with GFAP, nestin, desmin, and vimentin is used to define the quiescent phenotype of PSCs (Nielsen et al., 2017). The detailed mechanisms about the PSCs activation and disappearance of lipid droplets have not yet well understood. In addition to a large number of cytokines, other known activators include alcohol and its metabolites, endotoxin, oxidative stress, hyperglycemia, and some factors pertinent to pancreatic injury (Bynigeri et al., 2017). The physiological and pathophysiological functions of different phenotypes PSCs were shown in **Figure 1**.

PHYSIOLOGICAL FUNCTION OF PSCs

Much attention has been paid to exploring the behavior of activated PSCs as a negative regulator cell type for pancreatic diseases through the production of multiple inflammatory cytokines, enhanced self-proliferation, and fibrogenesis (Bynigeri et al., 2017). However, quiescent PSCs, which proliferate rarely and express few cell-specific markers, appear stagnant. Currently very little knowledge is shown about their biological significance for tissue homeostasis.

Current opinion holds that quiescent PSCs function as intermediary cells that contribute to the parenchymal function and cell structure through maintenance of the normal basement membrane (Means, 2013). These cells often show supportive effects such as supplying blood flow and providing scaffolding for epithelial integrity (Riopel et al., 2013; Sekiguchi and Yamada, 2018). Pancreas is completely different from other organs such as the intestines, which are responsible for barrier functions and nutrient absorption. Pancreas is short of stromal layer whose vasculature travels spreads along between major ducts and acini where PSCs are located. In addition, PSCs can regulate ECM turnover by regulating synthesis via matrix degrading enzymes (Riopel et al., 2013). Quiescent PSCs also partially maintain ECM components through secretion of metalloproteinases (MMP), such as MMP-2, MMP-9, and MMP-13, as well as their inhibitors (Phillips et al., 2003). These results strongly support that the effect of PSCs in the production of the acinar basement membrane but leave the question of how much effect of quiescent PSCs has in basement membrane maintenance under homeostatic conditions.

The description above is unlikely to capture the all aspect of physiological functions of quiescent PSCs. As rat PSCs were shown to express toll-like receptors (TLR), one might suppose that stellate cells play a role in innate immunity by phagocytosis of exo- and endogenous antigens (Masamune et al., 2008). Shimizu et al. (2005) found that PSCs act as resident phagocytic cells, and that CD36 promotes peroxisome proliferator-activated receptor γ transactivation. Hepatic stellate cells (HSCs), which have many biological features in common with PSCs, expressed the MHC class II proteins required for interaction with T cells (Yin et al., 2013; Weiskirchen and Tacke, 2014). Chen et al. (2006) reported

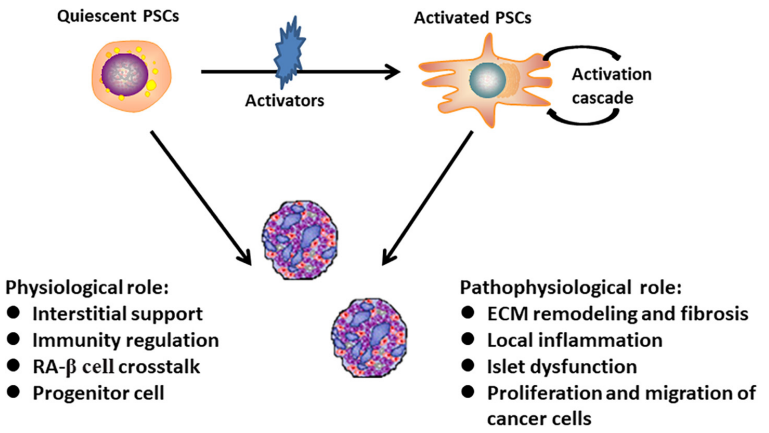

FIGURE 1 | The above summarizes physiological and pathophysiological functions of different phenotypes pancreatic stellate cells. Quiescent PSCs undergo morphologic and functional changes to become activated myofibroblast-like cells. Studies have identified a variety of physiological and pathophysiological functions of PSCs in pancreas homeostasis (Masamune et al., 2008; Means, 2013; O'Byrne and Blaner, 2013; Zang et al., 2015).

co-transplanted HSCs effectively protected isletallografts from rejection and formed as a multi-layered capsule that reduced allogeneic immunocyte infiltration by enhancing apoptosis in the islet transplantation model. These findings suggest that PSCs and phagocytic immune cells have several functions in common.

Quiescent PSCs are rich in droplets containing retinoid, predominantly as retinyl palmitate cytosolic droplets. Similarly, HSCs are responsible for up to 80% of the retinoid storage in the adult normal liver, and they can respond to VA metabolic needs in target tissue (Blaner et al., 1985; Hendriks et al., 1985). The exact function of retinol in PSCs has not been fully explored. Evidence has shown that VA and its derivatives play an important role in tissue homeostasis and the pathophysiology of pancreatic diseases, influencing cellular immunity and differentiation as well as cell apoptosis (Ross, 2012; O'Byrne and Blaner, 2013; Tejon et al., 2015). During the early days of development of the fetal pancreas, VA deficiency reduced the β-cell mass, which is attributed to a decrease in β-cell neogenesis, leading to impaired glucose tolerance in late adulthood (Matthews et al., 2004; Duester, 2008; Rhinn and Dolle, 2012). In the adult pancreas, retinoic acid (RA) is required for maintaining normal islet structure, endocrine cell mass, and endocrine function in non-diabetic mice (Kane et al., 2010; Raghow, 2012; Iqbal and Naseem, 2015). Dietary VA deprivation resulted in α-cell programmed cell death and islet remodeling, preventing the growth and proper functioning of pancreatic islets (Trasino et al., 2015). Retinoid can promote maintenance of the quiescent phenotype of PSC by inhibiting the activation of α-SMA and decreasing the expression of collagen synthesis (McCarroll et al., 2006; Chronopoulos et al., 2016; Sarper et al., 2016). All-trans RA (ATRA) has been proposed to restore and maintain the quiescent state of PSCs, suppressing their capacity to assist pancreatic exocrine and endocrine cells (Sarper et al., 2016).

The current evidence about the relationship between PSCs and islets has shown that PSCs not only promote islet fibrosis, but also contribute to endocrine functions in pancreatic diseases such as glucose intolerance, as well as in islet survival (Zha et al., 2014; Zang et al., 2015; Lee et al., 2017). Zang et al. (2015) found activated PSCs affected islet insulin release in short period researches in co-culture system, as measured by the secretion percentage of the cell insulin content, and increased islet cell death during prolonged culture periods. Mechanistic analyses have suggested that the production of local cytokines from macrophages in islet is the cause of this (Donath et al., 2009; Xue et al., 2015; Zang et al., 2015). We believed that the type of PSCs associated with islet dysfunction may be classified as ISCs, because activated ISCs were conducive to islet fibrosis and pancreatitis (Li F.F. et al., 2016). Therefore, more studies are needed to further evaluate the distribution of PSCs in the normal endocrine and exocrine tissues and in transplanted islets. The multipotential differentiation abilities of PSCs were shown in **Figure 2**.

PSCs AND PANCREATIC STEM CELLS

Numerous studies have identified that there are undifferentiated adult stem cells in very small numbers in the pancreas that can self-renew and differentiated into some or all of specialized cell types (Scharfmann, 2003; Pagliuca et al., 2014). Adult stem cells have been found in the intestinal epithelium, liver, skin, and hematopoietic tissue. However, the identity of pancreatic stem cells is largely unknown (Jiang and Morahan, 2014). There are various types of pancreatic interstitial cells that have been identified as potential adult pancreatic stem cells, such as nestin-positive cells (Feng and Wang, 2007), "side population" (SP) cells (Augstein et al., 2018) and so on. More recent studies have characterized a population of PSCs as likely to share a biological phenotype with these pancreatic stem cells.

Co-localization

One reason why the identity of pancreatic stem cells cannot currently be determined with certainty is that the location

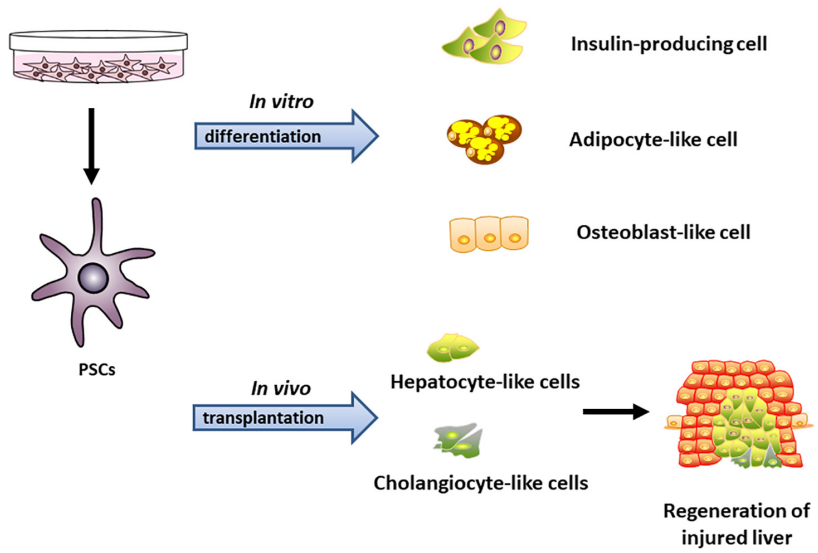

FIGURE 2 | The multipotential differentiation abilities of PSCs *in vitro* and *in vivo*. PSCs can be induced to differentiate toward insulin-producing cell, adipocyte-like cell, and osteocytes-like cell *in vitro*. Transplanted PSCs were able to repopulate large areas of the host liver after partial hepatectomy in rats through differentiation into hepatocytes and cholangiocytes *in vivo* (Mato et al., 2009; Kordes et al., 2012; Zha et al., 2016).

of pancreatic stem cells is debated. Some researchers hold the view that pancreatic stem cells may be a subset of the mesenchymal cells that are activated during the re-emergence of embryonic islets during maintenance and repair of the pancreas. These cells are located in the pancreas interstitial or surrounding islets.

One potential stem cell type-nestin positive cells, derived from human fetal pancreas, could formed three-dimensional insulin-producing cell clusters *in vitro* and reversed hyperglycemia in animal models (Huang and Tang, 2003; Zhang et al., 2005). Eberhardt et al. (2006) have also isolated nestin positive stem cells from human islets. In accordance with a mesenchymal phenotype, the cells also able to adopt pancreatic endocrine, adipocytic, or osteocytic phenotype *in vitro* and a hepatic phenotype *in vivo*. Recent single-cell genetic analysis showed that isolated nestin positive cells have multi-mesodermal potential and would therefore conform to the definition of teno-lineage stem cells (Yang et al., 2014; Yin et al., 2016; Bai et al., 2018). Furthermore, 5-bromo-2′-deoxyuridine labeling and immunohistochemical staining revealed that nestin positive cells first appeared in the area surrounding the interlobular region and then were diffusely distributed and filled the pancreatic lobules, after which they migrated toward the pancreatic lobules using the interlobar vessels as channels and penetrated through the vascular endothelium into the pancreatic acinar tissues. A portion of the stem cells eventually penetrated into islet tissue (Gong et al., 2014). The other known potential stem cell type-SP cells-has been identified in non-hematopoietic tissues including the human pancreas (Lechner et al., 2002). Freshly SP cells did not contain insulin protein or RNA, but expressed the homeobox transcription factor *Pdx1* required for development of β-cell. SP cells also could be differentiated into insulin-expressing cells that could response to glucose (Challen and Little, 2006). In the adult pancreas, SP cells expand in response to β-cell injury and are a source of β-cell progenitors with potential for the treatment of diabetes (Banakh et al., 2012; Augstein et al., 2018). Recent evidence has shown that SP cells can be detected as a low fluorescence by flow cytometry. The fluorescence of SP cells was quite similar to PSCs, which elicit a transient blue-green fluorescence typical of VA under UV light exposure. Meanwhile on the flow two-dimensional analysis of the dot matrix, SP cells are distributed in a comet-like form on the side of the main group of parenchymal cells, whose location is very similar to that of PSCs.

Pancreatic stellate cells are located near the basolateral side of pancreatic acinar cells, around small pancreatic ducts and blood vessels (Apte et al., 2012). A single stellate cell usually has multiple processes which extend across the space of disse to make contact with parenchymal cells. This intimate contact between PSCs and their neighboring cell may promote the intercellular transport of soluble mediators and cytokines. In islets, ISCs endings are directly adjacent to endocrine cell, which is consistent with functional studies confirming the hormone responsiveness of stellate cells (Li F.F. et al., 2016; Zha et al., 2016). Therefore, SP cells, nestin-positive cells, and PSCs may have the same location. Considering the characteristics including nestin expression and auto-fluorescence in SP cells and nestin-positive cells, PSCs may represent a similar cell type in the adult pancreas that functions as stem/progenitor cells.

Co-origin

Pancreatic development is a series of bifurcating lineage processes: ectoderm vs. mesoderm and endoderm, exocrine

vs. endocrine and hormone-positive cell types vs. non-hormone-positive cell types. Throughout the pancreatic development, pancreatic epithelium is sheathed in layers of mesenchyme (Jennings et al., 2015). There are a variety of cell types in this mesenchyme, including smooth muscle, stroma, and endothelial cells (Cleaver and Dor, 2012). In terms of potential pancreatic stem cells, nestin-positive cells as well as SP cells appear to be likely possibilities given their shared mesenchymal phenotype, combined expression of several dry effectors, and close proximity *in situ*.

Since PSCs were first identified, their embryologic origin has been elusive. Thus far, no direct evidence has been obtained to identify the origin of PSCs. The results of transcriptomics and proteomics analyses showed that PSCs and HSCs have significant similarities (Paulo et al., 2013). A recent study provided striking evidence to support mesodermal origin of HSCs using a lineage analysis approach (Asahina et al., 2009; Asahina et al., 2011; Simsek et al., 2016). As most of the characteristic features and functions of PSCs are similar to those of HSCs, this suggests that both might have evolved from a mesodermal origin. However, a recent study showed PSCs was in a very small possession of VA and a low expression of lecithin retinol acyltransferase compared to those of HSCs. The microstructure of PSCs was entirely different from that of HSCs as observed by electron microscopy (Yamamoto et al., 2017). There is still no firm experimental evidence for the origin of PSCs. Nevertheless, at least a subpopulation of PSCs in the normal pancreas has been shown to have a mesodermal origin. These seemingly contradictory studies favor progenitor cells as important players in pancreatic regeneration based on stem cell.

During development, endocrine and exocrine lineages arise from the embryonic endodermal epithelium. However, the property of lineage switching in pancreatic stem cells was observed both in altered culture conditions *in vitro* and spontaneous processes *in vivo* (Cheng et al., 2010, 2015). We believe PSCs, which may derive from a common precursor cell with pancreatic stem cells, may share this property of lineage switching.

Co-stem Cell Markers

Stem cell transcription factors are key factors that govern cell differentiation and maturation. Adult β-cell neogenesis is a dynamic process controlled by extrinsic signals from intrinsic transcription factors and non-insulin-producing cells. At present, pancreatic stem cell markers are not yet fully established. However, there is some consensus concerning a "universal" marker for the identification of pancreatic stem cells. Substantial evidence has shown that PSCs express multiple markers in common with pancreatic stem cells, including nestin, CK-19, and ATP-binding cassette superfamily G member 2 (ABCG2), which were the most commonly mentioned (Lardon et al., 2002; Yang et al., 2007; Mato et al., 2009).

Nestin, a type VI intermediate filament protein and essential transcriptional factor for the generation of endocrine cells, was originally detected in neural stem cells during development (Bernal and Arranz, 2018). Interestingly, nestin is also expressed in a variety of adult stem/progenitor cell populations (Calderone, 2012), meets criteria for adult stem cells, including proliferation, migration, and multipotency (Park et al., 2010), and shares many phenotypic markers with mesenchymal stem cells derived from the bone marrow (Bernal and Arranz, 2018). Nestin expression in the pancreas has been detected in stellate cells, pericytes, and endothelial cells (Lardon et al., 2002; Kawamoto et al., 2009). There is no consensus on the location of nestin-positive cells in different parts of the pancreas. It is generally believed that nestin is mainly expressed in islet, while CK-19 is expressed in the ductal epithelium. During embryonic development, nestin appears earlier than CK-19. Therefore, nestin-positive cells can be identified as primitive progenitor cells earlier than CK-19-positive cells.

CK-19, a family of keratins, is generally considered to be epithelial cell-specific markers. The expression of CK-19 first appears during the early embryonic stages and continues through the late embryonic and postpartum stages. After 12 weeks of embryonic development, CK-19 positive cells are concentrated in the ducts of the exocrine glands and in the undifferentiated ductal epithelium (Lane et al., 1983). It has been suggested that during pancreatic development, high levels of CK-19 expression are first present in duct-like cells, and these CK-19-positive cells are subsequently transformed into cells with endocrine and exocrine functions. Studies have shown that CK-19 is not expressed in β-cells, but more than 90% of pancreatic ductal epithelial cells express CK-19, and only 1–2% of cells can express CK-19 and nestin simultaneously. Recent studies have shown that a subset of CK-19-positive pancreatic stem cells can

TABLE 1 | A summarizing signaling pathways of PSCs and stem cells.

Signaling pathways	Functions	
	PSCs	**Stem cells**
MAPK	α-SMA expression, proliferation, migration, collagen synthesis	Proliferation, self-renew, differentiation
PI3K	Proliferation, migration, collagen synthesis	Proliferation, self-renew, differentiation
PKC	Collagen synthesis	Migration, self-renew
JAK/STAT	Proliferation	Proliferation, differentiation
Smads	Collagen synthesis	Migration, differentiation
Hhh	Migration	Differentiation
Shh	Migration	Differentiation
Wnt/β-catenin	Proliferation, collagen synthesis	Proliferation, self-renew, differentiation
TGF-β	Proliferation, collagen synthesis	Proliferation, self-renew, differentiation, ECM perturbation

differentiate into islet-like cells with insulin production and acinar epithelial cells with exocrine function (Gao et al., 2003; Yan et al., 2005; Yang et al., 2007). Therefore, a small number of CK-19 positive cells in pancreatic tissue have the potential to differentiate into pancreatic endocrine and exocrine cells, which is consistent with the basic characteristics of pancreatic stem cells.

ABCG2, one of the human ABC transporters, is an important molecule in both innate and acquired multidrug resistance, in regulation of drug bioavailability, in prognostic prediction of solid malignancies, and in protecting cancer stem cells (Mo and Zhang, 2012). Present studies found that ABCG2 is expressed in the stem cells of various tissues such as bone marrow, skeletal muscle, and nerve, but not in mature cells (Fatima et al., 2012; Padmanabhan et al., 2012). These results suggested that ABCG2 may be a stem cell marker. Recently, the presence of ABCG2 has also been found in islets and acini. Others have found ABCG2/BCRPI-expressing cells among nestin-positive islet-derived progenitor cells of embryonic pancreatic tissue, which can differentiate into islet-like cell clusters (Fetsch et al., 2006). Mato et al. (2009) found that mitoxantrone-resistant cells expressing the ABCG2 transporter obtained from rat pancreata have a PSCs phenotype and are capable of secreting insulin after cell differentiation. These data indicate that ABCG2 can be used as a marker for pancreatic stem cells. However, the relationship between ABCG2-positive cells and cells positive for other pancreatic stem cell markers, such as nestin and CK-19 is not yet well understood, and further study is needed to determine whether ABCG2 is a marker for more primitive stem cells.

Co-signaling Pathways

Signaling pathways which have major impact on stem cell differentiation is essential to help to integrate the signal inputs in order to initiate into specific lineage (Lin and Hankenson, 2011). During the past decade, Studies have several shared signaling pathways in PSCs and pancreatic stem cells. A variety of developmentally conserved signaling pathways are currently known as important control devices of stem cell fate (Blank et al., 2008). TGFβ, Wnt, Hhh, MAPK, and other pathways are significant during embryonic developmental stage for stem cell maintenance and equal proportionate growth rate among all lineages such as body patterning, cell fate determination, organogenesis, and so on (Guo and Wang, 2009). For example, TGFβ/Smad signaling pathway effect embryonic stem cells (ESCs) differentiation in the very early lineage decisions, which provides the origin of mesodermal and endodermal cells, represents the first step of ESCs differentiation (Sakaki-Yumoto et al., 2013; Xu et al., 2018).

For PSCs, the majority of intracellular signaling pathways that regulate physiological and pathological PSCs functions have also been detected. In addition to the common proliferation and migration signaling pathways, such as MAPK family, including ERK, JNK and p38 (Masamune et al., 2003; Uchida et al., 2013), the complex signaling networks involved in organogenesis, differentiation and maturation including Hhh, Shh, TGFβ, Smad and Wnt/β-catenin pathways was also determined in PSC (Lee et al., 2008; Xu et al., 2015). These signaling pathways also play a crucial role in various physiological functions and of PSCs. MAPK families are the initial pathways that precede proliferation and migration abilities of PSCs (Masamune et al., 2003). The TGFβ-Smad2/3 pathways were involved in preserving the activated phenotype and collagen synthesis (Lee et al., 2008). Other signaling pathways, such as Wnt/β-catenin, Hhh, and Shh pathways, were shown to influence mobility and differentiation of PSCs (Bynigeri et al., 2017).

To summarize these above experimental evidence, several signaling pathways were co-detected in both PSCs and pancreatic stem cells (**Table 1**). Thus, while these co-signaling pathways function in PSCs have not yet been established, the available data suggested these signaling pathways may the basis for PSCs to play stem cell function.

Co-multipotential Differentiation Abilities

Multipotential differentiation ability is an important measure to assess whether adult stem cells are able to generate an identical line of target cells with biological plasticity. Davani et al. (2007) used human islet-derived precursor cells to examine the multipotent differentiation characteristics. Under various differentiation conditions, these cells were separately induced into adipocytes, osteocytes, and chondrocytes.

For the multipotential differentiation abilities of PSCs, our previous study found that both PSCs and its subset of ISCs were able to differentiate *in vitro* into adipocyte- and osteoblast-like cells with Oil Red O or Alizarin Red S staining (Zha et al., 2016). Kordes et al. (2012) found that PSC-derived hepatocyte-like cells appeared typical hepatocyte functions including synthesize albumin along with stem/progenitor cell factors-vimentin. Transplanted PSCs isolated from enhanced green fluorescent protein-expressing rats reached the host liver and repopulated large areas of the injured organ through differentiation into hepatocytes and cholangiocytes along with long-lasting survival ability. These findings demonstrate that PSCs fulfill the essential characteristics of stem cells and can promote the regeneration of damaged organs through differentiation across tissue boundaries.

CONCLUSION

In recent years, most research interest in PSCs has focused on their central role in pancreatitis and pancreatic cancer. It has become clear, however, that the function of PSCs is no longer limited with the regulation of pathologic fibrosis in the pancreas. Its important roles in health as immune and/or progenitor cells and as intermediary cells may be more significant. Given the

accumulating evidence regarding the differentiation potential of PSCs, further work is necessary to fully elucidate the delicate balance between PSCs and β-cells, to determine when and how signals are exchanged between them, and to clarify the effects of these signals. An improved understanding of the supportive physiological role of PSCs would not only improve our understanding of their biological function in pancreas homeostasis, but would likely yield new therapeutic strategies for β-cell regeneration.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

YZ conceived and wrote the manuscript. JZ, XW, and MC critically collected and reviewed articles. WL and FG provided the data. BS and ZS conceived and designed the manuscript.

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Ginsenoside Rb2 Alleviates Obesity by Activation of Brown Fat and Induction of Browning of White Fat

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Ginsenoside Rb2 (Rb2), the most abundant saponin contained in Panax ginseng, has been used to treat variety of metabolic diseases. However, its effects in obesity and potential mechanisms are not well-understood. In the present study, we investigated metabolic performance with a Rb2 supplement in diet-induced obese (DIO) mice, focusing on the effects and mechanisms of Rb2 on brown and beige fat functions. Our results demonstrated that Rb2 effectively reduced body weight, improved insulin sensitivity, as well as induced energy expenditure in DIO mice. Histological and gene analysis revealed that Rb2 induced activation of brown fat and browning of white fat by reducing lipid droplets, stimulating uncoupling protein 1 (UCP1) staining, and increasing expression of thermogenic and mitochondrial genes, which could be recapitulated in 3T3-L1, C3H10T1/2, and primary adipocytes. In addition, Rb2 induced phosphorylation of AMP-activated protein kinase (AMPK) both *in vitro* and *in vivo*. These effects were shown to be dependent on AMPK since its inhibitor blocked Rb2 from inducing expressions of Pgc1α and Ucp1. Overall, the present study revealed that Rb2 activated brown fat and induced browning of white fat, which increased energy expenditure and thermogenesis, and consequently ameliorated obesity and metabolic disorders. These suggest that Rb2 holds promise in treating obesity.

Keywords: Ginsenoside Rb2, obesity, browning, UCP1, AMPK

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INTRODUCTION

Obesity, manifested as excessive fat accumulation, has become a global epidemic disorder that contributes to the development of several chronic diseases, including diabetes, cardiovascular diseases, and metabolic syndrome (1). As the central player in energy homeostasis, adipose tissues could be divided into the following subsets: white adipose tissue (WAT) which is characterized by large unilocular lipid-droplets-containing and functions as an active endocrine organ to regulate diverse activities, such as insulin sensitivity and brown adipose tissue (BAT) which dissipates energy as heat via the uncoupling protein 1 (UCP1) (2). Recently, a newly defined type of white adipocytes, called brite or beige adipocytes, has been shown to be reprogrammed to be brown-like adipocytes under cold or β-agonists and exhibit similar energy-consuming characteristic of brown adipocytes, which is known as the process of "browning" (3, 4). Numerous studies have suggested that genetic and pharmacological activation of brown and beige fat in mice led to enhancement of energy expenditure, thermogenesis and leanness (5). In addition to the direct regulation of

energy homeostasis, brown and beige fats also function as metabolic sinks to modulate glucose and lipid metabolism, independent of their effects on weight loss (6). Importantly, these functional brown and beige adipocytes are found to exist in adults while obese and aging individuals exhibit functional defects in these adipocytes (7–9), suggesting the importance of targeting brown and beige adipocytes for treating obesity and metabolic disorders.

With the plasticity of beige adipocytes to enhance energy expenditure and thermogenesis under stimuli, massive efforts have been made to look for potent inducers for the browning effects of white fat. Apart from a few promising candidates such as fibroblast growth factor 21 (FGF21) and irisin (10, 11), active ingredients from traditional Chinese medicine (TCM) provide a wide spectrum of choices and show great potential. For example, independent studies have shown that compounds such as berberine, resveratrol, artemisinin, and celastrol coming from TCM could enhance the function of brown fat and induce browning of WAT via the transcriptional and post-transcriptional regulation of critical metabolic regulatory molecular nodes such as AMP-activated protein kinase (AMPK), silent information regulator 1 (SIRT1), peroxisome proliferator-activated receptor gamma coactivator-1a (PGC1a), and heat shock factor 1 (HSF1) (12).

Ginseng plants have been used as an ancient medicine in China for thousands of years to strengthen holistic health. As major pharmacological ingredients in ginseng plants, ginsenosides possess multiple pharmacological properties, including anti-oxidative, anti-aging, anti-cancer, and other health-improving actions (13). For metabolic aspects, research showed that ginsenosides reduced body weight (14), prevented hepatic steatosis (15), increased insulin sensitivity (16), restored mitochondrial dynamics (17), and attenuated hepatic glucagon response (18). As the most abundant saponin contained in Panax ginseng (19), Ginsenoside Rb2 (Rb2) has been reported to alleviate hepatic lipid accumulation in high-fat diet (HFD)-induced obese mice (20), decrease glycaemia in streptozotocin-induced diabetic rats (21), and lower triacylglycerol levels in 3T3-L1 adipocytes (22). We recently showed that Rb2 reduced adiposity and improved insulin sensitivity in obese mice via phosphorylation of AKT and inhibited NF-κB signaling pathway in white adipose tissues and 3T3-L1 adipocytes (23). However, the effects and mechanisms by which Rb2 alleviates obesity have not been fully elucidated, especially regarding the effects of Rb2 on brown and beige fat.

In the present study, we investigated the metabolic performances with Rb2 supplement in diet-induced obese (DIO) mice and demonstrated that Rb2 effectively reduced body weight, improved insulin sensitivity, and increased energy expenditure in DIO mice. Detailed analysis demonstrated that Rb2 induced activation of brown fat and browning of white fat as shown by reducing lipid droplets, increasing UCP1 staining and increasing brown gene programs, which could be recapitulated *in vitro*. In addition, Rb2 induced AMPK phosphorylation while AMPK activation is dispensable for the beneficial effects of Rb2. Overall, the present study revealed that Rb2 ameliorated obesity and metabolic disorders by activating brown fat and inducing browning of white fat, leading to increase in energy expenditure and thermogenesis. It was suggested that Rb2 was a promising beneficial compound treating obesity.

MATERIALS AND METHODS

Chemicals and Antibodies

Ginsenoside Rb2 (purity >98.0%) was purchased from Shanghai Yuanye Biotech Co, Ltd (Shanghai, MO, China). The AMPK inhibitor compound C (10 mM; purity = 99.67%) was from Selleck Chemicals (S7306). Mice HFD (60% kcal from lard; MD12033) and sucrose matched low-fat control diet (10% kcal from lard; MD12031) were obtained from Research Diets. Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle Media: Nutrient Mixture F-12(DMEM/F12) Medium and Fetal Bovine Serum (FBS) were obtained from Sigma-Aldrich (San José, CA, USA). Insulin powder, T3 powder, clostridium histolyticum type II collagenase, indomethacin, isobutyl-1-methylxanthine (IBMX), and dexamethasone were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Primary antibodies (anti-AMPK and anti-p-AMPK) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Animals

All C57BL/6J mice (male, 8-weeks old) were housed under a constant 12 h light/dark cycle with free access to water and standard chow or HFD for 9 weeks. The mice were obtained from Shanghai Slake Experimental Animal CO.LTD., and were maintained at 22 ± 2°C with 60 ± 5% relative humidity. The HFD and chow diet-fed mice were administrated an intraperitoneal injection of Rb2 (40 mg/kg/day) or PBS (vehicle) between 16:00 and 17:00 daily. Body weight was monitored once a week between week 9 and once a day over a 10-day course of treatment. Tissues and serum were collected, snap-frozen in liquid nitrogen, and stored at −80°C. BAT was separated from the interscapular region; eWAT (epididymal WAT) was obtained from the epididymis; iWAT (subcutaneous WAT) was dissected from the layer under the skin and outside the abdominal cavity at the hips. Fat mass was the sum of BAT, eWAT, and iWAT. The experiments were randomized. All animal experiments were approved by the Institutional Animal Care and Use Committee in Wenzhou Medical University (No: SYXK-2015-0009).

3T3-L1, C3H10T1/2 Adipocytes, and Mice Primary Stromal-Vascular Fraction (SVF) Culture and Differentiation

3T3-L1 and C3H10T1/2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM with 10% FBS mixture at 37°C in a 5% CO_2 environment. Mice SVF was isolated from the iWAT of male C57BL/6J mice aged 6–8 weeks. SVF isolation was performed previously (24, 25). Briefly, tissues were sheared, digested with 1 mg/ml collagenase in PBS supplemented with 0.5% bovine serum albumin (BSA), and 10 mM Hepes mixture for 30 min, with 300

rpm shaking at 37°C. The suspension was strained through a 40 μM nylon mesh (Falcon), collected by centrifugation. Finally, the adipocytes were then cultured in DMEM/F12 containing 10% FBS mixture and placed inside an incubator at 37°C with a 5% CO_2 environment.

After reaching 100% confluence, 3T3-L1, C3H10T1/2 cells, and mice SVF were stimulated to differentiate a hormone cocktail containing 50 nM insulin, 100 nM T3, 0.125 mM indomethacin, 2 μg/ml dexamethasone, and 0.5 mM IBMX. After 48 h, the adipocytes were moved to the medium containing 50 nM insulin and 1 nM T3. The media was replaced every 2 days before Rb2 treatment. Dimethyl sulfoxide (DMSO) was used as the vehicle treatment. Finally, the mature adipocytes were confirmed by light microscopy and Oil Red O staining, and then used for further analysis.

Oil Red O Staining

Lipid droplets present within the adipocytes were proven by Oil Red O staining (Sigma). Oil Red O was diluted into water to make a 60% working solution, and then filtered through a filter paper. Adipocytes were fixed in 4% neutral buffered formalin for 30 min at room temperature. The Oil Red O solution was added (1.5 mL) and maintained at room temperature for 15 min. The solution was refused,

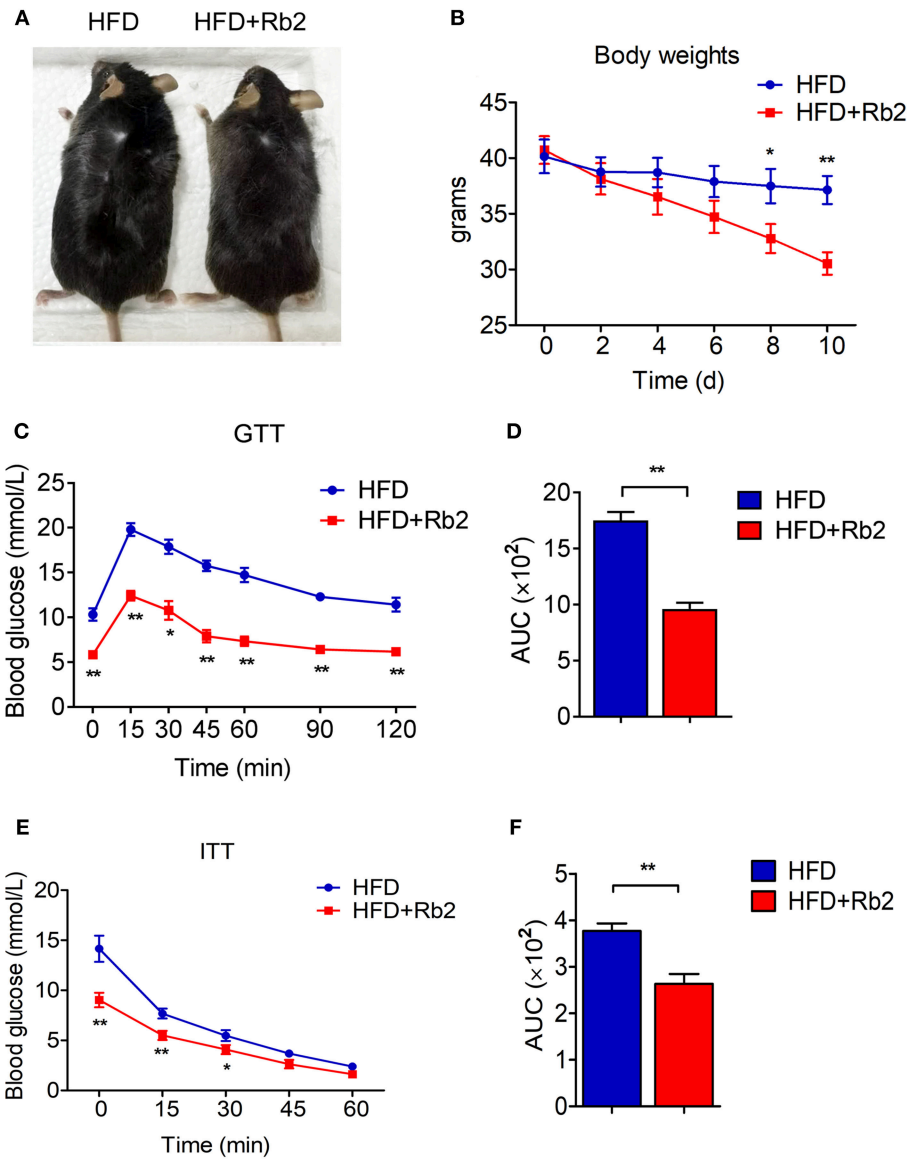

FIGURE 1 | Rb2 treatment reduced body weight and improved insulin sensitivity in DIO mice. **(A)** Representative photograph of DIO mice after intraperitoneal injection of PBS or Rb2 for 10 days. **(B)** Body weight of DIO mice treated with or without Rb2 for 10 days ($n = 6$). **(C–F)** Performances of GTT **(C)** and ITT **(E)** of DIO mice treated with or without Rb2. Area under the curve (AUC) of GTT and ITT was also shown as D and F. $N = 6$ per group. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to HFD group.

and the wells were washed with PBS several times until the background was clear. Finally, the adipocytes were imaged using light microscopy.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Previous methods were followed to implement GTT and ITT. GTT was performed in male C57BL/6J mice after an overnight fast. Blood glucose concentrations were measured from the tail vein immediately at 0, 15, 30, 45, 60, 90, and 120 min after a 0.75 g/kg i.p. injection of glucose (7) was administered. Food was removed for 2 h before the ITT was performed. After a 0.75 U/kg i.p. injection of human insulin (Eli Lilly) was administered to the mice, glucose concentrations were assessed at 0, 15, 30, 45, and 60 min (26).

Energy Expenditure Analysis

Energy expenditure was determined using Comprehensive Lab Animal Monitoring System (CLAMS system, Columbus Instruments) according to the manufacturer's instructions. The animals were acclimated to the system for 24 h before measurement of V_{O2} and V_{CO2}. The mice were maintained at 22°C under a 12 h light/dark cycle. Food and water were available *ad libitum*. Heat production was calculated according to the

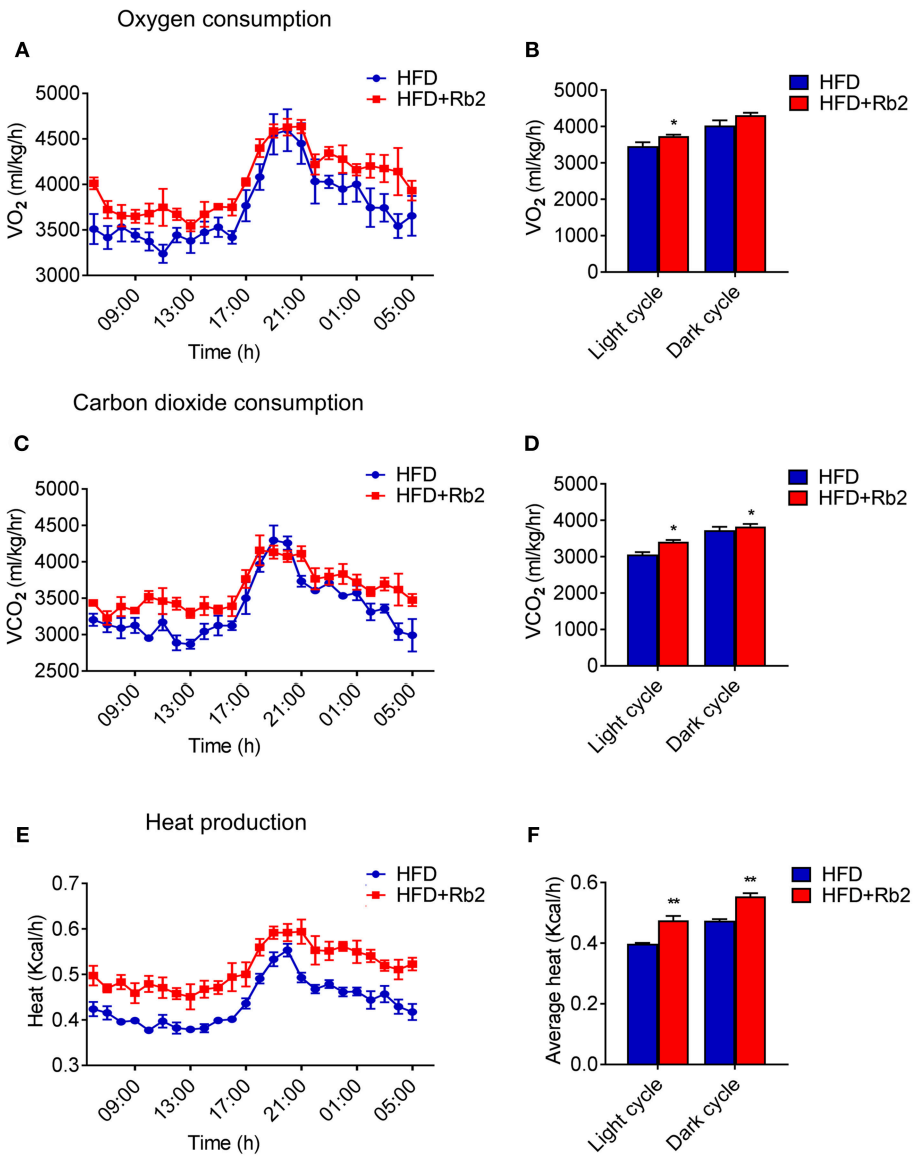

FIGURE 2 | Rb2 treatment increased energy expenditure in DIO mice. **(A–F)** Energy expenditure was evaluated by measurement of oxygen consumption (V_{O2}) **(A)** carbon dioxide release (V_{CO2}) **(C)** heat production **(E)** over a 24 h period in DIO mice after 10 days of PBS or Rb2 treatment. The average numbers calculated as day and night were shown in **(B,D,F)**. $N = 6$ per group. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to HFD group.

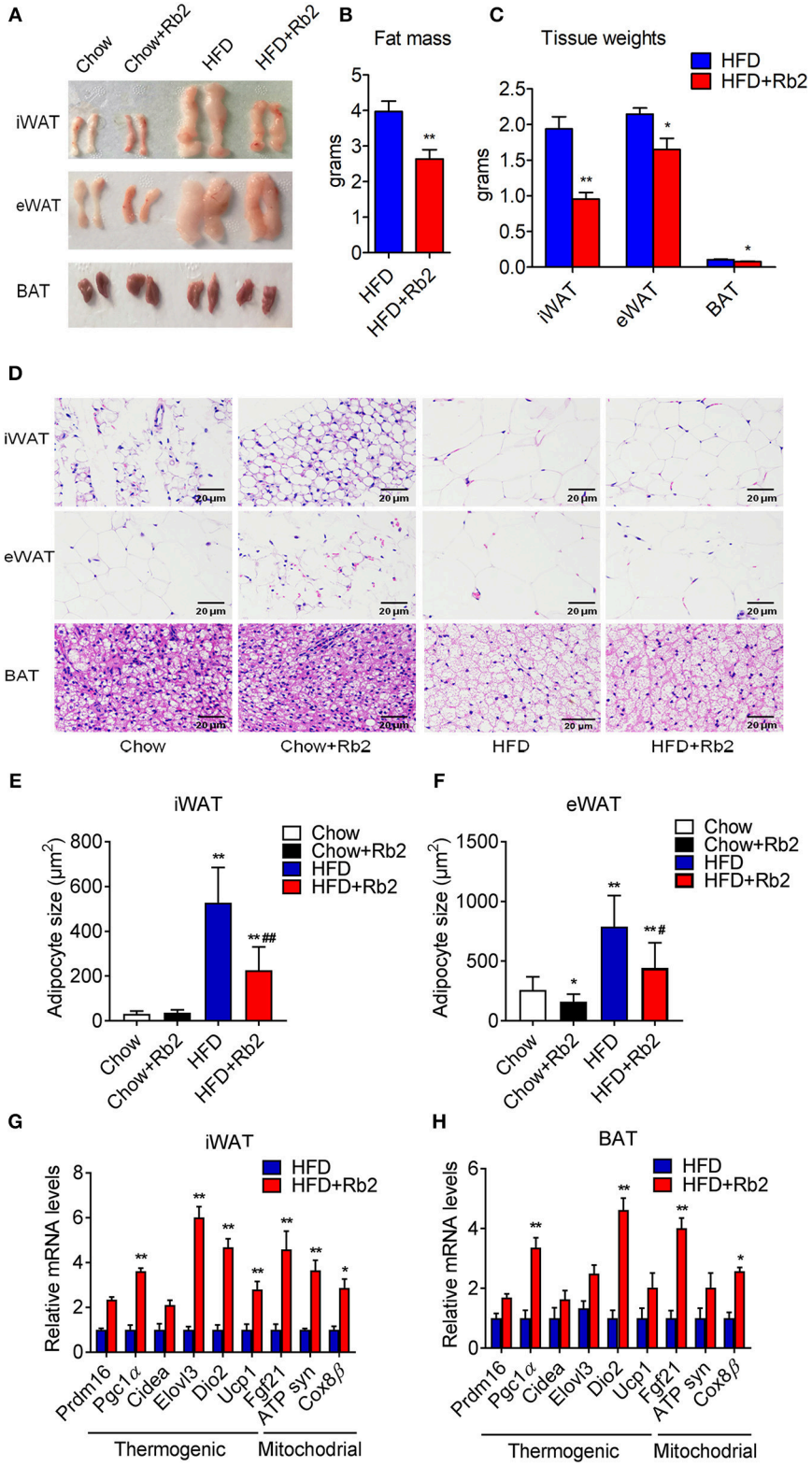

FIGURE 3 | Rb2 treatment reduced adiposity and induced brown fat gene programs in adipose tissues. **(A–C)** Representative photographs, fat mass and tissue weight of iWAT, eWAT, and BAT of DIO mice treated with Rb2 and PBS for 10 days. **(D–F)** Representative hematoxylin and eosin staining from iWAT, eWAT, and BAT sections and quantifications of adipocytes sizes of white fat. **(G,H)** gene expression analysis of thermogenic and mitochondrial genes in iWAT and BAT. $N = 6$ per group. Data are presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to chow group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to HFD group.

following equation:

$$\text{Heat} = [3.815 + 1.232(V_{CO2}/V_{O2})] \times V_{O2} \times \text{body weight}$$

where heat is measured in kcal h^{-1}, V_{O2}, and V_{CO2} are measured in liters kg^{-1} h^{-1} and body weight is measured in kg (27).

Histological Analysis and Immunofluorescence

Tissues fixed in 4% paraformaldehyde were sectioned after being paraffin embedded. Multiple sections were prepared and stained with hematoxylin and eosin for general morphological observations. Immunofluorescence staining was performed according to standard protocols using the following antibodies and dilutions: UCP1 (Abcam) and 1:400, respectively. The incubations were performed overnight in a humidified chamber at 4°C. The secondary antibodies for immunofluorescence staining were purchased from Invitrogen. Hematoxylin staining was used to mark cell nuclei. Immunofluorescence staining was performed first, and immunofluorescent images were captured. Finally, the images were acquired using an Olympus BX51 system. And Image J was used to calculate the adipocytes size.

Cold Tolerance Test

At the 10th day after PBS or Rb2 treatment, the mice were then subjected to a cold room (4°C) for 24 h without access to food or water. The rectal temperature of the mice was measured at 0, 30, 60, and 120 min begin at cold exposure with Th5 Thermalert Monitoring Thermometer (Braintree, US). The histological and molecular analyses were performed after the 24 h cold exposure.

Western Blot Analysis

Adipocytes were harvested in protein extraction solution (Solarbio) and incubated for 30 min at 4°C. After cell debris was removed, supernatant containing proteins were collected and determined by using the Pierce BCA protein assay kit according to manufacturer instructions. Next, 40 μg proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. Blots were incubated by using 5% milk blocking solution at room temperature overnight with a primary antibody

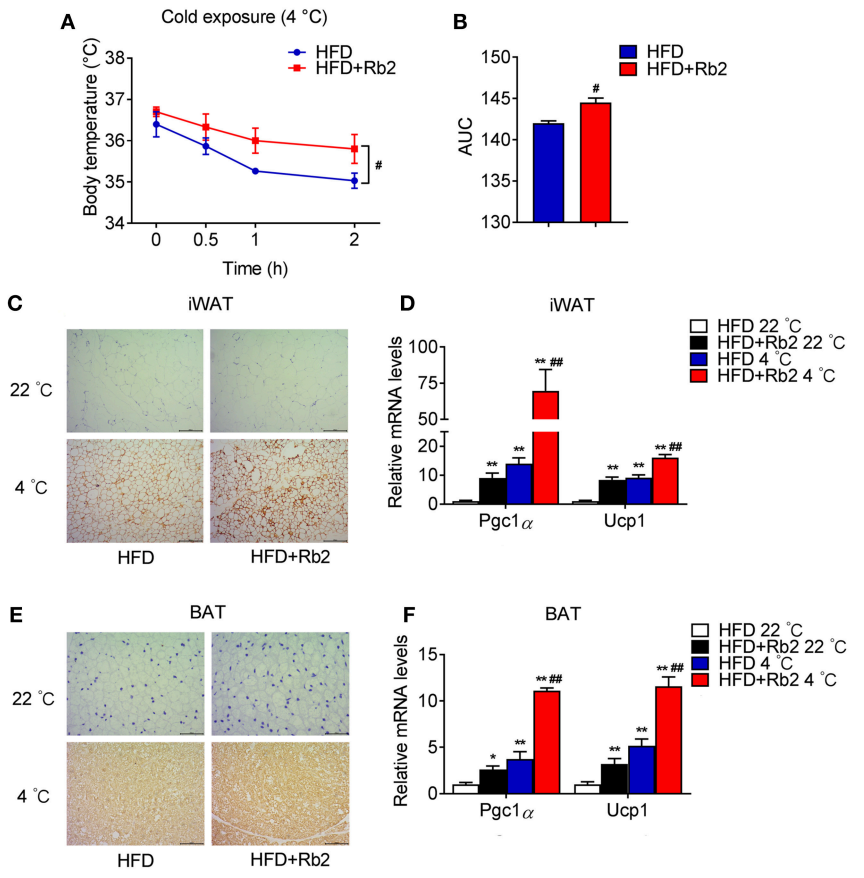

FIGURE 4 | Rb2 treatment increased adaptive thermogenesis. **(A,B)** Rectal body temperature and related AUC of DIO mice treated with or without Rb2 during cold tolerance test. **(C–F)** Immunohistochemistry for UCP1 staining and gene expression of Pgc1α and Ucp1 in iWAT and BAT of DIO mice treated with or without Rb2 under cold stimuli for 24 h. $N = 6$ per group. Data are presented as mean ± SEM and $^{\#}P < 0.05$ compared to HFD group in **(A,B)**, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to HFD 22°C group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to HFD 4°C group in **(D,F)**.

against AMPK and p-AMPK. The blots were then washed with TBST and incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000) for an hour. Finally, the blots were developed using an enhanced chemiluminescence kit (Salarbio) according to manufacturer protocols.

Quantitative Realtime PCR Analysis

Total RNA was isolated from cells by using Trizol (Invitrogen, Carlsbad, CA) and was reverse transcribed to first-strand cDNA using the Reverse Transcription System (A3500, Promega, Madison, WI). To analyze gene expression, real-time quantitative PCR was conducted by using an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA). Primers were provided upon requests.

Statistical Analysis

Data were expressed as the mean ± SEM from at least three independent experiments. Analyses were performed by using Graph Pad Prism 5 and SPSS version 20.0. For repeated measurements (for example, body weight), Dunnett's multiple comparisons test were performed. For single time-point measurement, statistical analyses were performed by using an unpaired Student's *t*-test for two groups and a one-way analysis of variance (ANOVA) for more than two groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

Rb2 Treatment Reduced Body Weight and Improved Insulin Sensitivity in DIO Mice

To assess the effects of Rb2 in treating obesity, we firstly established a DIO mice model by feeding mice for 9 weeks. This led to a significant increase in body weight to around 40 grams compared with chow feeding (**Figures S1A,S1B**). Importantly, 10 days of Rb2 treatment largely decreased body weight in these DIO mice, while not affecting body weight in mice fed by chow diet (**Figures 1A,B**, **Figure S1C**). The food intake in mice treated with Rb2 showed a slight downtrend but no significant differences when compared to the control group (**Figure S1D**). Meanwhile, we found that DIO mice supplemented with Rb2 had better tolerance to glucose load and were more sensitive to insulin addition, which were shown in GTT and ITT analyses (**Figures 1C–F**, **Figures S1E–S1H**). Therefore, these results demonstrated that Rb2 could reduce body weight and improve insulin sensitivity in DIO mice.

Rb2 Treatment Increased Energy Expenditure in DIO Mice

In order to better understand the mechanism of Rb2 in ameliorating obesity and improving insulin sensitivity, we systematically analyzed the actions of Rb2 on energy

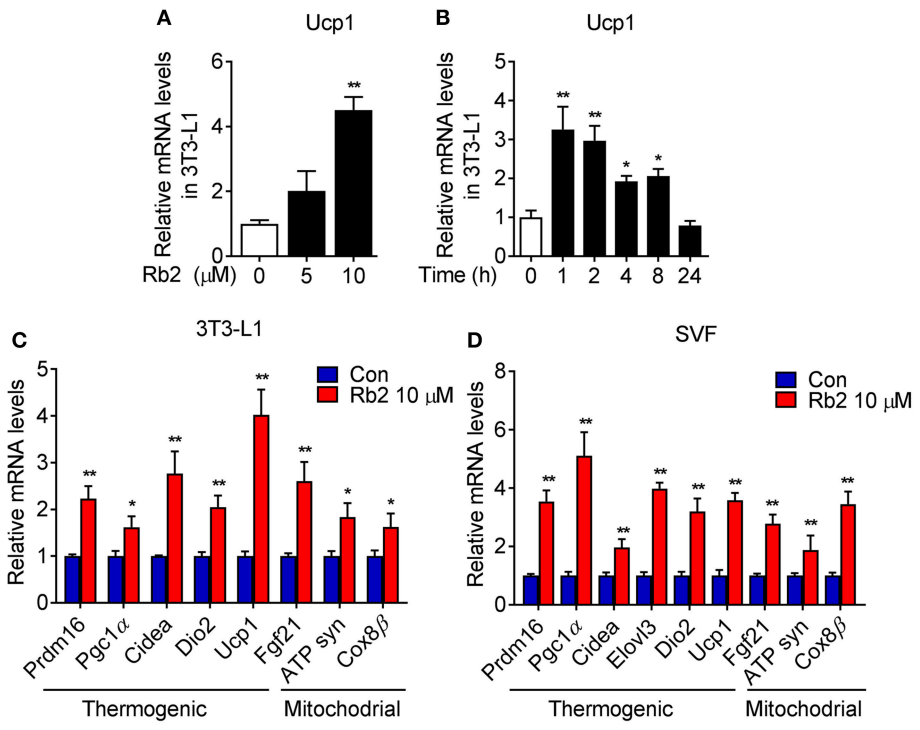

FIGURE 5 | Rb2 treatment induced brown gene programs in white adipocytes. **(A,B)** mRNA levels of Ucp1 in 3T3-L1 cells treated with or without Rb2 in dose and time dependent manner. **(C,D)** mRNA levels of thermogenic and mitochondrial genes in 3T3-L1 adipocytes and differentiated adipocytes from primary iWAT SVF. $N = 6$ per group. Data are presented as mean ± SEM and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

expenditure using comprehensive lab animal monitoring system (CLAMS). The Rb2 treated mice showed markedly higher oxygen consumption and carbon dioxide production rates through a 12 h light/dark cycle than the control group (**Figures 2A–D**). Besides, DIO mice received Rb2 exhibited a significant increase in the whole-body energy expenditure shown as heat production (**Figures 2E,F**). These results suggested that Rb2 increased energy expenditure in DIO mice.

Rb2 Treatment Reduced Adiposity and Induced Brown Fat Gene Programs in Adipose Tissues

To further explore the effect of Rb2 on adiposity, we closely examined the adipose tissue morphology and molecular signatures. We found that Rb2 treatment significantly decreased fat mass and weights of iWAT, eWAT, and BAT in DIO mice compared with control groups (**Figures 3A–C**). Consistently, Rb2 treated mice showed smaller adipocytes sizes as shown by histological analysis (**Figures 3D–F**). Moreover, we performed gene expression analysis on adipose tissues of DIO mice treated with or without Rb2. As shown in **Figures 3G,H**, **Figure S2**, several thermogenic and mitochondrial genes were significantly increased in iWAT and BAT of Rb2 treated mice with HFD while mildly elevated in mice with chow diet.

Rb2 Treatment Enhanced Adaptive Thermogenesis and Induced Browning of White Fat

Cold exposure is known to induce adaptive thermogenesis, brown fat activation, and browning of white adipose tissues. Thus, we performed cold tolerance tests to assess the thermogenic capacity of mice treated with Rb2 and found that Rb2 treatment

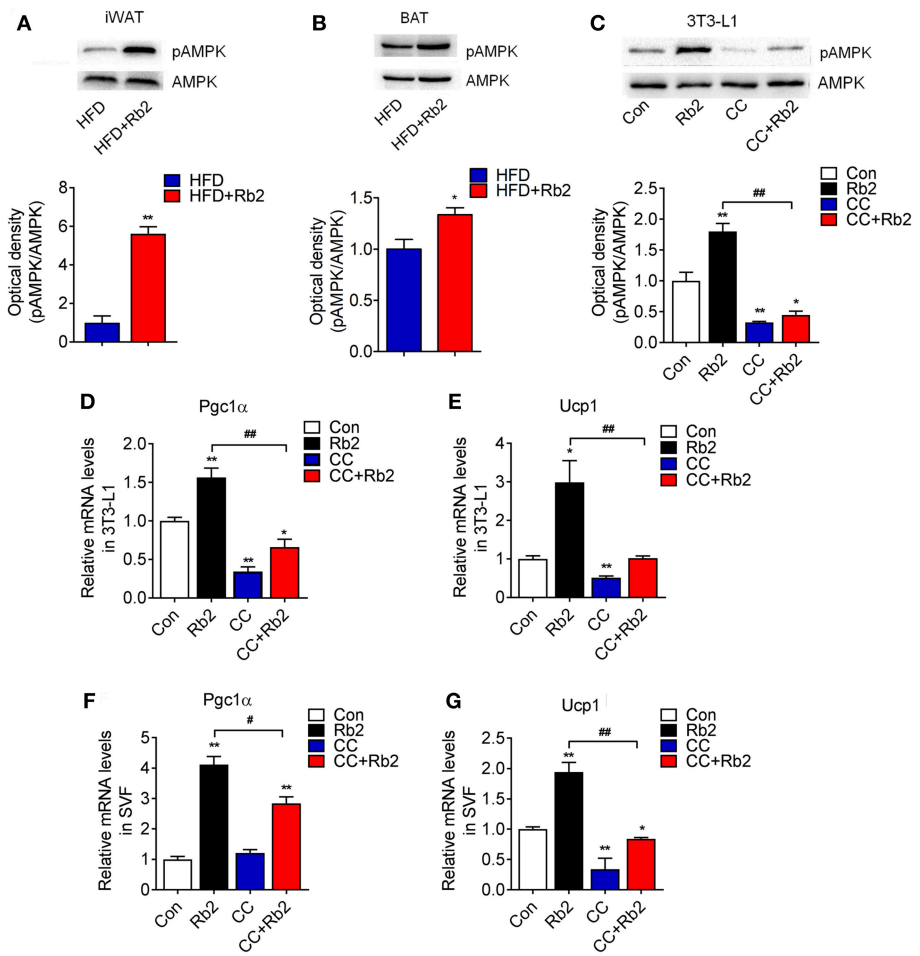

FIGURE 6 | AMPK signaling pathway was dispensable for the beneficial effects of Rb2 in browning. **(A,B)** Phosphorylation and total protein levels of AMPK in response to Rb2 treatment in the iWAT and BAT of DIO mice. **(C)** Phosphorylation and total AMPK levels in 3T3-L1 adipocytes treated with or without Rb2 in the absence or presence of compound C (10 μM). mRNA levels of Pgc1α and Ucp1 in 3T3-L1 adipocytes **(D,E)** and differentiated adipocytes from primary iWAT SVF **(F,G)** treated with or without Rb2 in the absence or presence of compound C. Data are presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to Rb2 group.

significantly ameliorated the cold-induced reduction in rectal temperatures (**Figures 4A,B**). In addition, we found profound morphological transformation toward a brown fat phenotype and increased UCP1 staining in iWAT of Rb2 treated mice (**Figures 4C,E**), accompanied with the synergistic effects of Rb2 and cold in the induction of Pgc1α and Ucp1 in both iWAT and BAT (**Figures 4D,F**). These results suggested that Rb2 treatment enhanced adaptive thermogenesis and induced browning of white fat.

Rb2 Treatment Induced Browning of White Adipocytes

To further examine the cell autonomous effects of Rb2 on adipocytes, we examined the Rb2 treatment on differentiated 3T3-L1 and C3H10T1/2 adipocytes and differentiated SVF from iWAT of mice. As shown in **Figures 5A,B**, Rb2 dose- and time-dependently induced Ucp1 transcription in 3T3-L1 adipocytes. Furthermore, treatment of Rb2 in adipocytes strongly induced thermogenic and mitochondrial genes, including Pgc1α and Ucp1 levels in 3T3-L1 and C3H10T1/2 adipocytes, and also differentiated SVF from iWAT of mice (**Figures 5C,D**, **Figure S3**). Therefore, the data suggested the effect of Rb2 on browning of white adipocytes.

AMPK Signaling Was Dispensable for the Effects of Rb2 on Browning of White Adipose Tissues and White Adipocytes

AMPK signaling has been shown to play a critical role in energy homeostasis and obesity, which is associated with reduced AMPK activity in both WAT and BAT (28–30). Therefore, we examined AMPK signaling in Rb2 treated mice and adipocytes. Western-blot analysis showed that Rb2 supplementation increased phosphorylation of AMPK in iWAT and BAT of both chow diet-fed and DIO mice, which suggested the activation of AMPK signaling in these adipose tissues (**Figures 6A,B**, **Figure S4**). Consistently, Rb2 administration enhanced the phosphorylation of AMPK, as well as its downstream thermogenic genes Pgc1α and Ucp1 in differentiated adipocytes (**Figures 6C–E**). Furthermore, when using AMPK inhibitor compound C, the induction of AMPK phosphorylation, as well as Pgc1α and Ucp1 mRNA levels were diminished in differentiated adipocytes (**Figures 6C–E**) and SVF from iWAT of mice (**Figures 6F,G**), suggesting that AMPK was dispensable for the beneficial effects of Rb2.

DISCUSSION

Obesity is characterized by the reduced metabolic activity of brown and beige fat (31, 32), which suggests that therapies to restore brown/beige fat functionality may be effective to overcome obesity. To date, researches have shown that monomers of TCM, such as celastrol and berberine, play important roles in the remodeling of adipose tissue and prevention of obesity, suggesting the vast choices of TCM in the frontline combating obesity. Ginsenosides, as a class of natural product steroid glycosides and triterpene saponins, are able to improve lipid and glucose metabolism and reduce obesity (33). However, the molecular mechanisms have not been fully understood. Previous reports on the *in vivo* metabolic effects of ginsenosides focused mainly on liver, skeleton muscle and found that in liver, Rg1, Rg3, Rg5, and Rb2 prevented hepatic steatosis with AMPK as their possible common target (15, 18, 20, 34). Besides, Ginsenosides Rb1, Rg1, Rg5, and Re target skeleton muscle for enhancing insulin sensitivity (35–38) and ginsenosides Rb1 may target the central nervous system in obese mice to improve leptin sensitivity (39). While the effects of Ginsenosides on adipocytes are mainly recorded in the 3T3-L1 system, the present study focusing on the brown/beige fat functionality provides both *in vitro* and *in vivo* evidences that Rb2, one of the major effective monomers in ginsenosides, could reduce body weight and improve glycemic and lipid metabolism by activating brown fat functionality, promoting white adipose browning and increasing energy expenditure. Whether other ginsenosides also contribute to the browning process still needs further investigation. In our study, an appropriate dose of Rb2 supplement could be considered as a possible approach to reduce the risk of diet-induced obesity and insulin intolerance.

A complication of obesity is hyperinsulinemia. Specifically, HFD feeding can cause primary hyperinsulinemia, develop insulin resistance, and increase blood glucose levels (40). It is well-established that metabolic organs including pancreas, liver, skeleton muscle and adipose tissues are involved in glycemic control and all contribute to insulin sensitivity (41). Although we found that the Rb2 treatment led to brown fat activation and browning of white fat, it is not clear which is the major and primary organ responsible for improving insulin sensitivity observed in Rb2 treated mice, especially considering previous reports recording the beneficial effects of Rb2 in improving hepatic steatosis. Future studies with euglycemic clamp would be more informative to distinguish the role of Rb2 in glycemic control.

Obesity develops when the energy intake exceeds the energy expenditure, treatment for obesity must either reduce energy intake or increase energy expenditure, or have an effect on both at the same time. In our study, the food intake in mice treated with Rb2 showed no significant differences from the control group. Our findings indicated that Rb2 reduced the adipocytes area in DIO mice and eWAT of chow mice, but it had no effect on weight loss compared to the control group, which was also observed in other studies (42)—it further confirmed the safeness of Rb2 in chow animals. Several researches have shown that Rb2 lowered cholesterol and triacylglycerol levels in 3T3-L1 (22). This might be another important mechanism of Rb2 limits weight gain.

AMPK is a highly conserved master regulator of metabolism at both the cellular and organismal levels. AMPK is actively involved in multiple physiological and pathological processes and regulates food intake, glucose homeostasis (43), hepatic glucose production, lipid metabolism (44), energy homeostasis, body weight (45), browning of WAT, and BAT thermogenesis (46). Specifically, AMPK extensively regulates thermogenesis by

modulating the sympathetic nervous system. Genetic evidence with the specific deletion of AMPK in adipocytes led to defects in BAT mitochondrial structure, function, and reduced oxidative metabolism in response to cold exposure or β-adrenergic stimulation. Besides, loss of adipocytic AMPK exacerbated the development of hepatic steatosis and insulin resistance (47). Thus, AMPK agonists such as resveratrol and A-769662 have been applied to alleviate obesity and metabolic disorders. To investigate whether Rb2 inducing brown gene programs in adipocytes was dependent on AMPK, we inhibited the activity of AMPK via compound C in the presence or absence of Rb2. Our results showed that supplement with compound C significantly suppressed the expressions of PGC-1α and UCP1, suggesting that AMPK was at least partially responsible for the effects of Rb2 in adipocytes. Compound C is one of the most widely used compounds as AMPK inhibitor to assess AMPK signaling pathway alternations, but it has limitations. And more analysis on Rb2 mediated signaling pathways may be applied to fully elucidate the mechanisms of Rb2 in adipocytes.

In summary, the present study with both *in vivo and in vitro* evidence highlighted the beneficial effects of Rb2 in treating diet-induced obesity and insulin resistance. Our results demonstrated that Rb2 activated brown fat functionality, induced browning of white fat, and consequently increased thermogenesis and energy expenditure. We further showed that the protective effect of Rb2 against obesity might be potentially modulated by inducing AMPK phosphorylation and its downstream thermogenic and mitochondrial genes. These findings provide rationality to study the effects of other ginsenosides on the brown/beige fat functionality, and suggest the importance of potentially applying Rb2 to the treatment of obesity and metabolic functions.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found online at https://pan.baidu.com/s/1qMWWmEX-OJEdXTIcEBQ5tg.

AUTHOR CONTRIBUTIONS

XG conceived and carried out the experiments. YH and YL conceived the experiments and analyzed data. All the authors have significantly contributed to this work. Other authors were involved in writing the paper and searching literature.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00153/full#supplementary-material

Figure S1 | The effects of Rb2 treatment in chow diet-fed mice. **(A,B)** Body weights of chow diet and high fat diet-fed mice for 9 weeks. **(C)** Body weights of chow diet-fed mice treated with or without Rb2 for 10 days. Average of food intakes (g) of DIO mice during the Rb2 treatment **(D)**. **(E–H)** GTT **(E)** and ITT **(G)** analysis of chow diet-fed mice treated with or without Rb2 for 10 days. Area under the curve (AUC) of GTT and ITT was also shown as **(F,H)**. $N = 6$ per group. Data are presented as mean ± SEM.

Figure S2 | The mRNA levels of brown gene programs in iWAT and BAT of chow diet-fed mice treated with or without Rb2. **(A,B)** mRNA levels of thermogenic and mitochondrial genes in iWAT **(A)** and BAT **(B)** of chow diet-fed mice treated with or without Rb2. $N = 6$ per group. Data are presented as mean ± SEM and $^{**}P < 0.01$ compared to control group.

Figure S3 | The mRNA levels of Pgc1α and Ucp1 in differentiated C3H10T1/2 adipocytes treated with or without Rb2. Data are presented as mean ± SEM, $^{*}P < 0.05$ compared to control group.

Figure S4 | p-AMPK and AMPK protein levels in iWAT and BAT of chow diet-fed mice treated with or without Rb2. **(A,B)** Phosphorylation and total protein levels of AMPK in response to Rb2 treatment in the iWAT and BAT of chow diet-fed mice. Data are presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

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Etiology and Pathogenesis of Latent Autoimmune Diabetes in Adults (LADA) Compared to Type 2 Diabetes

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As the heterogeneity of diabetes is becoming increasingly clear, opportunities arise for more accurate assessment of factors influencing disease onset, which may lead to more efficient primary prevention. LADA – latent autoimmune diabetes in adults – is a common, hybrid form of diabetes with features of both type 1 and type 2 diabetes. This review aims to summarize current knowledge on the pathophysiological and etiological overlap and differences between LADA and type 2 diabetes, discuss similarities between LADA and type 1 diabetes and point at future research needs. Studies conducted to date show a clear genetic overlap between LADA and type 1 diabetes with a high risk conferred by variants in the human leukocyte antigen (HLA) region. In contrast, data from the limited number of studies on lifestyle factors available indicate that LADA may share several environmental risk factors with type 2 diabetes including overweight, physical inactivity, alcohol consumption (protective) and smoking. These factors are known to influence insulin sensitivity, suggesting that insulin resistance, in addition to insulin deficiency due to autoimmune destruction of the beta cells, may play a key role in the pathogenesis of LADA. Moreover, this implies that onset of LADA, similar to type 2 diabetes, to some extent could be prevented or postponed by lifestyle modification such as weight reduction and increased physical activity. The preventive potential of LADA is an important topic to elucidate in future studies, preferably intervention studies.

Keywords: LADA, type 2 diabetes, lifestyle, epidemiology, prevention

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INTRODUCTION

Diabetes is the world's fastest growing disease and a major threat to public health worldwide (NCD Risk Factor Collaboration [NCD-RisC], 2016). Since it is a chronic disease, first and secondary prevention is key in reducing its burden on individuals and society. A prerequisite for efficient primary prevention is knowledge about factors influencing disease onset. Diabetes is a more heterogeneous disease than the crude subdivision into type 1 and type 2 diabetes implies (Tuomi et al., 2014; Leslie et al., 2016; Ahlqvist et al., 2018); with a finer classification of diabetes based on differences in pathophysiology, opportunities arise for more accurate assessment of factors influencing disease onset and prognosis. This may allow us to identify etiological factors potentially obscured in analysis of heterogeneous patient groups with different pathogenesis and help us identify modifiable factors which may eventually lead to improved primary prevention. LADA – latent autoimmune diabetes in adults – is a common but understudied form of diabetes with features of both type 1 and type 2 diabetes. The aim of this review is to summarize findings regarding the role of environmental and genetic factors in the etiology of LADA, discuss potential

etiological and pathophysiological overlap between LADA and type 2 diabetes, point at similarities between LADA and type 1 diabetes and the preventive potential of LADA.

LADA

In 1977, Irvine et al. showed that 11% of individuals initially diagnosed with type 2 diabetes have antibodies against the insulin producing beta-cells, which is a characteristic of type 1 diabetes and indicative of an autoimmune pathogenesis (Irvine et al., 1977). The term LADA was introduced in 1993 by Tuomi et al. to describe this subgroup of patients that seems to share phenotypical features with type 2 diabetes and immunological features with type 1 diabetes (Tuomi et al., 1993). LADA has been estimated to account for 3–12% (Turner et al., 1997; Tuomi et al., 1999; Takeda et al., 2002; Zinman et al., 2004; Buzzetti et al., 2007; Maioli et al., 2010; Hawa et al., 2013; Rasouli et al., 2013b; Zhou et al., 2013; Maddaloni et al., 2015) of all diabetes in adults, being more frequent in Europe (Turner et al., 1997; Tuomi et al., 1999; Buzzetti et al., 2007; Maioli et al., 2010; Hawa et al., 2013; Rasouli et al., 2013b) than in other parts of the world including Asia (Takeda et al., 2002; Zhou et al., 2013; Maddaloni et al., 2015) and North America (Zinman et al., 2004). The majority of LADA studies conducted to date comes from European countries but an increasing number of studies are being conducted also outside Europe (Mishra et al., 2018).

There is no uniform agreement on the definition of LADA but diagnosis is usually based on three criteria (Fourlanos et al., 2005; Naik et al., 2009); (1) adult age at onset, (2) islet autoantibodies as a marker of autoimmune activity, (3) insulin independence. However, the exact application of these criteria varies; Onset should be in adulthood but the actual age limit varies, although 30 or 35 years is most commonly used. The slow onset that distinguishes LADA from type 1 diabetes with adult onset is typically defined as absence of insulin treatment during the first 6 or 12 months following diagnosis, but this criterion has been questioned since it is subjective and depends on the judgment of the treating physician (Brophy et al., 2008). One alternative is to use fasting C-peptide levels as an indicator of remaining insulin secretion and a "latent" onset (Takeda et al., 2002; Rasouli et al., 2016). The least controversial criterion is the assessment of autoantibodies as a marker of the autoimmune activity that separates LADA from type 2 diabetes. Glutamic acid decarboxylase antibodies (GADA) are most frequently used due to the fact that this antibody is far more common in patients with adult onset autoimmune diabetes than other autoantibodies often found in children with type 1 diabetes (Tuomi et al., 1999; Sørgjerd et al., 2012; Hawa et al., 2013).

PATHOGENESIS

Type 1 and Type 2 Diabetes

The main pathophysiological features of type 2 diabetes are insulin resistance in skeletal muscle, liver and adipose tissue, together with impaired insulin secretion (DeFronzo et al., 2015). Insulin resistance reduces peripheral glucose uptake and stimulates hepatic glucose output which leads to elevated blood glucose levels. The ensuing hyperglycemia increases the demand on the beta-cells for a compensatory rise in insulin secretion. This may consequently exhaust the beta-cells and lead to a progressive loss of beta-cell function, resulting in insulin deficiency and subsequent diabetes (DeFronzo et al., 2015). Type 1 diabetes on the other hand seems to result from a distinct pathophysiological process; its main feature is insulin deficiency which is caused by autoreactive T-cells of the immune system that destroy the pancreatic beta-cells. The autoimmune reaction leads to a progressive loss of functional beta-cell mass and declining insulin production (Burrack et al., 2017).

LADA

Similar to type 1 diabetes, LADA patients display autoantibodies which is indicative of an autoimmune pathogenesis. However, the autoimmune process seems to be milder and the progression of beta-cell failure slower; this is evidenced by the fact that LADA patients consistently display higher levels of C-peptide as indicator of insulin secretion (Carlsson et al., 2000; Hosszúfalusi et al., 2003; Hernandez et al., 2015) and furthermore, they are not insulin-requiring for some time following diagnosis. Compared to patients with type 2 diabetes, those with LADA have less insulin secretion (Turner et al., 1997; Carlsson et al., 2000; Hernandez et al., 2015; Hjort et al., 2018b) and progress to insulin dependence faster (Turner et al., 1997; Zampetti et al., 2014). As an example, data from the United Kingdom Prospective Diabetes Study (UKPDS) showed that among individuals initially diagnosed with type 2 diabetes, 84% of those who were GADA positive at diagnosis progressed to insulin dependence within 6 years compared to 14% of antibody negative individuals (Turner et al., 1997). Similar to what is seen in type 1 diabetes (Insel et al., 2015), autoimmunity seems to be the first pathological symptom in LADA; data from the prospective HUNT Study show that 64% of LADA patients where GADA positive several years prior to diagnosis (Sørgjerd et al., 2012) and similar findings were reported by Lundgren et al. in the Finnish Botnia Study (Lundgren et al., 2010).

Whereas type 1 diabetes typically is characterized by a clustering of different islet autoantibodies (Regnell and Lernmark, 2017), LADA patients tend to be positive primarily for GADA (Tuomi et al., 1999; Sørgjerd et al., 2012; Hawa et al., 2013); In Action LADA, a European multicenter study, 90% of the antibody positive patients were positive for GADA, whereas only 10% could be detected merely through antibodies to insulinoma-associated antigen-2 (IA-2A) or zinc transporter 8 (ZnT8A) (Hawa et al., 2013). Moreover, multiple antibodies were found in 24% of antibody positive patients in Action LADA (Hawa et al., 2013) and in 10% of LADA patients in the Norwegian HUNT Study (Sørgjerd et al., 2012). This indicates that at least for research purposes, it is enough to measure GADA in order to separate LADA from type 2 diabetes. The level of GADA is inversely related to C-peptide levels as a marker of insulin secretion (Radtke et al., 2009) and GADA may therefore serve as an indicator not only of the presence of autoimmune activity but also to some extent, of the magnitude of such activity.

The fact that LADA patients have more insulin production than patients with type 1 diabetes at time of diagnosis indicates that other mechanisms besides autoimmune destruction of the beta-cells may play a role in the pathogenesis. In line with this, it has been shown that LADA patients display insulin resistance (Carlsson et al., 2000; Behme et al., 2003; Chiu et al., 2007; Juhl et al., 2014; Hjort et al., 2018b), although not as pronounced as in type 2 diabetes (Zinman et al., 2004; Lundgren et al., 2010; Hjort et al., 2018b), which has been attributed to differences in BMI (Chiu et al., 2007; Juhl et al., 2014). The relative contribution of insulin resistance to the development of LADA may depend on the degree of underlying autoimmunity; both Swedish and Norwegian data show that LADA patients with less autoimmune activity as indicated by low GADA levels, tend to be more insulin resistant than those with high GADA levels (Hjort et al., 2018b).

ETIOLOGY

Genetic Factors

In type 1 diabetes, the strongest genetic influence is conferred by genes in the HLA complex which are responsible for approximately half of the genetic susceptibility (Noble et al., 1996). In contrast, the genetic influence on type 2 diabetes risk seems to be spread all across the genome and attributed to a large number of common, genetic variants – each contributing a small amount to heritability of the disease (Fuchsberger et al., 2016). The strongest effect is conferred by variants in the transcription factor 7-like 2 (*TCF7L2*) gene, which is associated with a 30% risk increase. The majority of known type 2 diabetes-related genetic risk variants are associated with insulin secretion rather than insulin resistance (Fuchsberger et al., 2016). Similar to type 1 diabetes, LADA is closely linked to genes in the HLA complex, and furthermore, the highest risk is seen in carriers of the HLA haplotypes HLA-DRB1*04-DQB1*03:02 and HLADRB1*03:01-DQB1*02:01, which also confer the highest risk of type 1 diabetes (Desai et al., 2007; Andersen et al., 2010; Pettersen et al., 2010; Mishra et al., 2017). The HLA genes encode the major histocompatibility complex (MHC) that regulates the immune system, consequently these findings thus point to a strong involvement of the immune system in the pathogenesis of LADA (Andersen and Hansen, 2018). In addition, LADA is linked with type 1 diabetes associated variants outside of the HLA region including *PTPN22*, *INS*, and *SH2B3* (Mishra et al., 2017). A genetic overlap with type 2 diabetes has also been reported; the most consistent finding is an association with type 2-associated variants of *TCF7L2* (Cervin et al., 2008; Andersen et al., 2014). This evidence comes primarily from candidate gene studies of which the largest was based on 978 LADA cases of European descent (Mishra et al., 2017) however, recently the first GWAS study on LADA was published including pooled data from 2634 patients (Cousminer et al., 2018). This study confirms that the genetic basis of LADA primarily resembles that of type 1 diabetes, but also includes genetic variants associated with type 2 diabetes. Importantly, studies conducted to date consistently show that the excess risk of LADA conferred by HLA genotypes is far stronger than the risk observed for type 2-associated genetic variants (Andersen and Hansen, 2018). In support hereof, a recent study of family history of diabetes indicated that the risk of LADA is increased six-fold in individuals with family history of type 1 diabetes compared to 2-fold in those with family history of type 2 diabetes (Hjort et al., 2017a).

Lifestyle

Type 1 and Type 2 Diabetes

Strong support for a role of environmental factors in the etiology of autoimmune diabetes is provided by the world wide rise in incidence of type 1 diabetes in children (Diamond Project Group, 2006; Mayer-Davis et al., 2017), which proposedly reflects an increasingly diabetogenic environment rather than changes in the genetic makeup of the population. Still, the environmental triggers of autoimmunity and type 1 diabetes has proved difficult to map. Associations with a number of lifestyle factors have been reported, including exposure to enterovirus, several dietary factors, weight gain and psychological stress (Rewers and Ludvigsson, 2016). Nevertheless, attempts to replicate findings across studies have often failed and intervention studies have been largely unsuccessful in preventing type 1 diabetes in children (Skyler, 2013).

More is known about the etiology of type 2 diabetes; the risk is closely linked to a number of unhealthy lifestyle factors and among those, excess weight is the strongest risk factor; obesity is associated with a seven-fold increased risk according to a meta-analysis based on 18 prospective cohort studies (Abdullah et al., 2010). Low birth weight, proposedly reflecting fetal malnutrition, is also associated with an increased risk of type 2 diabetes (Whincup et al., 2008). In contrast, physical activity is associated with a reduced risk (Smith et al., 2016), whereas sedentary time (Biswas et al., 2015) and hours of TV viewing (Patterson et al., 2018) is positively associated with incidence of type 2 diabetes. Smokers have excess risk that increases in a dose-response manner by number of cigarettes (Pan et al., 2015). Several dietary factors have also been linked to type 2 diabetes even after adjustment for BMI; Food groups associated with a reduced risk include whole grain (Schwingshackl et al., 2017), fruit and vegetables (Schwingshackl et al., 2017), alcohol (moderate vs. no intake) (Li et al., 2016) and coffee (Schwingshackl et al., 2017), while an increased risk is associated with consumption of sugar-sweetened beverages (Schwingshackl et al., 2017), and red and processed meat (Schwingshackl et al., 2017). The preventive potential of type 2 diabetes seems to be substantial; a recent study based on the China Kadoorie Biobank including >400 000 participants estimated that the combination of healthy BMI, waist-hip ratio (WHR) and diet together with non-smoking could prevent 3/4 of all cases of type 2 diabetes (Lv et al., 2017). Similar findings have been reported in Europe (Laaksonen et al., 2010) and in different ethnic groups in the United States (Steinbrecher et al., 2011). The biological mechanism linking the majority of these lifestyle factors to type 2 diabetes primarily involves promotion of insulin resistance or beneficial effects on insulin sensitivity (Petersen and Shulman, 2006). Since LADA, in addition to autoimmunity, seems to be characterized by insulin resistance it may be hypothesized that environmental or lifestyle

factors known to promote insulin resistance may also increase the risk of LADA.

LADA

There are few studies on the influence of lifestyle factors on the risk of LADA (Carlsson, 2018) and the reason for this is most likely lack of data. Prerequisites for such studies are; (a) information on incident cases of LADA which requires antibody testing in order to separate LADA from type 2 diabetes, (b) a suitable non-diabetic population for comparison, (c) detailed information on lifestyle factors from the time before diagnosis, and (d) enough patients for viable analyses. Characteristics of some of the largest individual LADA studies to date are presented in **Table 1**. From the table it is evident that few studies fulfill these criteria and consequently, the risk of LADA in relation to lifestyle factors has so far only been investigated with data from the Norwegian HUNT (Nord-TrØndelag Health) Study (Krokstad et al., 2013) which is the world's only prospective cohort study of LADA (Hjort et al., 2018b), and the Swedish ESTRID (Epidemiological Study of Risk Factors for LADA and Type 2 diabetes) Study (Rasouli et al., 2016) which is a case-control study with incident cases recruited from the ANDIS (All new diabetics in Scania) biobank (Ahlqvist et al., 2018) and incidence density sampled controls (Vandenbroucke and Pearce, 2012). Both studies are population-based and according to the most recent publication based on these data bases, the number of LADA patients is 147 in the HUNT Study and 425 in the ESTRID Study (Hjort et al., 2018b). With data from these two studies, it is possible to calculate separate risk estimates for LADA and type 2 diabetes using the same reference population, exposure and confounding assessment. Unfortunately, patients with type 1 diabetes are not included in the ESTRID-study, and the HUNT-study has too few incident cases of adult onset type 1 diabetes for viable analyses. For that reason, comparisons between LADA and type 1 diabetes has to be based on findings in children from other cohorts. There are additional, larger LADA studies based on pooled data (e.g., 40 and 46) that are not described in **Table 1**, however, neither of these studies report information on lifestyle factors. ESTRID and HUNT studies have been approved by ethical boards for medical research in Sweden and Norway and all participants gave informed consent.

Environmental factors in LADA associated with insulin resistance and type 2 diabetes

Findings of the studies on lifestyle and risk of LADA compared to type 2 diabetes conducted to date are summarized in **Figure 1**. In support of a role for insulin resistance and a type 2-like etiology, these studies indicate that LADA may share several risk factors with type 2 diabetes; Excess risk of LADA is seen in relation to overweight (Hjort et al., 2018b), adiposity (Hjort et al., 2018b), low birth weight (Hjort et al., 2015), and sweetened beverage intake (Löfvenborg et al., 2016), whereas the risk is reduced in those with moderate alcohol consumption (Rasouli et al., 2013a, 2014) and high physical activity (Carlsson et al., 2007; Hjort et al., 2018a; **Figure 1**). The associations are generally weaker for LADA than for type 2 diabetes (**Figure 1**), which is not surprising since insulin resistance can be expected to be less important in disease progression in the presence of autoimmunity and a more pronounced insulin deficiency. This is particularly evident for BMI; as shown in **Figure 2**, data from the ESTRID study (data set described in Hjort et al., 2018b) indicate that the risk of both LADA and type 2 diabetes increases progressively with BMI but more dramatically for the latter. Furthermore, the excess risk of LADA conferred by high BMI is stronger for less autoimmune LADA (GADA < median), even though it is also seen for more autoimmune LADA (GADA > median) (**Figure 2**). This is in line with findings of cross-sectional studies, including the NIRAD (Non-Insulin Requiring Autoimmune Diabetes) (Buzzetti et al., 2007) and Action LADA (Hawa et al., 2013) studies showing that LADA patients with low GADA have higher BMI and higher prevalence of the metabolic syndrome than those with high GADA levels.

With regard to underlying mechanisms, results based on the ESTRID Study indicate that BMI (Hjort et al., 2018b) and smoking (Rasouli et al., 2016) are positively associated with insulin resistance calculated by homeostasis model assessment (HOMA-IR) (The Diabetes Trials Unit, 2017) in LADA, with a similar non-significant tendency for sweetened beverage intake (Löfvenborg et al., 2016), whereas there is an inverse association between alcohol consumption (Rasouli et al., 2014), physical activity (Hjort et al., 2018a) and insulin resistance (**Table 2**). In contrast, there is no indication that the mechanism linking any of these factors to LADA includes a triggering or exacerbating effect on autoimmunity (**Table 2**). **Figures 3**, **4** displays the association between BMI and HOMA_IR and GADA in LADA and type 2 diabetes based on the ESTRID-study (data set described in Hjort et al., 2018b). These data indicate that BMI is positively associated with HOMA_IR both in patients with LADA and type 2 diabetes (**Figure 3**) which fits with previous observations (Chiu et al., 2007). GADA, on the other hand, was inversely associated with BMI among LADA patients (**Figure 4**).

Environmental factors in LADA associated with autoimmunity and type 1 diabetes

What triggers autoimmunity besides genetic factors is not clear; Psychological stress has been linked to type 1 diabetes in children (Rewers and Ludvigsson, 2016) but no such association was seen with LADA (Rasouli et al., 2017b). In line with some findings regarding type 1 diabetes in children (Stene et al., 2003; Norris et al., 2007; Niinistö et al., 2017), fatty fish is associated with a reduced risk of LADA but unrelated to type 2 diabetes in the ESTRID Study (Löfvenborg et al., 2014). A beneficial effect could hypothetically be attributed to omega 3-fatty acids which are abundant in fatty fish and possessing anti-inflammatory and immunomodulatory properties (Calder, 2013). Moreover, coffee consumption is positively associated with the risk of LADA in carriers of HLA high risk genotypes, as well as with GADA levels (Rasouli et al., 2018), which is in contrast with the reduced risk of type 2 diabetes consistently shown in high consumers of coffee (Carlström and Larsson, 2018). This may be a spurious finding but interestingly, an earlier study on type 1 diabetes with onset in adolescence showed a similar association (Virtanen et al., 1994). A mechanism remains to be established but as outlined in a recent review, several components of coffee may

TABLE 1 | Description of some of the largest individual LADA studies to date.

Reference	Study	Setting	Time period	Study design	Study participants	No. cases	LADA definition	Time since diagnosis	Genetic information	Lifestyle information
Andersen et al., 2010	Botnia	Finland	1990	Cross-sectional (with prospective part)	Patients recruited through primary care and their family members	213	≥35 years, GADA positive, no insulin ≤6 months	Not mentioned	Yes	None presented
Buzzetti et al., 2007	NIRAD	Italy	2001–2004	Cross-sectional with prevalent cases	Patients recruited through 83 diabetes clinics	193	Adult onset, GADA or IA-2A positivity, non-insulin requiring,	6 months to 5 years	Yes	None presented
Hawa et al., 2013	Action LADA	Europe	2004–2007	Cross-sectional with prevalent cases	Patients recruited through primary care or hospital settings.	598	30–70 years, GADA/ IA-2A or ZnT8A positivity, no insulin ≤6 months	<5 years	None presented	None presented
Hjort et al., 2018b	ESTRID	Sweden	2010	Case-control with incident cases and matched controls	Patients recruited through primary care and controls from population registry	425	≥35 years, GADA positive, remaining insulin production indicated by C-peptide	Median 5 months	Yes	Yes
Maddaloni et al., 2015	ICLDC database	United Arab Emirates	2013	Cross-sectional with prevalent cases	Patients attending the Imperial College London Diabetes Centre	437	>30 years, GADA or IA-2A positivity, non-insulin requiring	≤10 years	None presented	None presented
Maioli et al., 2010	–	Sardinia	2000–2005	Cross sectional	Patients recruited through five diabetes units	251	35–70 years, GADA positivity, no insulin ≤8 months	0–5 years	Yes	None presented
Rasouli et al., 2013b	HUNT	Norway	1984–2008	Prospective cohort study with incident cases	General population in the County of Nord-TrØndelag in Norway	140	≥35 years, GADA positivity	Incident cases	Yes	Yes
Takeda et al., 2002	Ehime study	Japan	1998–99	Cross-sectional study with prevalent cases	Patients recruited through hospitals	97	>20 years, GADA positivity, remaining insulin secretion indicated by C-peptide	Mean > 10 years	Yes	None presented
Turner et al., 1997	UKPDS	United Kingdom	1977–1991	Cross-sectional	Patients recruited through 23 centers	430	25–65 years, GADA/ autoantibodies toislet-cell cytoplasm (ICA) positivity	"newly diagnosed"	No	None presented
Wod et al., 2018	–	Denmark	1997–2013	Cross-sectional with prevalent cases	Patients referred to the University hospital in Odense	327	≥18 years, GADA positivity, remaining insulin secretion indicated by C-peptide	Not mentioned	None presented	None presented
Zhou et al., 2013	LADA China Study	China	2006–2010	Cross-sectional with newly diagnosed cases	Patients recruited through 46 hospitals	287	≥30 years, GADA positive, non-insulin requiring	<1 year	Yes	None presented
Zinman et al., 2004	ADOPT	United States, Europe	2000–2002	Cross-sectional with prevalent cases	Patients recruited through 488 center	174	Adult onset, GADA positivity, non-insulin requiring	3 years	None presented	None presented

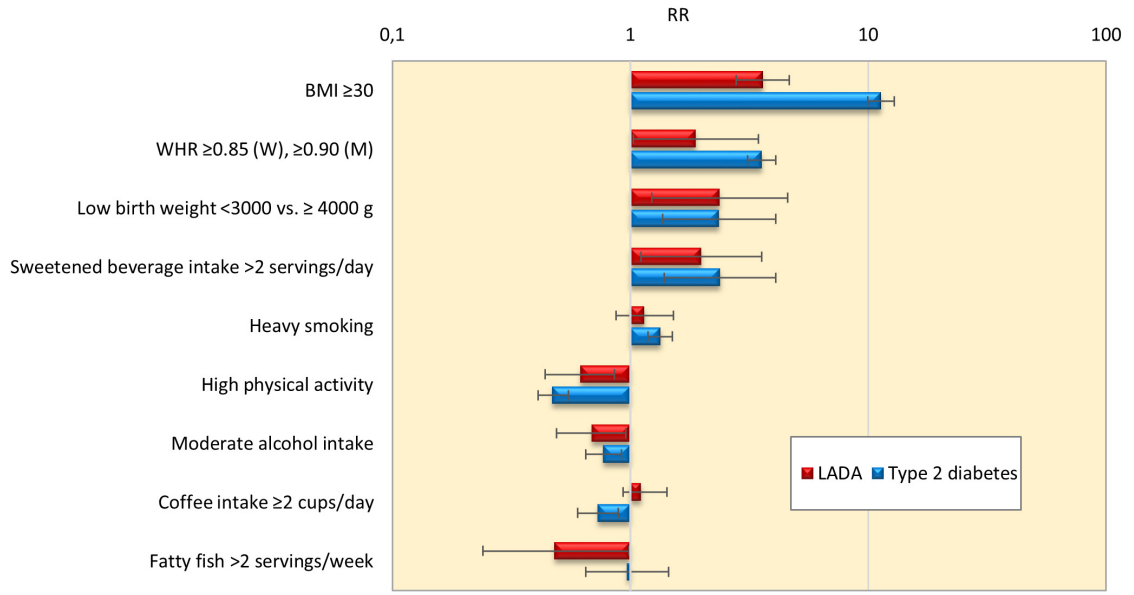

FIGURE 1 | Relative risk and 95% confidence interval for LADA and type 2 diabetes in relation to lifestyle factors. Results from ESTRID and HUNT studies. Estimates for low birth weight, sweetened beverages, coffee intake and fatty fish are based on data from the ESTRID Study (extracted from Löfvenborg et al., 2014, 2016; Hjort et al., 2015; Rasouli et al., 2018); estimates for BMI, smoking, physical activity and alcohol intake are based on pooled data from ESTRID and HUNT studies (extracted from Rasouli et al., 2013a,b, 2014, 2016; Hjort et al., 2018a,b); estimates for WHR is based on the HUNT Study (Hjort et al., 2018b).

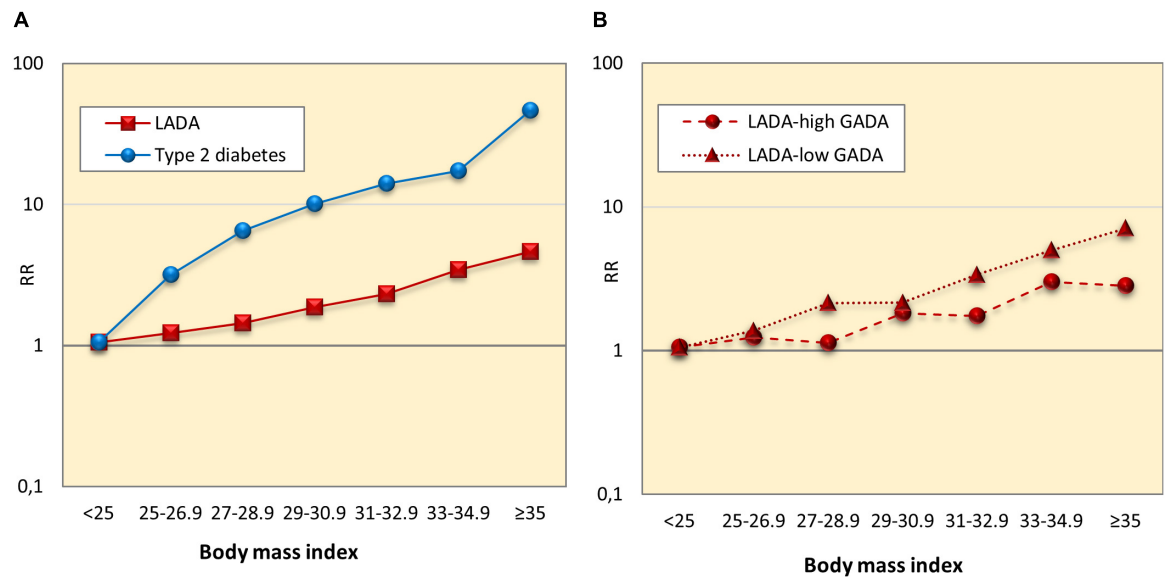

FIGURE 2 | Relative risk of LADA and type 2 diabetes **(A)**, and LADA with high and low GADA levels **(B)** in relation to Body mass index.

have immunomodulatory effects (Sharif et al., 2017). Since the HUNT Study has limited information on dietary factors, these findings are solely based on data from the ESTRID Study and should clearly be interpreted with caution.

Inconsistencies

Results were consistent across the HUNT and ESTRID-studies for BMI, physical activity and alcohol intake (**Table 2**) but not for smoking which was associated with a significantly increased risk in the Swedish data (Rasouli et al., 2016) and a reduced risk in the Norwegian data (Rasouli et al., 2013b). Previous studies have shown that whereas smoking is associated with an increased risk of type 2 diabetes, primarily attributed to negative effects of nicotine on insulin sensitivity, several studies have linked parental smoking to a reduced risk of type 1 diabetes in the offspring (Dahlquist and Källén, 1992; Magnus et al., 2018),

TABLE 2 | Lifestyle factors and associations with risk of LADA, insulin resistance and GADA, results based on HUNT and ESTRID studies.

		Norwegian HUNT Study	Swedish ESTRID Study		
		Association with LADA incidence	Association with LADA incidence	Association with insulin resistance*	Association with GADA levels*
Increased risk	Overweight/obesity (Hjort et al., 2018b)	↑	↑	↑	↓
	Low birth weight (Hjort et al., 2015)		↑		
	Smoking (Rasouli et al., 2013b, 2016)	↓	↑	↑	↓
	Coffee (Rasouli et al., 2018)		↑		↑
	Sweetened beverages (Löfvenborg et al., 2016)		↑	↑ ±	
Reduced risk	Alcohol (Rasouli et al., 2013a, 2014)	↓	↓	↓	
	Fatty fish (Löfvenborg et al., 2014)		↓		
	Physical activity (Carlsson et al., 2007; Hjort et al., 2018a)	↓	↓	↓	
No association	Serious life events (Rasouli et al., 2017b)		→		
	Smokeless tobacco use (Rasouli et al., 2017a)		→		

*↑, increased risk; ↓, reduced risk; →, no association. *,↑, Positive association or higher mean/median level in exposed than unexposed; ↓, Negative association or lower mean/median in exposed than unexposed. ± HOMA-IR 3.7 vs. 2.5, P = 0.0561 in high (>2 servings per day) compared to low consumers.*

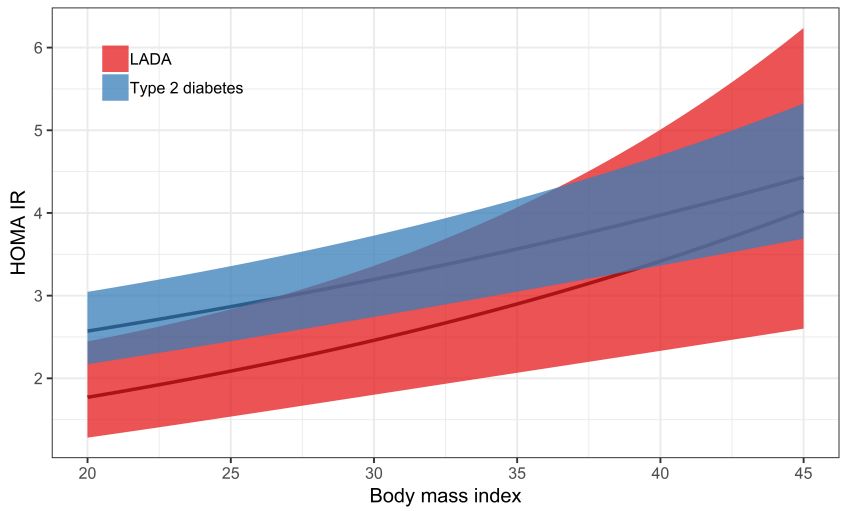

FIGURE 3 | Association between BMI and HOMA-IR in LADA and type 2 diabetes. The curved line represents results of a regression and the shaded surface represents 95% confidence intervals. Data from the ESTRID-study (data set described in Hjort et al., 2018b).

including a recent study on maternal smoking during pregnancy based on data from three different cohorts (Magnus et al., 2018). The authors speculate that a beneficial influence may be due to immune suppressive effects of nicotine (Magnus et al., 2018). Interestingly, in LADA patients, smoking is positively associated with insulin resistance and negatively associated with GADA levels (Rasouli et al., 2013b, 2016). This indicates that smoking may confer both positive and negative effects and population characteristics including degree of underlying autoimmunity may determine whether the net effect is beneficial or detrimental. The effects may also cancel out each other which may explain why we did not find an association between use of Swedish oral moist snuff and LADA (Rasouli et al., 2017a). Interaction with genetic factors may account for some of this heterogeneity; notably, strong interaction between smoking and HLA genotypes has been demonstrated in relation to rheumatoid arthritis (Kallberg et al., 2007) but this remains to be explore in relation to LADA.

DISCUSSION

Despite the autoimmune nature of LADA and clear genetic overlap with type 1 diabetes, studies on lifestyle and LADA risk indicate that factors such as overweight and physical inactivity that are associated with insulin resistance and type 2 diabetes may also promote LADA. This indicates that insulin resistance may play a key role in the pathogeneses of LADA together with autoimmune destruction of the insulin producing beta-cells. Based on current knowledge, one can create a model over LADA development where the first step in disease development is genetically triggered autoimmunity that slowly destroys the beta-cells and reduces insulin release. At a second stage, exposure to unhealthy lifestyle factors leads to insulin resistance and increased demand on the beta-cells for a compensatory rise in insulin production. Eventually the beta-cells will fail to meet the increasing

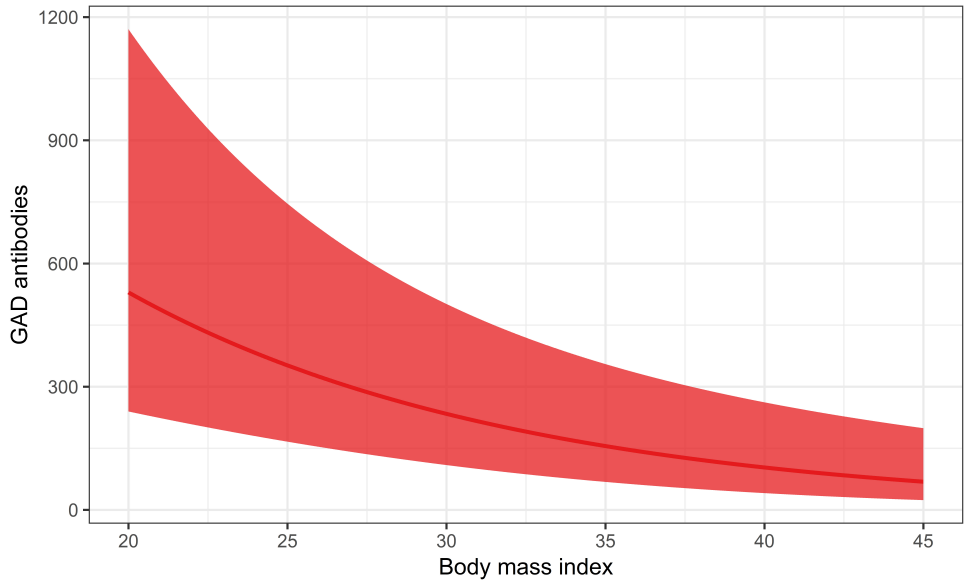

FIGURE 4 | Association between BMI and GADA levels in LADA. The curved line represents results of a regression and the shaded surface represents 95% confidence intervals. Data from the ESTRID-study (data set described in Hjort et al., 2018b).

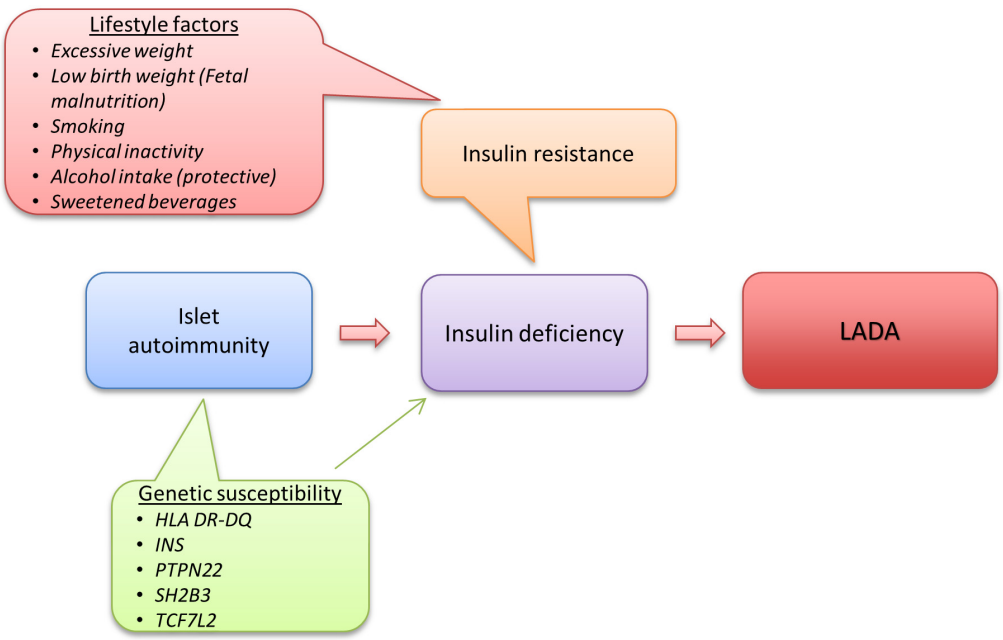

FIGURE 5 | Etiology of LADA, model based on current knowledge.

insulin need, resulting in hyperglycemia and LADA becomes manifest (**Figure 5**).

This fits with the accelerator hypothesis (Wilkin, 2001), which proposes that insulin resistance may be involved in the promotion of all forms of diabetes by stressing the beta-cells, possibly accelerating the autoimmune process as well as beta-cell apoptosis. In line with this reasoning, excessive weight has also been linked to the risk of type 1 diabetes in children (Verbeeten et al., 2011). The relative importance of insulin resistance for disease progression will most likely depend on the degree of underlying autoimmunity; if autoimmunity is severe enough, the influence of factors promoting insulin resistance is most likely minor. Consistent with this hypothesis, data from the ESTRID Study (Hjort et al., 2018b) show a negative association between GADA and HOMA-IR in LADA patients at time of diagnosis (**Figure 6**).

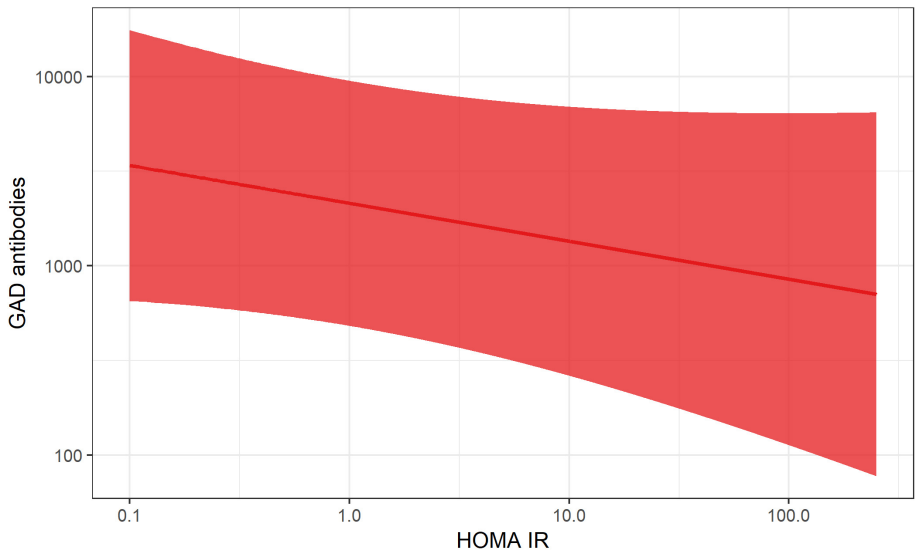

FIGURE 6 | Association between HOMA-IR and GADA levels in LADA. The curved line represents results of a regression and the shaded surface represents 95% confidence intervals. Data from the ESTRID-study (data set described in Hjort et al., 2018b).

This also fits with reasoning by Naik et al. who proposed that insulin resistance will determine at what point in the autoimmune process LADA will become manifest (Naik et al., 2009).

Opportunities for Prevention

Intervention studies show that it is possible to prevent type 2 diabetes by lifestyle modification (Tuomilehto et al., 2001; Knowler et al., 2002). In the Finnish Diabetes Prevention Study and the United States Diabetes Prevention Program, a 58% risk reduction among individuals with pre-diabetes was achieved by diet modification (e.g., increase in fiber intake and reduction of fat intake) and increased physical activity, with weight loss as the key factor behind the protective effect. A partly similar etiology of LADA suggests that it may also be possible to prevent or postpone LADA through the same lifestyle modifications. The fact that GADA has been detected in LADA patients several years prior to diagnosis suggests that LADA, similar to type 2 diabetes, has a long pre-diabetic phase (Lundgren et al., 2010; Sørgjerd et al., 2012) during which it may be possible to intervene. The preventive potential can be expected to be smaller for LADA than for type 2 diabetes where insulin resistance is the main driver of disease development. In line with this, the population attributable fraction of cases attributed to overweight/obesity in the ESTRID Study was estimated at 31% for LADA and 82% for type 2 diabetes (Hjort et al., 2018b). In order to investigate whether lifestyle intervention may reduce LADA risk, intervention studies are needed and such studies could target people with high risk, e.g., those with high genetic risk or antibody positivity similar to what has been done in type 1 diabetes (Skyler, 2013). Unfortunately, it has proved to be very difficult to prevent type 1 diabetes in children (Skyler, 2013). However, the trials conducted to date have primarily targeted factors with hypothesized effect on development and progression of autoimmunity and not insulin resistance which could prove to be fruitful in the prevention of LADA. Consistent with findings in children, adult carriers of autoantibodies are at increased risk of developing diabetes (Lundgren et al., 2010), and screening of non-diabetic individuals indicate that 0.7–4.7% of the general population display such autoantibody positivity (Ruige et al., 1997; Lundgren et al., 2010; Rolandsson et al., 2015; Sørgjerd et al., 2015). Such individuals may be suitable targets for intervention studies, under the assumption that whether a person acquires insulin resistance determines if and when the autoimmune reaction will result in hyperglycemia. Furthermore, preliminary findings based on the ESTRID and HUNT studies (Hjort et al., 2017b) indicate that the combination of HLA genotypes, in particular DR4/4, and overweight dramatically increases the risk of LADA. Carriers of these genotypes may also be considered for intervention studies focusing on weight reduction.

Much Remains to Be Explored

At this point it has to be stressed that current knowledge of the role of lifestyle factors in the etiology of LADA is still very limited. There are few studies and they are all based on data from two Scandinavian studies. The HUNT and ESTRID studies have limitations; HUNT has the advantage of being a prospective cohort study, at the same time the number of patients is small. ESTRID on the other hand, is based on more than twice as many cases but uses a case-control design which is more efficient for rare conditions, but implies that lifestyle information is collected retrospectively and this may introduce recall bias. Strengths of the studies include the detailed information on lifestyle and genetic information, careful characterization of cases and

population-based design. Moreover, the consistency of findings across the two cohorts provides support for the validity of the data at hand. Interestingly, data from the Swedish ESTRID-study suggests a protective effect of fatty fish (Löfvenborg et al., 2014) and a harmful effect of coffee consumption (Rasouli et al., 2018) on the risk of LADA, that is not seen for type 2 diabetes. Moreover, findings regarding smoking seemed to go in opposite directions in the Swedish (Rasouli et al., 2016) and Norwegian data (Rasouli et al., 2013b). Replications of these findings in other populations are clearly warranted and there are many potential risk factors that remain to be investigated. This is especially true for dietary factors. Moreover, gene*environment interaction in LADA is an important but currently unexplored field. Since lifestyle factors are likely to act on genetic susceptibility in the promotion of LADA, this is important to address if we want to understand how LADA develops.

What About Prognosis?

So what does the specific pathogenesis of LADA entail in terms of prognosis and disease management? Data from a small number of studies indicate that LADA patients have worse glycemic control than patients with type 2 diabetes (Olsson et al., 2013; Hawa et al., 2014) which may be due to the limited endogenous insulin production. Lack of established treatment guidelines may also play a role; as outlined in a recent review there is a shortage of randomized clinical trials in LADA and the optimal treatment regimen is still unknown (Hals, 2018). Moreover, in the absence of autoantibody testing, LADA patients are likely to be diagnosed as having type 2 diabetes and treated according to such recommendations, which may not be optimal. The combination of poor glycemic control and insulin resistance, together with other features of the metabolic syndrome may put individuals with LADA at high risk of complications. As summarize in a recent review (Buzzetti et al., 2017), data from the few studies conducted to date indicate that the risk of both micro- and macro vascular complications is at least as high in LADA as in type 2 diabetes patients, in spite of their generally healthier metabolic profile. The long term consequences of LADA and the role of different prognostic factors such as treatment and lifestyle are important to elucidate in order to improve secondary prevention. As pointed out by Buzzetti et al. (2017), future studies need to take into account the heterogeneous nature of LADA; the degree of underlying autoimmunity and subsequent beta-cells loss will most likely affect response to treatment as well as prognosis.

CONCLUSION

Taken together, studies conducted to date indicate that LADA, similar to type 2 diabetes, results from an imbalance between insulin sensitivity and insulin secretion. However, the relative contribution of insulin deficiency to disease progression is greater in LADA and of different origin, namely due to the same autoimmune pathogenesis underlying type 1 diabetes. The findings of an association between unhealthy lifestyle factors and LADA opens up the possibility that LADA to some extent may be prevented through the same lifestyle modifications as type 2 diabetes, including healthy diet, increased physical activity and subsequent weight loss. Intervention studies to test this hypothesis are needed and such studies may proposedly target GADA positive individuals without manifest diabetes or individuals with high genetic risk.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the datasets analyzed during the current study are available from the corresponding author on reasonable request (ESTRID) and with permission of the HUNT Study by applying to the HUNT Study data access committee. Requests to access the datasets should be directed to sofia.carlsson@ki.se.

AUTHOR CONTRIBUTIONS

SC conceived and wrote the manuscript and agreed to be accountable for its content.

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REVIEW
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Role of Bile Acids in Bariatric Surgery

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Bariatric surgery has been proved to be effective and sustainable in the long-term weight-loss and remission of metabolic disorders. However, the underlying mechanisms are still far from fully elucidated. After bariatric surgery, the gastrointestinal tract is manipulated, either anatomically or functionally, leading to changed bile acid metabolism. Accumulating evidence has shown that bile acids play a role in metabolic regulation as signaling molecules other than digestive juice. And most of the metabolism-beneficial effects are mediated through nuclear receptor FXR and membrane receptor TGR5, as well as reciprocal influence on gut microbiota. Bile diversion procedure is also performed on animals to recapitulate the benefits of bariatric surgery. It appears that bile acid alteration is an important component of bariatric surgery, and represents a promising target for the management of metabolic disorders.

Keywords: bariatric surgery, bile acids, metabolic disorders, diabetes type 2, gut microbiota

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INTRODUCTION

Bariatric surgery has been proved to be both effective and sustainable in the long-term weight-loss and remission of metabolic disorders (Adams et al., 2017; Schauer et al., 2017). However, the underlying mechanisms are still far from fully elucidated. One interesting phenomenon observed after bariatric surgery is the increase of serum total bile acids. Actually, apart from the purely restrictive procedure, adjustable gastric banding (AGB) (Kohli et al., 2013a), it has been reported that all other types of bariatric surgery are associated with postsurgical increase of serum total bile acids (Steinert et al., 2013; Albaugh et al., 2015; Ferrannini et al., 2015; Han et al., 2015). And this phenomenon has raised concern about the relationships between bile acids and bariatric surgery. Bile acids are known as the "intestinal soap," for their physiological effect on facilitating dietary fat and fat-soluble vitamin absorption. It is not until the late 1990s that bile acids had been found taking effect as signaling molecules (Makishima et al., 1999). Most of the metabolic benefits of bile acids are mediated through nuclear or membrane receptors, or interaction with gut microbiota. In the present review, we focus on two major questions: (i) why serum bile acids are increased after bariatric surgery? (ii) how the altered bile acid metabolism contributes to the metabolic benefits of bariatric surgery?

MECHANISMS OF SERUM BILE ACID ELEVATION AFTER BARIATRIC SURGERY

Bile acids are synthesized from cholesterols in the liver, stored in the gallbladder and expelled into the duodenum in response to enteral stimuli. Approximately, 95% of luminal bile acids are recycled

into the portal vein in the terminal ileum and reutilized by the liver, forming the "enterohepatic circulation." Serum bile acids represent the excess bile acids that cannot be recycled by the liver during the enterohepatic circulation and thus present in the peripheral circulation. Increased serum bile acids may derive from increased synthesis in the liver, more reabsorption in the ileum and/or decreased excretion in the feces.

In Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion (BPD), shortened route of the enterohepatic circulation (the biliopancreatic limb and the common limb) expedites the contact of luminal bile acids with the ileum, a major area for bile acid reabsorption, leading to earlier and more active bile acid reabsorption (Ferrannini et al., 2015). This explanation is also supported by an experimental procedure performed on rats termed ileal interposition (IT), in which the interposition of a segment of the ileum into the proximal jejunum shows greater bile acid reabsorption and increased serum bile acids (Cummings et al., 2013). It has been speculated that the length ratio of the biliopancreatic limb and the common limb represent a major determinant of postoperative serum bile acid concentrations. Postoperative serum bile acid concentrations are likely to be greater with longer biliopancreatic limb and shorter common limb, as evidenced by both animal (Han et al., 2015; Miyachi et al., 2016) and human studies (Mika et al., 2018). The precise mechanism has yet been elucidated. In BPD, the functional common limb left is less than one meter (Tacchino et al., 2003), and the increased luminal bile acid reabsorption cannot compensate for substantial fecal bile acid loss. Therefore, the increased hepatic bile acid synthesis also contributes to increased serum bile acids as a complementary mechanism (Scopinaro, 2006; Ferrannini et al., 2015). Compared to BPD, RYGB as well as duodenal-jejunal bypass (DJB, an experimental procedure designed to investigate the mechanism of diabetes remission with no effect on weight-loss), has enough intestinal bile acid reabsorption area, with either decreased (de Siqueira Cardinelli et al., 2019) or unchanged fecal bile acid excretion (Li et al., 2011; Bhutta et al., 2015), and the hepatic bile acid synthesis is decreased (Ferrannini et al., 2015; Zhang et al., 2016), probably due to a feedback regulation in response to the increased serum bile acids.

For vertical sleeve gastrectomy (VSG), only limited data are available regarding postsurgical serum bile acids. One human study showed immediate increase of serum bile acids after surgery (Kindel et al., 2018), while two other human studies showed unchanged serum bile acids until 1 or 2 years after surgery, with only statistically non-significant increase (Nakatani et al., 2009; Haluzikova et al., 2013). In contrast, animal studies showed consistently increased serum bile acids after VSG (Cummings et al., 2012; Myronovych et al., 2014a,b). Due to the lack of direct anatomical manipulation of the gut, the increased serum bile acids after VSG are likely to be a secondary effect of altered gastrointestinal function (Sips et al., 2018) and progressively intestinal adaption. It has been reported that VSG accelerates gastric emptying and intestinal motility (Mans et al., 2015). And Myronovych et al. (2014b) has found decreased hepatic bile acid synthesis, increased intestinal villus length and enlarged bile acid reabsorption intestinal area, which might explain the increased serum bile acids after VSG.

BILE ACIDS, FXR AND BARIATRIC SURGERY

Role of FXR in Maintaining Bile Acid Homeostasis

The farnesoid X receptor (FXR, NR1H4), an orphan member of the nuclear receptor family, was first identified as a natural receptor for bile acids in 1999 (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Subsequently, bile acids were found transcriptionally regulating their own synthesis and enterohepatic transport by repressing CYP7A1 (a rate-limiting enzyme for bile acid synthesis in the liver) via hepatic FXR-SHP-CYP7A1 pathway (Goodwin et al., 2000; Lu et al., 2000). Later, fibroblast growth factor 19 (FGF19, in rodents ortholog FGF15), secreted mainly from the ileum in response to intestinal FXR activation, was found as an enterohepatic signal to regulate bile acid homeostasis as well (Inagaki et al., 2005). Circulating FGF15/19 within the portal venous system targets FGF receptor 4 (FGFR4) in the liver, and inhibits CYP7A1 expression and bile acid synthesis, thus working as components of a gut-liver signaling pathway that synergizes with hepatic FXR-SHP-CYP7A1 pathway to regulate bile acid homeostasis (Inagaki et al., 2005).

Role of FXR in Metabolism Regulation

The metabolism-regulating effect of FXR is more complex compared to bile acid homeostasis maintenance. A variety of genetic and pharmacological animal models have been established to investigate the role of FXR signaling in body weight management and metabolism regulation. Whole body FXR knock-out mice ($FXR^{-/-}$) displayed impaired glucose disposal and elevated blood glucose concentrations, as a result of blunted insulin pathway in the liver, peripheral adipose tissue and skeletal muscles, although FXR is not expressed in skeletal muscles (Cariou et al., 2006; Ma et al., 2006). Meantime, plasma triglycerides, cholesterols and free fatty acids were elevated, with severe liver fat accumulation, however, surprisingly, this kind of mouse model was protected from diet- or genetically-induced obesity, probably because the adipocyte differentiation was impaired in the absence of FXR (Sinal et al., 2000; Cariou et al., 2006). In contrast, supplementation of whole-body FXR agonist (GW4064) in *ob/ob* mice has multiple beneficial effects, indicating that FXR could be a potential therapeutic target for metabolic disorders and bariatric surgery (Cariou et al., 2006).

Since FXR is abundant in the liver and small intestine, some studies concentrate on its tissue-specific signaling. In the liver, FXR activation lowers plasma triglycerides via FXR-SHP-(SREBP-1c) pathway (Watanabe et al., 2004), and improves hyperglycemia by inhibiting gluconeogenesis and improving insulin sensitivity (Zhang et al., 2006). In contrast, liver-specific $FXR^{-/-}$ mice showed increased plasma triglycerides, and unlike

whole-body FXR knock-out, were not protected from diet-induced obesity and insulin resistance (Watanabe et al., 2004). In the small intestine, the physiological role of FXR is primarily mediated by FGF15/19 whose metabolism-beneficial functions resemble those of insulin in stimulating protein and glycogen synthesis and inhibiting gluconeogenesis, but does not lead to body weight gain or hepatic fat accumulation (Kir et al., 2011).

Bile Acid-FXR Pathway in Bariatric Surgery

Since bile acids are natural ligands for FXR, it has been believed that bile acid-FXR pathway contribute to the beneficial effects of bariatric surgery. However, no consensus has been reached so far. The essential role of FXR in bariatric surgery was first demonstrated by a milestone research published in 2014. The ability of VSG to reduce body weight and improve glucose tolerance was substantially reduced in the state of genetic disruption of FXR (Ryan et al., 2014). Nevertheless, the results were later questioned by the authors themselves, as the whole body FXR knock-out mice *per se* were protected from diet-induced obesity as well as glucose intolerance, and the observational window might not be long enough (Bozadjieva et al., 2018). Bariatric surgery alters bile acid drainage, either anatomically or functionally, and changes luminal bile acid concentrations and composition. Activation of intestinal FXR by bile acids increases FGF19 following bariatric surgery in humans and represents one mechanism accounting for postsurgical metabolic improvement (Jansen et al., 2011; Pournaras et al., 2012; Gerhard et al., 2013; Haluzikova et al., 2013; Jorgensen et al., 2015; Sachdev et al., 2016). One human study did not support the contribution of FGF19 to the benefit of RYGB, as the increase of FGF19 did not parallel the improvement of glucose tolerance (Jorgensen et al., 2015). And animal studies have shown that both intestine-specific FXR agonist (Fexaramine) (Fang et al., 2015) and antagonist (Glycine-muricholic acid) (Jiang et al., 2015) have the capacity of reducing body weight and improving metabolism. Interestingly, alteration of luminal bile acids after bariatric surgery did not guarantee activation of intestinal FXR. On the contrary, intestinal FXR was intact or even repressed (Kohli et al., 2013b; Goncalves et al., 2015). These unexpected phenomena have been thought closely related to the intestinal bile acid milieu, as the physiological effect of each individual bile acid differs in interacting with FXR, with chenodeoxycholic acid (CDCA) being a potent agonist, deoxycholic acid (DCA) being a moderate agonist (Parks et al., 1999), yet ursodeoxycholic acid (UDCA) (Mueller et al., 2015), and muricholic acid (MCA, only in rodents) being antagonists (Jiang et al., 2015). Surprisingly, intestinal FXR repression also leads to metabolic benefits. One study reported that FXR repression within intestinal L cells led to glucagon-like peptide 1 (GLP-1) secretion hence improving glucose metabolism (Trabelsi et al., 2015). This finding may also partly explain the paradox between intestinal bile supplementation and bile deprivation (e.g., bile sequestrant), both of which have been confirmed effective in enhancing GLP-1 secretion (Trabelsi et al., 2015; Zhang et al., 2016).

It should be noted that most of the studies identifying intestinal FXR repression as a positive metabolic regulator are from animal models, suggesting that the gut may respond differently to FXR-specific targeting between animals and humans.

BILE ACIDS, TGR5, AND BARIATRIC SURGERY

G protein coupled receptor 1, also known as TGR5 (GPR131), was discovered as a membrane-type receptor for bile acids in 2002 (Maruyama et al., 2002) and has been confirmed as another essential receptor in maintaining glucose homeostasis after sleeve gastrectomy (McGavigan et al., 2017). However, one latest report revealed that the metabolic benefits were still maintained in $Tgr5^{-/-}$, but not $Fxr^{\Delta/E}$ mice after a bariatric procedure, suggesting a less important role for TGR5 as opposed to intestinal FXR (Albaugh et al., 2018). Unlike FXR, each individual bile acid is capable of activating TGR5, only with different potency (conjugated form > unconjugated form, LCA > DCA > CDCA > CA) (Maruyama et al., 2002). Therefore, after bariatric surgery, the increased luminal or serum bile acids are able to elicit TGR5-mediated metabolic benefits, and appear to make a complementary contribution to the metabolic benefits of bariatric surgery.

Luminal Bile Acids, TGR5, and Incretins

In the distal small intestine, TGR5 activation within enteroendocrine L cells in response to luminal bile acids have been confirmed as a critical signaling pathway in promoting GLP-1 secretion in both animal models (Thomas et al., 2009) and humans (Wu et al., 2013). Following bariatric surgery, as a result of gut manipulation and increased luminal bile acids, serum GLP-1 has been found ubiquitously elevated, either acutely (le Roux et al., 2007) or gradually (Borg et al., 2006), and is now referred to as an important, albeit not exclusive, mechanism of bariatric surgery in improving glucose tolerance (Madsbad and Holst, 2014). GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are known as "incretin hormones" as they mediate the incretin effect, defined as the increased stimulation of insulin secretion elicited by oral compared with intravenous administration of glucose under similar plasma glucose levels (Wu et al., 2016). In health, the incretin effect accounts for 70% of postprandial insulin secretion after oral ingestion of glucose in a glucose-dependent fashion (Nauck et al., 1986). In T2DM, the incretin effect is markedly impaired as a result of the loss of insulinotropic effect of GIP, while GLP-1 still maintains its pleiotropic physiological functions including stimulating insulin secretion, improving hepatic insulin sensitivity and suppressing appetite (Wu et al., 2014, 2016), which might account for the partially restored incretin effect after bariatric surgery (Laferrere et al., 2007). A latest "proof of concept" study

has proved that the distal, as opposed to proximal, small intestine is superior in modulating postprandial glucose metabolism in both health and T2DM, of which GLP-1 plays a major role (Zhang et al., 2019). Currently, GLP-1 analogs as well as dipeptidyl peptidase-4 (DDP4, an enzyme for degradation of incretins) inhibitors have been commercialized for the management of T2DM.

Serum Bile Acids, TGR5, and Energy Expenditure

In the skeletal muscle and brown adipose, increased serum bile acids have the capacity of converting tetraiodothyronine (T4) to triiodothyronine (T3) through TGR5, leading to more energy expenditure, which might facilitate weight loss (Kohli et al., 2013a; Broeders et al., 2015; Watanabe et al., 2006). However, the relevance between TGR5-mediated energy expenditure and bariatric surgery has not been clarified. In a human study, increased skeletal gene expressions of TGR5 downstream targets (mitochondrial COX IV and Kir6.2) were detected after RYGB, but no correlation with resting energy expenditure was found (Kohli et al., 2013a). In addition, the majority of studies suggest decreased energy expenditure after bariatric surgery, which might be a secondary effect of weight loss (Benedetti et al., 2000; Carrasco et al., 2007). In contrast, increased weight-adjusted energy expenditure was also present in some studies (Faria et al., 2012; Rabl et al., 2014). As energy metabolism is related to many factors, such as body composition, exercise or hormones, whether TGR5-mediated energy regulation plays a role in weight-loss after bariatric surgery still warrants further research.

MICROBIOTA AND BILE ACIDS

Role of Gut Microbiota in Metabolic Disorders

Gut microbiota, also named as "The Second Genome," consists of more than 10^{14} microorganisms, most of which have not been fully investigated (Turnbaugh et al., 2007). Five phyla dominate human gut community, including Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia, of which Bacteroidetes and Firmicutes account for over 90% of the whole gut microbiota (Tilg and Kaser, 2011). Along the whole gut, microbiota is not uniformly distributed. Both the abundance and diversity are increased from the proximal to distal gut (Prakash et al., 2011). In the stomach, only bacteria resistant to acidic environment survive (e.g., *Lactobacillus* or *Streptococcus* or *Veillonella*) (Dicksved et al., 2009), while in the large intestine, where the intrinsic environment is suitable for bacterial growth, lives most of the gut bacteria (Prakash et al., 2011). Gut microbiota begins to colonize since newborns, and is related to certain pathological states in adulthood. An identical twin study showed that different physiologic states (obese versus lean) are associated with distinct gut microbiota, despite the same genetic background (Turnbaugh et al., 2009). In obesity, the majority of animal and human studies have demonstrated that the diversity of gut microbiota is decreased, and Phylum Firmicutes is increased at the expense of Phylum Bacteroidetes, independent of food consumption (Ley et al., 2006; Turnbaugh et al., 2006, 2009). In T2DM, inconsistent results have been reported. Larsen et al. found that the proportions of Phylum Firmicutes and Class Clostridia were significantly reduced in T2DM, and Bacteroidetes/Firmicutes ratio was correlated positively and significantly with plasma glucose concentrations (Larsen et al., 2010). Qin et al. used a deep shotgun sequencing method analyzing gut microbial DNA from 345 Chinese individuals, and found that type 2 patients had a moderate degree of gut bacterial dysbiosis, a decline in butyrate-producing bacteria and an increase in diverse opportunistic pathological bacteria (Qin et al., 2012). They also pointed out that compared to inflammatory bowel disease (IBD), the degree of gut microbiota changes in T2DM was not that substantial. Since gut microbiota is influenced by various factors such as life style, dietary, disease, medication, and surgery, the currently available results should be interpreted with caution, and may not be used as a marker for T2DM monitoring at the moment.

Changes of Gut Microbiota After Bariatric Surgery

Bile acids and gut microbiota are reciprocally affected and are both altered by bariatric surgery. Within the enterohepatic circulation, bile acids in the distal ileum and colon undergo hydrolysis (deconjugation) and subsequent transformation into secondary bile acids. These processes require the catalysis of bile salt hydrolases (BSHs, produced by certain gut bacteria) and the bacterial activity of 7α-dehydroxylation (Ridlon et al., 2006). Meantime, both gut bile acid concentrations and compositions have direct influence on gut microbiota. Increased luminal bile acid concentrations have anti-bacterial effects, killing certain strains of bacterium and make some other bacteria (e.g., *Bilophila wadsworthia*) thrive (Noh and Gilliland, 1993; Devkota et al., 2012). Furthermore, some individual bile acids are capable of activating enteral FXR, thus leading to enteroprotection by promoting related gene expressions and inhibits bacterial overgrowth and mucosal injury in the ileum. All these effects indicate the existence of indirect influence on gut microbiota by gut bile acids (Inagaki et al., 2006). Following bariatric surgery, gut microbiota is changed. In RYGB, at the phylum level, Proteobacteria has been reported to be increased at the expense of Firmicutes (Zhang et al., 2009; Tremaroli et al., 2015), while Bacteroidetes has been found inconsistently changed, either increased (Furet et al., 2010) or decreased (Li et al., 2011; Graessler et al., 2013). In VSG, both animal and human studies showed increased Bacteroidetes and decreased Firmicutes, and rebuilt of the gut microbiota diversity (Jahansouz et al., 2017; Murphy et al., 2017; Shao et al., 2018). At the species level, the outcomes have been inconsistently reported across different studies, further increasing the difficulty of gut microbiota research.

TABLE 1 | Bile diversion procedures.

Year	Author	Journal	Model	Diversion site	Weight	Fasting glucose	Glucose tolerance	Insulin	GLP-1
1985	Manfredini G	Am J Physiol	Wistar rats	Second jejunal loop	↓ (week 1)	↓ (week 1)	↓ (week 1)	—	N/A
1991	Ermini M	Acta Diabetol Lat	STZ-induced diabetic rats	Second jejunal loop	↓ (instantly)	↓ (instantly)	N/A	↓ (instantly)	N/A
2013	Kohli R	Endocrinology	Long-Evans obese rats	15, 30 cm distal to Treitz ligament	— (week 5)	↓ (week 5)	↓ (week 5)	↓ (week 4)	↑ (week4)
2015	Flynn CR	Nat Commun	Diet-induced obese mice	Ileum	↓ (week 8)	↓ (week 4)	↓ (week 2)	↓ (week 8)	N/A
2015	Goncalves D	Ann Surg	Sprague-Dawley rats	Mid-jejunum and mid-ileum	— (day 9)	— (day 10)	↓ (day 10)	↑ (day 10)	N/A
2015	Zhang X	Obes Surg	STZ-induced diabetic rats	25 cm distal to Treitz ligament	↓ (week 8)	↓ (week 2)	↓ (week 8)	—	↑ (week8)

As to the interaction of bile acids and gut microbiota after bariatric surgery, it's similar to an old question: "which came first, the chicken or the egg?" We do not have direct and strong evidence at the moment, but assume that the instantly altered luminal bile acids following bariatric surgery might be the driving force, and the final phenotype of luminal bile acids and gut microbiota is a result of gradual interaction and adaption of each other.

Gut Microbiota After Bariatric Surgery: A Signal Carrier?

Another question to be answered is how gut microbiota affects the host after bariatric surgery. In fact, the contribution of gut microbiota to the metabolic benefits after bariatric surgery has not been clarified. Based on currently available reports, gut microbiota is likely to be a signal carrier and transmits signals that are able to play a role even in a different host. Direct evidence comes from fecal microbiota transplantation (FMT). Patients with metabolic syndrome experienced significantly increased insulin sensitivity after receiving gut microbiota from lean donors (Vrieze et al., 2012). However, the improvement of insulin sensitivity disappeared at 12 weeks after FMT, suggesting that the beneficial effect of FMT might not last in absence of sustainable exogenous stimulations. Metabolic benefits were also observed in FMT from bariatric surgery recipient donors to mice (Tremaroli et al., 2015), but we do not know whether the effect of bariatric surgery could be recapitulated by FMT in human recipient as well.

Due to technical reasons, it's difficult to observe the dynamic change of luminal bile acids and gut microbiota frequently after bariatric surgery. And a Dutch population-based cohort showed that the influence of currently realized exogenous and intrinsic host factors could only explain 18.7% of the inter-individual variation of microbial composition (Zhernakova et al., 2016). Therefore, the knowledge of gut microbiota is far from enough and warrants future research.

BILE DIVERSION PROCEDURES

In order to isolate the contribution of bile acids to the metabolic benefits of bariatric surgery, some studies have tried to manipulate bile flow directly without altering the gastrointestinal tract (**Table 1**). Bile diversion (BD) procedure in rats was performed as early as the 1980s, even before the discovery of GLP-1, to investigate the influence of re-routing of bile flow on glucose homeostasis. This early study showed 10% less weight of rats undergoing BD than control, decreased fasting blood glucose and improved glucose tolerance, with no effect on insulin secretion (Manfredini et al., 1985). Another study in 1991 using streptozocin (STZ) -induced diabetic rats showed similar results, except an immediate return of insulin to normal level after surgery (Ermini et al., 1991). It is not until 2013 that the BD procedure had regained attention. Subsequent studies have demonstrated comparable effects of BD with gastric bypass procedure in glucose control

in normal rats (Goncalves et al., 2015), diet-induced obese rats (Kohli et al., 2013b), and mice (Flynn et al., 2015). However, under diabetic state, the effects of BD were attenuated compared to DJB, with limited glucose-lowering effect observed. Therefore, to our knowledge, bile acid manipulation appears to be capable of recapitulating the effects of bariatric surgery only in non-diabetic state.

CONCLUSION

Bile acids play a role in metabolic regulation through various pathways. Alteration of bile acid metabolism is an important component of bariatric surgery, and represents a promising target for the management of metabolic disorders.

AUTHOR CONTRIBUTIONS

WW was involved in literature searching and writing of the manuscript. ZC, YW, YD, XZ, and SH were involved in conception and assist in writing. XZ was involved in conception, design, and coordination of the work. All authors critically reviewed the manuscript and have approved the publication of this final version of the manuscript. XZ and SH are the guarantors of this work.

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The Influence of the Gut Microbiome on Host Metabolism Through the Regulation of Gut Hormone Release

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The microbial community of the gut conveys significant benefits to host physiology. A clear relationship has now been established between gut bacteria and host metabolism in which microbial-mediated gut hormone release plays an important role. Within the gut lumen, bacteria produce a number of metabolites and contain structural components that act as signaling molecules to a number of cell types within the mucosa. Enteroendocrine cells within the mucosal lining of the gut synthesize and secrete a number of hormones including CCK, PYY, GLP-1, GIP, and 5-HT, which have regulatory roles in key metabolic processes such as insulin sensitivity, glucose tolerance, fat storage, and appetite. Release of these hormones can be influenced by the presence of bacteria and their metabolites within the gut and as such, microbial-mediated gut hormone release is an important component of microbial regulation of host metabolism. Dietary or pharmacological interventions which alter the gut microbiome therefore pose as potential therapeutics for the treatment of human metabolic disorders. This review aims to describe the complex interaction between intestinal microbiota and their metabolites and gut enteroendocrine cells, and highlight how the gut microbiome can influence host metabolism through the regulation of gut hormone release.

Keywords: enteroendocrine cells, microbiome, metabolism, GLP-1, PYY, GIP, serotonin, CCK

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INTRODUCTION

The gastrointestinal (GI) tract is host to a highly complex microbial ecosystem, comprising of bacteria, yeast, fungi, bacteriophages, and other viruses (Scarpellini et al., 2015), as well as protozoa and archaea (Koskinen et al., 2017; Laforest-Lapointe and Arrieta, 2018). Commensal bacteria, hereto referred to as gut microbiota, are found along the length of the GI tract and at greatest density within the caecum and colon, and along with their genes and gene products (collectively referred to as the gut microbiome), perform several functions that heavily influence host physiology. Not only does the gut microbiota play a critical role in modulating host immune defense (Belkaid and Hand, 2014) and brain function (Rogers et al., 2016), it also plays a role in regulating host

metabolism (Turnbaugh et al., 2006; Turnbaugh and Gordon, 2009; Vrieze et al., 2012, 2014; Le Chatelier et al., 2013; Nieuwdorp et al., 2014; Blaut, 2015; Hartstra et al., 2015; Seeley et al., 2015; Suarez-Zamorano et al., 2015; Aguirre et al., 2016; Pedersen et al., 2016; Molinaro et al., 2017; Rodrigues et al., 2017; Brubaker, 2018; Fabbiano et al., 2018). This is well-illustrated by the transfer of microbiota from lean and obese human twins into germ-free (GF) mice lacking a native gut microbiome, resulting in the conveyance of the metabolic phenotype of the host (Ridaura et al., 2013). Microbiota depletion in mice confers significant protection against metabolic dysregulation induced by a high-fat diet such as obesity, glucose intolerance and insulin resistance (Suarez-Zamorano et al., 2015), all of which are hallmarks of metabolic diseases including type 2 diabetes (T2D). While bacteria-mediated inflammation is associated with detrimental metabolic effects in mice (Lam et al., 2012; Molinaro et al., 2017), the underlying mechanisms by which gut microbiota influence metabolism are still not fully understood. The gut microbiome contributes significantly to host energy harvest by converting inaccessible nutrient sources such as plant polysaccharides and other complex carbohydrates, into readily absorbable metabolites (Tremaroli and Backhed, 2012). Moreover, a key link has been established between the gut microbiome and the release of several gut hormones that are important regulators of peripheral metabolism.

Within the mucosal lining of the gut, specialized enteroendocrine (EE) cells synthesize and secrete several hormones that facilitate a range of key physiological processes. Collectively, EE cells constitute the largest endocrine organ in the body (Ahlman and Nilsson, 2001), despite making up less than 1% of the total epithelial cell population in the gut. A broad number of EE cell subpopulations have been defined, based largely on their hormone expression profile (Fothergill et al., 2017). EE cells have the capacity to sense the luminal nutrient environment of the gut and are differentially responsive to many dietary compounds and luminal conditions within the intestine. Mounting evidence has highlighted that the gut microbiome influences EE cell hormone release, with downstream consequences for host metabolism and metabolic disease progression (**Figure 1**). There is also recent evidence that microbial-mediated release of gut hormones may influence other EE cell types (Lund et al., 2018), demonstrating the complexity that is the relationship between the gut microbiome, gut hormone release, and host metabolism. This review aims to describe how intestinal microbiota and their metabolites can influence host metabolism through the regulation of gut hormone release.

MICROBIAL METABOLITES SIGNAL WITH HOST CELLS

Short Chain Fatty Acids

The gut microbiota produces an array of metabolites through the breakdown of indigestible carbohydrates (**Figure 2**). The most abundant of these metabolites are the short chain fatty acids (SCFAs) acetate, propionate and butyrate (Topping and Clifton, 2001), which exist at a ratio of approximately 3:1:1 in the human intestinal lumen, respectively (Cummings et al., 1987; Mowat and Agace, 2014), however, this ratio is, at least in part, dependent upon both diet and microbial composition. The fate of these bacteria-derived SCFAs differs substantially: acetate is readily absorbed into the circulation for distribution to peripheral tissues. Propionate, on the hand, is metabolized by the liver upon absorption (Koh et al., 2016), while the majority of butyrate is consumed locally by colonocytes as their primary fuel source. While the majority of bacteria-derived SCFAs are present in the colon, lesser amounts have also been detected in the ileum of pigs, as a result of cecoileal reflux (Cuche and Malbert, 1999), and to a lesser extent, the proximal small intestine. The relative abundance of SCFA is also likely to differ along the length of the gut as a result of the region-specific microbial composition, substrate exposure, and absorption (Gu et al., 2013). For example, genes encoding carbohydrate metabolism pathways are enriched in members of the Bacteroidetes phylum, while genes encoding bile acid metabolism pathways are enriched in the bile acid-tolerance Firmicutes (David et al., 2014).

Receptors present on EE cells allow these cells to sense luminal and possibly circulating SCFA, which triggers the release of several metabolically active gut hormones. SCFA signal via two predominant mechanisms: (1) inhibition of nuclear histone deacetylase (HDAC) (Waldecker et al., 2008; Fellows et al., 2018; Larraufie et al., 2018) to alter gene transcription and expression, and (2) stimulation of G-protein-coupled free fatty acid receptors 2 and 3 (FFAR3, FFAR3), expressed throughout the length of the GI tract in distinct regional patterns. FFAR2 has equal affinity for acetate, propionate and butyrate, while the affinity of FFAR3 for acetate is substantially lower than for propionate and butyrate (Offermanns, 2014). Signaling of SCFA via FFAR2/3 is therefore dependent upon the combination of receptor type and specific metabolite abundance.

Secondary Bile Acids

Bile acids are amphipathic molecules synthesized by hepatocytes from cholesterol and are released into the GI lumen to aid the solubilization, and thus absorption, of dietary lipids. It has long been appreciated that the intestinal microbiota is directly involved in host bile acid metabolism, as effective enterohepatic recycling of bile salts is heavily reliant on deconjugation and dihydroxylation of bile acids by microbial-derived bile salt hydrolases (BSH). This gives rise to secondary bile acids (Jones et al., 2008), which are more hydrophobic and can thus be reabsorbed via passive diffusion, limiting bile acid loss through feces. Specific activity of BSH in human gut microbiota differs between different phyla, with BSH in the Firmicutes and Actinobacteria capable of metabolizing all conjugated bile salts and Bacteroidetes BSH activity being specific to tauro-conjugated bile acids (Jones et al., 2008). In addition to this role in the GI tract, bile acids are signaling molecules that are implicated in peripheral metabolism. Bile acids have major roles in peripheral metabolism through their action on two bile acid receptors, the G-protein coupled receptor TGR5 (formerly known as Gpbar1), and the nuclear receptor FXR, both

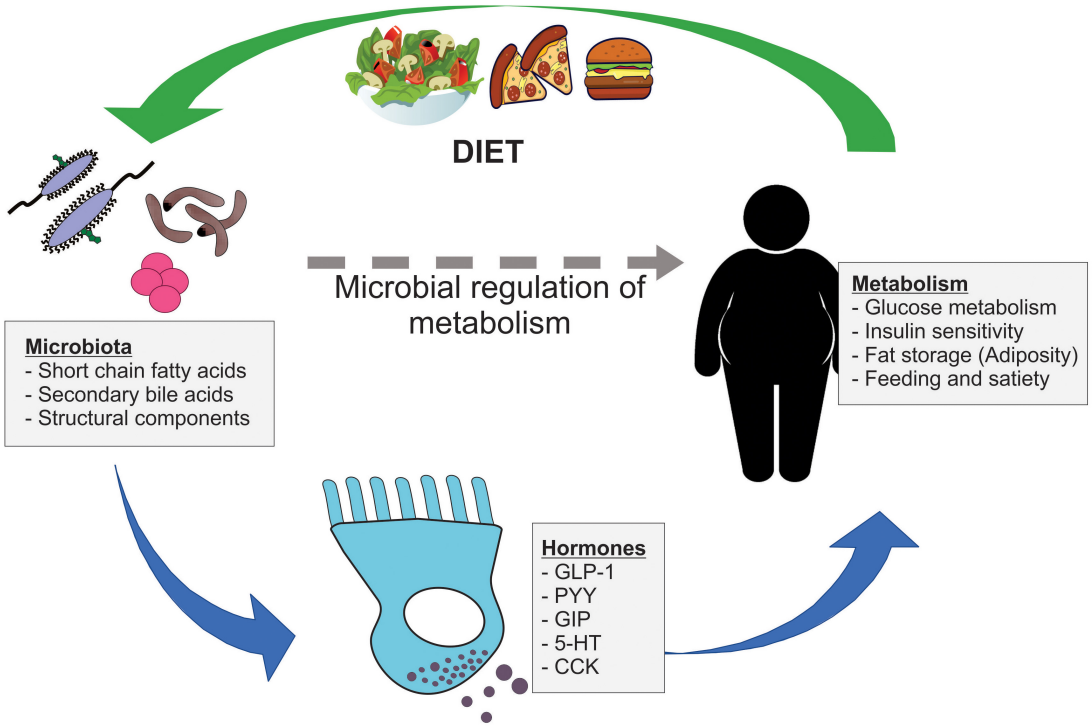

FIGURE 1 | Microbial regulation of host metabolism via gut hormone release. Gut microbiota signal to nearby enteroendocrine (EE) cells via a range of microbial metabolites, including short chain fatty acids and secondary bile acids, and structural components. These EE cells release important metabolically active hormones, such as GLP-1, PYY, GIP, 5-HT, and CCK, which influence key metabolic processes including glucose metabolism, insulin sensitivity, adiposity, and feeding behavior. In turn, dietary components impact the composition of gut microbiota, which may have further downstream consequences on gut hormone secretion and host metabolism.

of which are expressed in EE cells (**Figure 2**). Receptor affinity and potency varies substantially between different bile acids. As such, the gut microbiome can exert profound influence on host metabolism by altering the composition of the bile acid pool, through altered bile acid synthesis and re-uptake.

Cellular Recognition of Microbial Structural Components

Structural components of the microbial membrane, such as flagella and membrane-bound lipopolysaccharide (LPS), act as signaling molecules through a number of cellular pattern recognition proteins (Gordon, 2002). LPS is a cell-wall component of Gram-negative bacteria, such as members of the Bacteroidetes phylum, and is a potent ligand for toll-like receptors (TLRs), particularly toll-like receptor 4 (TLR4). In addition to powerful immunity- and inflammation-inducing effects (Takeuchi and Akira, 2002; Lancaster et al., 2018), the expression of TLRs has been demonstrated in a number of EE cells and activation of this receptor triggers secretion of a number of metabolically active hormones such as GLP-1 (Lebrun et al., 2017), 5-HT (Kidd et al., 2009), and PYY (Larraufie et al., 2017; **Figure 2**). Clinically, elevated levels of LPS (endotoxemia) are closely associated with obesity and insulin resistance (Cani et al., 2007a). Mechanisms by which LPS contribute to perturbed glycemic control and adiposity likely involve complex interactions between gut hormone secretion, mucosal barrier integrity, and host inflammation and immune pathways.

THE MICROBIOME REGULATES HOST METABOLISM VIA GUT HORMONE RELEASE

Serotonin

Enterochromaffin (EC) cells are the source of almost all (about 95%) serotonin (5-HT; 5-hydroxytryptamine) within the body. These cells constitute almost half of all EE cells and are dispersed throughout the length of the GI tract in varying densities (Raghupathi et al., 2013). EC cells have long been known to be important in many intrinsic gut mechanisms associated with motility (Keating and Spencer, 2010; Spencer et al., 2011, 2015; Spencer and Keating, 2016; Keating and Spencer, 2018) and EC cells are able to sense their local nutrient environment and respond by secreting 5-HT in a unique manner (Zelkas et al., 2015; Raghupathi et al., 2016; Thorn et al., 2016). There is now firm evidence that gut-derived 5-HT is a key driver for dysregulation of peripheral metabolism (Sumara et al., 2012; Watanabe et al., 2014; Crane et al., 2015; Young et al., 2015, 2018; Martin et al., 2017c). The absence of gut-derived 5-HT,

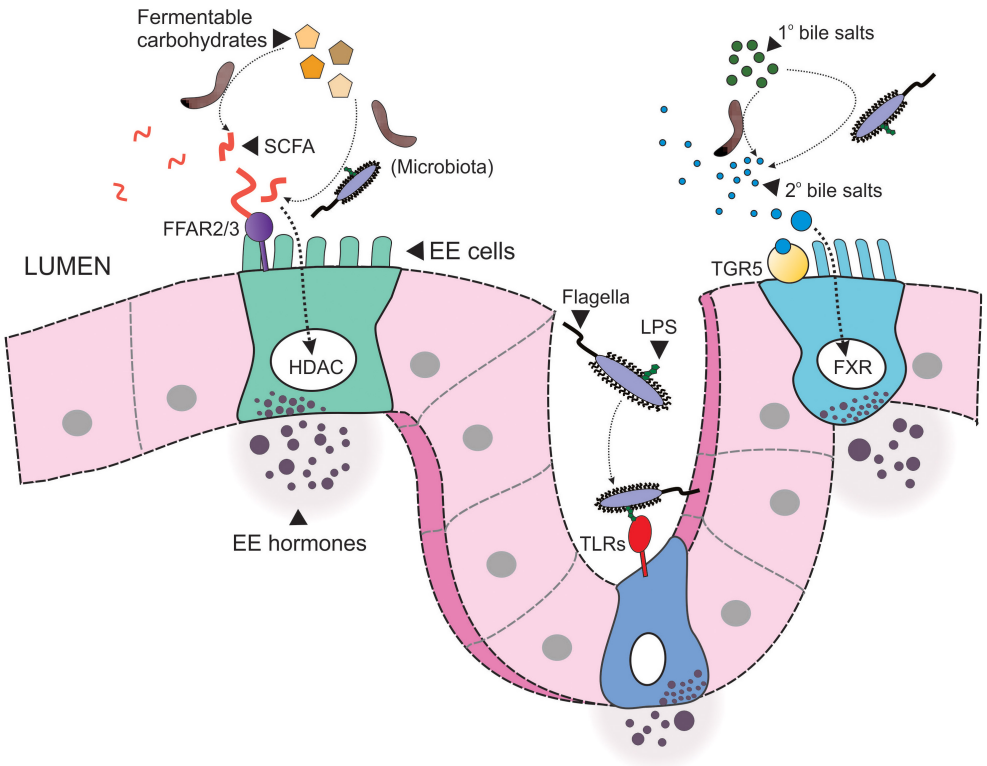

FIGURE 2 | Microbial signaling to enteroendocrine cells. Resident microbiota within the intestinal lumen signal to enteroendocrine (EE) cells via multiple pathways. Firstly, microbiota convert indigestible carbohydrates to short chain fatty acids (SCFA), which in turn signal to EE cells via free fatty acid receptors 2 or 3 (FFAR2/3) or by activation of nuclear histone deacetylases (HDAC). Secondly, microbiota convert primary (1°) bile acids to secondary (2°) bile acids, such as deoxycholate, which then signal to EE cells via the membrane G protein-coupled bile acid receptor, TGR5, or nuclear FXR. Finally, structural components of microbiota, such as flagella, and the endotoxin lipopolysaccharide (LPS), signal to toll-like receptors (TLRs).

through pharmacological inhibition or genetic ablation of the rate-limiting enzyme for 5-HT synthesis in the gut, tryptophan hydroxylase 1 (TPH1), conveys protection from diet-induced obesity in mice (Crane et al., 2015). Moreover, circulating 5-HT is increased in obese humans and is positively correlated with body mass index (Young et al., 2018) and poor glycaemic control (Takahashi et al., 2002).

The gut microbiome influences 5-HT levels in the host. GF and antibiotic-treated mice have substantially lower levels of EC cell-derived 5-HT when compared to conventionally raised (CONV-R) controls, which are restored by colonization of GF mice with donor gut microbiota (Yano et al., 2015). EC cells have the capacity to sense microbial metabolites, as they express both FFAR2 and FFAR3 (Akiba et al., 2015; Martin et al., 2017b), and a number of olfactory receptors (Bellono et al., 2017; Lund et al., 2018). Acute exposure of mouse primary EC cells to SCFA in culture does not, however, elicit an increase in 5-HT secretion (Martin et al., 2017a). Rather, the increase in 5-HT observed in the presence of a gut microbiome (Reigstad et al., 2015; Yano et al., 2015) is likely due to the chronic exposure mediating an increase in the biosynthesis of 5-HT, contributed to by increased EC cell proliferation (Yano et al., 2015). In addition, luminal butyrate infusion restores intestinal motility in GF mice, which is blunted in TPH1-KO mice, indicating the effects of butyrate may be mediated by EC cell 5-HT (Vincent et al., 2018). Acute responses to aromatic metabolites, such as isobutyrate and isovalerate, have been observed in EC cells within intestinal organoid preparations (Bellono et al., 2017), likely via olfactory receptor activation. However, it is plausible that this is an indirect response, due to the cross-talk with other gut-derived hormones such as GLP-1, which are also increased following exposure to microbial metabolites and have the capacity to signal to EC cells (Lund et al., 2018).

GLP-1

Glucagon-like peptide 1 (GLP-1), a cleavage product of proglucagon, is secreted by L-cells predominantly located in the ileum and colon. GLP-1 is an incretin hormone (Kreymann et al., 1987), released postprandially and in response to nutrients such as glucose (Sun et al., 2017) to augment insulin and inhibit glucagon secretion from the pancreas (Grondahl et al., 2017). In addition, GLP-1 inhibits gastric emptying and influences satiety and food intake (Holst, 2007). Together with PYY, GLP-1 is thought to underlie some of the metabolic gains observed following gastric bypass surgery (Madsbad and Holst, 2014) and the action of GLP-1 underlies some of the glucose-lowering ability of the diabetes therapy, metformin (Bahne et al., 2018). As such, GLP-1-targeted therapeutics including GLP-1 analogs

and inhibitors of GLP-1 degradation by the enzyme dipeptidyl peptidase IV (DPP-4) have been extensively exploited for their anti-diabetic properties (Aroda et al., 2012). Interestingly, the microbiota possess DPP-4-like activity (Olivares et al., 2018b), which in mice is reduced by administration of the DPP-4 inhibitor, vildagliptin, and is accompanied by a shift in microbial composition that is independent of the direct effects of DPP-4 inhibition on microbiota function (Olivares et al., 2018a). Specifically, vildagliptin treatment is associated with a decrease in the abundance of *Oscillibacter* and increased in the abundance of *Lactobacillus*, with a reduction in TLR ligands and an increase in propionate (Olivares et al., 2018a). Thus, the DPP-4-like activity of intestinal bacteria can potentially influence the levels of circulating GLP-1 and PYY, which in turn may exist as a feedback loop to influence microbial composition and microbial metabolite abundance.

A dynamic relationship exists between L-cells and the gut microbiome. Bile acid-mediated activation of TGR5 (Bala et al., 2014), SCFA signaling (Tolhurst et al., 2012), LPS, and other metabolites such as indole (Chimerel et al., 2014) are all potent GLP-1 secretagogues. The regulation of GLP-1 by secondary bile acids is dependent upon the receptor-signaling pathway involved, as activation of TGR5 increases GLP-1 secretion while, on the other hand, activation of FXR reduces GLP-1 secretion. This dynamic relationship is made even more complex by TGR5-FXR cross-talk that exists between these two receptors, particularly in the colon (Pathak et al., 2017). Luminal infusion of the bile acid chenodeoxycholic acid, in rats, triggers release of GLP-1 into the vasculature, in addition to PYY, via TGR5 (Kuhre et al., 2018). Indole, another major bacterial metabolite derived from dietary tryptophan, acutely stimulates GLP-1 by prolonging cellular action potential duration (Chimerel et al., 2014). Conversely, chronic exposure to indole dose-dependently decreases GLP-1 secretion in primary murine L cells by inhibiting ATP synthesis pathway (Chimerel et al., 2014). In addition, microbial LPS triggers GLP-1 secretion via TLR4 following mucosal barrier injury (Lebrun et al., 2017), and as such, glucose-stimulated insulin secretion in a mouse model of endotoxemia (Nguyen et al., 2014). Dietary prebiotics such as oligofructose, which increase bacterial SCFA production, are associated with upregulated L-cell differentiation and GLP-1 content in the rat proximal colon, and reduces weight gain when administered before and during a HFD (Cani et al., 2007b). Increasing L-cell numbers, whereby increasing post-prandial GLP-1 release, are also associated with enhanced satiety and reduced adiposity (Cani et al., 2007b). The mechanisms by which SCFA increase GLP-1 secretion are region-dependent, as signaling in the small intestine is predominantly via FFAR3, whereas FFAR2-mediated GLP-1 release occurs in the colon (Greiner and Backhed, 2016).

Paradoxically, GF and antibiotic-treated mice have higher circulating GLP-1 levels during fasting (Zarrinpar et al., 2018) and reduced mucosal GLP-1 content (Duca et al., 2012), compared to genetically identical CONV-R mice. A recent study (Arora et al., 2018) reported that the gene expression profile of ileal L-cells derived from GF mice differed substantially from CONV-R mice. Notably, many of the genes regulating L-cell functional capacity are upregulated in GF mice and L-cells have a greater number of secretory vesicles in GF mice. What underlies these differences in GF mice is unknown but may be a reflection of GLP-1 resistance, which is observed in diet-induced obesity and associated with altered microbiota composition, particularly in the ileum (Grasset et al., 2017).

PYY

Peptide tyrosine-tyrosine (PYY) is synthesized and secreted by L-cells, in addition to GLP-1, and is predominantly expressed in the lower small intestine and colon. PYY regulates food intake and satiety through activation of central G protein-coupled Y2 receptors on neuropeptide Y (NPY) and AgRP neurons in the hypothalamic arcuate nucleus (Dumont et al., 1995). This initiates a signaling cascade whereby appetite-stimulating NPY neurons are suppressed, allowing for the disinhibition of the satiety-inducing proopiomelanocortin (POMC)/α-MSH pathway (Loh et al., 2015). Obese humans have reduced circulating PYY (Batterham et al., 2006), as a result of attenuated colonic PYY secretion (le Roux et al., 2006), rather than PYY-resistance (Batterham et al., 2003). Circulating PYY exists as two forms: PYY_{1-36} and the DPP-4-cleaved PYY_{3-36}, with the latter being the most dominant postprandial circulating form (Grandt et al., 1994) and the most biologically potent with respect to its anorectic effects (Chelikani et al., 2006).

The ability of gut microbiota to influence PYY secretion therefore has significant implications for the development of obesity and metabolic disease. Microbial SCFAs, particularly butyrate, cause a dose- and time-dependent increase in *PYY* gene expression in two EE model cell lines and in primary human colonic cell cultures (Larraufie et al., 2018). In addition, oral administration of butyrate moderately increases circulating PYY (Lin et al., 2012). The mechanisms by which SCFA increase the biosynthesis of PYY appear to be via a combination of FFAR2/3 signaling by all SCFA, and inhibition of HCAD by propionate and butyrate (Larraufie et al., 2018). Although, these mechanisms appear to be species-specific (Larraufie et al., 2018) and were not accompanied by an increase in GLP-1 secretion that is seen following exposure of primary mouse colonic cultures to propionate (Psichas et al., 2015). The use of a FFAR2 knockout mouse demonstrates the involvement of SCFA signaling in increasing the number of PYY-containing cells, particularly in mice exposed to a diet rich in the SCFA-precursor, inulin (Brooks et al., 2017). Alteration of the human gut microbiota through a 4-day broad-spectrum antibiotic regimen, acutely and reversibly increased postprandial plasma PYY (Mikkelsen et al., 2015). However, the precise alterations in microbial metabolites and bacterial species that underlie this change are unknown. Secondary bile acids are also potent stimuli for PYY secretion and the mechanisms by which this occurs are consistent with those for GLP-1 secretion (Kuhre et al., 2018). Luminal perfusion of a mixture of both primary and secondary bile acids into a vascularly perfused rat lumen increases venous effluent PYY levels in a TGR5-dependent manner, while the same effect was observed with infusion of the secondary bile acid CDCA alone (Kuhre et al., 2018).

GIP

Glucose-dependent insulinotropic peptide (GIP), also known as gastric inhibitory peptide, is an incretin hormone released postprandially in the small intestine from classically defined K cells (Buffa et al., 1975). The activity of GIP is conveyed through GIP receptors (GIPR) expressed in pancreatic β-cells (Gremlich et al., 1995), adipocytes (Yip et al., 1998), bone cells (Bollag et al., 2000), and in neurons of the CNS (Paratore et al., 2011). Similar to GLP-1, the biological activity of GIP is rapidly attenuated by enzymatic breakdown by DPP-IV (Baggio and Drucker, 2007). Within the pancreas, GIP contributes significantly to postprandial insulin secretion, through increased insulin biosynthesis (Baggio and Drucker, 2007) and upregulated β-cell proliferation (Widenmaier et al., 2009). Defective GIP-signaling is believed to underlie, at least in part, the attenuated glucose-stimulated insulin secretion seen in T2D individuals (Vilsboll et al., 2002). GIP is also widely considered an adipogenic hormone (Thondam et al., 2017) as it promotes lipid uptake and storage in adipocytes (Getty-Kaushik et al., 2006).

Elevated GIP level was associated with the observed increased adiposity induced by a sub-therapeutic antibiotic regimen administered to mice at weaning for 7-weeks, as the treatment did not alter plasma levels of other gut hormones (Cho et al., 2012). The treatment significantly increased Firmicutes/Bacteroidetes ratio and caecal SCFA levels, which could potentially underlie the increased GIP level and thus, the increased adiposity. However, recent contradictory evidence demonstrates that carbohydrates within the lumen inhibit GIP secretion, via the microbial SCFA-FFAR3 signaling pathway (Lee et al., 2018). Whether the discrepancy seen across these studies is due to differential signaling via FFAR2 and FFAR3 is unknown. Consistent with specific receptor pathways for SCFA-mediated gut hormone release, oral administration of sodium butyrate into mice has been shown to transiently increase GIP and GLP-1 secretion, while sodium pyruvate and a SCFA cocktail are selective for increased GIP, but not GLP-1 or PYY (Lin et al., 2012).

CCK

Cholecystokinin (CCK) is derived from the classically named "I cells" predominantly localized to the upper small intestine (Dockray, 2012). CCK is released in response to dietary fat and protein intake. CCK has well-defined roles in appetite regulation (Ritter, 2004; Becskei et al., 2007), gastric emptying and motility (Raybould and Tache, 1988; Raybould, 1991; Ellis et al., 2013) and the release of bile acids and pancreatic enzymes that are important for digestion (Li and Owyang, 1994; Li and Owyang, 1996; Owyang and Logsdon, 2004), through activation of CCK receptors (Rogers and Hermann, 2008). Less is known about gut microbial regulation of CCK compared to other gut hormones, largely due to the exposure of CCK-containing cells to microbiota limited to the small intestine. In pigs, ileal infusion of the SCFAs acetate, propionate and butyrate during feeding increased plasma CCK levels and paradoxically inhibits pancreatic secretion (Sileikiene et al., 2008). Limited investigations have been undertaken into microbial regulation of CCK in humans, however. One report from Roux-en-Y gastric bypass patients has revealed no changes in circulating CCK levels across normal weight and obese individuals pre- and post-surgery, despite a significant shift in microbial composition following surgery (Federico et al., 2016). However, reduced CCK protein expression is observed in dissociated cells from the proximal small intestine of GF mice, which was not due to reduced numbers of EE cells (Duca et al., 2012).

Diet Influences Gut Microbiota Composition

The diversity of gut microbiota and relative abundance of microbial metabolites (metabolomic profile) is heavily dependent on specific dietary components (David et al., 2014), as evidenced by the substantial difference in microbial communities with consumption of plant-rich or protein-rich diets. This is due to the nutrient-induced selective pressures placed on microbiota, favoring bacterial species enrichened in the genes required for specific substrate metabolism. For example, plant-based diets and intake of probiotics increases luminal fiber and complex carbohydrate content, whereby selecting for species enriched in carbohydrate-active enzymes (David et al., 2014). Animal-based diets rich in fats and proteins and low in fiber increase luminal bile acid content, favoring bile acid-resistant microbes enriched with genes for bile acid metabolism, such as bile acid hydrosases and sulfite reductase (Devkota et al., 2012; David et al., 2014). Dietary fiber is also a major influence on gut transit (reviewed in detail by Muller et al., 2018), which, in turn, is an important determinant of fecal microbiome composition and metabolism (Roager et al., 2016; Vandeputte et al., 2016). In the absence of dietary fiber, however, a compensatory shift in the gut microbiome has been observed, with an increase in populations expressing mucin-degrading enzymes, suggesting an overall microbial preference for fiber-based substrates (Desai et al., 2016).

The increased consumption of non-nutritive sweeteners (NNS), such as saccharin, sucralose and aspartame, while being acutely beneficial for reducing caloric intake and blood glucose excursions, also has long-term consequences for microbiome composition and glucose intolerance. Notably, consumption of common NNS has been demonstrated in mice to exacerbate the development of glucose intolerance (Suez et al., 2014, 2015) and weight gain in males (Bian et al., 2017), which is mediated by distinct functional alterations to the gut microbiome (Suez et al., 2014). Specifically, the NNS saccharin and acesulfame-K increased the abundance of members of Bacteroidetes (Suez et al., 2014; Bian et al., 2017) and reduced abundance of Firmicutes (Suez et al., 2014). The effects of NNS on metabolism and microbial composition in humans is largely dictated by the native microbial composition prior to NNS exposure (Suez et al., 2014), while in mice, the response also appears to be gender-specific (Bian et al., 2017). Nevertheless, the NNS-induced changes in microbial composition observed in these studies is consistent with the microbial composition seen with obesity and metabolic disease (Ley et al., 2006; Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009).

The richness and diversity of the human gut microbiome correlates with metabolic function (Le Chatelier et al., 2013; David et al., 2014; Dao et al., 2016) and a core obesogenic microbiome has been established, characterized by a high ratio of Firmicutes to Bacteroidetes (Ley et al., 2006; Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009). Shifting the composition of the microbiome to reduce the abundance of Bacteroidetes, as seen with short-term caloric restriction, conveys improved metabolic outcomes in mice as a result of decreased LPS production and reduced TLR4 signaling (Fabbiano et al., 2018). There are also large-scale observational studies that linked antibiotic use to risk of type 2 diabetes (Boursi et al., 2015; Mikkelsen et al., 2015), although caution should be excised in interpreting these results, as hyperglycaemia is a risk factor for infection (Falagas and Kompoti, 2006) and may thus warrant increased antibiotic use. In addition, specific clinically used antibiotics, particularly vancomycin-imipenem and ciprofloxacin, induce differential effects on microbiome composition and microbial metabolite abundance following regrowth (Choo et al., 2017), which may have downstream implications for antibiotic-specific effects on metabolism. Ingestion of xenobiotics, such as pharmaceuticals and environmental chemicals, has the potential to modify gut microbial composition with downstream consequences for metabolism. This is evidenced by the diabetes drug, metformin, for which a shift in gut microbial composition is in part responsible for its therapeutic effects (Wu et al., 2017). The metabolism of xenobiotics by gut microbiota is also chemically distinct (Koppel et al., 2017), which highlights the gut microbiome as a possible tool for targeted drug design and delivery. Recent work by Vangay et al. (2018) has elegantly shown that migration from a non-Westernized culture to a Westernized culture rapidly and inter-generationally impacts the diversity of gut microbiota. This loss of microbial complexity and biodiversity resulted in a loss of key microbial enzymes required for plant fiber digestion, partly attributed to altered dietary composition and reduced food diversity, and may predispose individuals to metabolic disease (Vangay et al., 2018). As such, interventions to shift gut microbiota composition may be a powerful therapeutic tool for the treatment of obesity and metabolic disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Glucagon Receptor Signaling and Lipid Metabolism

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Glucagon is secreted from the pancreatic alpha cells upon hypoglycemia and stimulates hepatic glucose production. Type 2 diabetes is associated with dysregulated glucagon secretion, and increased glucagon concentrations contribute to the diabetic hyperglycemia. Antagonists of the glucagon receptor have been considered as glucose-lowering therapy in type 2 diabetes patients, but their clinical applicability has been questioned because of reports of therapy-induced increments in liver fat content and increased plasma concentrations of low-density lipoprotein. Conversely, in animal models, increased glucagon receptor signaling has been linked to improved lipid metabolism. Glucagon acts primarily on the liver and by regulating hepatic lipid metabolism glucagon may reduce hepatic lipid accumulation and decrease hepatic lipid secretion. Regarding whole-body lipid metabolism, it is controversial to what extent glucagon influences lipolysis in adipose tissue, particularly in humans. Glucagon receptor agonists combined with glucagon-like peptide 1 receptor agonists (dual agonists) improve dyslipidemia and reduce hepatic steatosis. Collectively, emerging data support an essential role of glucagon for lipid metabolism.

Keywords: glucagon, lipid, liver, adipose tissue, alpha cell

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INTRODUCTION

Glucagon is processed from its precursor, proglucagon, by prohormone convertase 2 and secreted from pancreatic alpha cells (Rouille et al., 1994). The role of glucagon in glucose metabolism has been intensively studied, and comprehensive reviews are found elsewhere (Jiang and Zhang, 2003; Ramnanan et al., 2011; Ahren, 2015; Holst et al., 2017a). In addition to regulating glucose metabolism, glucagon also seems important for minute-to-minute regulation of amino acid metabolism as part of the recently described liver-alpha cell axis (Solloway et al., 2015; Dean et al., 2017; Galsgaard et al., 2017; Holst et al., 2017b; Kim et al., 2017), in which amino acids stimulate glucagon secretion and glucagon in turn stimulates hepatic amino acid uptake and metabolism (ureagenesis) and, thus, circulating amino acid concentrations as well as increased hepatic NADH/NAD^+ ratio. The actions of glucagon are mediated via the glucagon receptor,

a seven transmembrane receptor coupled to $G_{\alpha s}$- and G_q-proteins, which regulate adenylate cyclase (AC) and phospholipase C activities when activated (Wakelam et al., 1986; Jelinek et al., 1993; Aromataris et al., 2006). The glucagon receptor is primarily expressed in the liver, but it is also expressed in varying amounts in the central nervous system, kidneys, gastro-intestinal tract, heart (controversial), and pancreas (Svoboda et al., 1994).

Glucagon receptor expression has been reported in rat adipocytes (Svoboda et al., 1994; Hansen et al., 1995), where a lipolytic effect of glucagon may be of physiological relevance. As type 2 diabetic hyperglucagonaemia (Faerch et al., 2016) contributes to the hyperglycemic state of patients with type 2 diabetes (T2D) (Unger and Orci, 1975; Baron et al., 1987), inhibition of glucagon receptor signaling has been investigated as glucose-lowering therapy in T2D patients (Kazda et al., 2016; Kazierad et al., 2016, 2018; Vajda et al., 2017; Pettus et al., 2018). Interestingly, potential adverse effects of this therapeutic approach include increased low-density lipoprotein (LDL) plasma concentrations and increased hepatic fat accumulation (Guzman et al., 2017). Furthermore, hepatocyte studies have shown that glucagon stimulates beta-oxidation (Pegorier et al., 1989), inhibits lipogenesis and decrease triglyceride (TG) and very-low-density lipoprotein (VLDL) secretion (Guettet et al., 1988; Bobe et al., 2003) emphasizing a potentially important role of glucagon in lipid metabolism.

GLUCAGON MIGHT STIMULATE LIPOLYSIS IN ADIPOSE TISSUE IN RODENTS BUT NOT IN HUMANS

Lipolysis in adipocytes depends on activation of AC and thereby increased protein kinase A (PKA) activity. PKA phosphorylates (hence activates) perilipins (Greenberg et al., 1991) and hormone-sensitive lipase (HSL) (Stralfors et al., 1984; Garton et al., 1988; Anthonsen et al., 1998), and two additional lipases, resulting in hydrolysis of TGs and release of glycerol and free fatty acids (FFAs), e.g., palmitate (Egan et al., 1992; Lass et al., 2006; Granneman et al., 2009; Shen et al., 2009; Wang et al., 2009; **Figure 1**). Circulating levels of FFAs and glycerol therefore reflect the rate of lipolysis (Schweiger et al., 2014). For glucagon to directly influence adipocyte function, its cognate receptor must be expressed. Glucagon receptor mRNA has been detected in rat adipocytes (Svoboda et al., 1994; Hansen et al., 1995), but to determine the physiological relevance of glucagon receptor mRNA expression, it is necessary to investigate whether the mRNA is actually translated into a functional receptor. Specific antibodies directed against the glucagon receptor are necessary in addressing this question, but development of specific antibodies against glucagon receptors has been challenging and the antibodies available are unspecific and therefore not suitable for receptor localization (van der Woning et al., 2016). As an example, one study reported localization of the glucagon receptor in rat adipocytes using a monoclonal antibody (Iwanij and Vincent, 1990) whereas another using autoradiography, glucagon receptors were not found to be expressed (Watanabe et al., 1998), and no studies have demonstrated presence of glucagon receptors on human adipocytes (Carranza et al., 1993). Clearly, future studies should investigate glucagon receptor expression using antibody and antibody-independent methods.

Glucagon has been reported to activate HSL (Vaughan et al., 1964; Slavin et al., 1994) and lipolysis in rat adipocytes (Vaughan and Steinberg, 1963; Rodbell and Jones, 1966; Prigge and Grande, 1971; Manganiello and Vaughan, 1972; Lefebvre et al., 1973; Livingston et al., 1974) within minutes (Honnor et al., 1985) at concentrations as low as 6×10^{-10} M (Lefebvre and Luyckx, 1969) and 10^{-11} M (Heckemeyer et al., 1983). Glucagon has also been shown to stimulate lipolysis in birds, rabbits (Richter et al., 1989; Wu et al., 1990), and human adipocytes *in vitro* (Perea et al., 1995) at concentrations near 10^{-8} M (Richter et al., 1989). At physiological plasma concentrations (1–40 pM), a lipolytic effect of glucagon in human adipocytes has been difficult to demonstrate (Mosinger et al., 1965; Vizek et al., 1979; Gravholt et al., 2001). One of the first human studies reporting a lipolytic effect of glucagon, demonstrated that an injection of 7.5 μg glucagon into the branchial artery resulted in a rapid increase in FFA plasma concentrations in the corresponding vein (Pozza et al., 1971) but this was not replicated in a similar study with mean increases of glucagon plasma concentrations by 237 pM in overnight fasted subjects (Pozefsky et al., 1976). An increase in FFA plasma concentrations has been demonstrated upon glucagon infusion (mean glucagon increment 209 ± 15 pM) (Schneider et al., 1981) and intravenous injection of glucagon [reaching plasma concentrations of >1,000 pM (Schade and Eaton, 1975)]. Since supra-physiological glucagon concentrations were applied, these studies may lack specificity because of interaction of glucagon with other related G protein-coupled receptors (e.g., the glucagon-like peptide 1 (GLP-1) receptor) (Hjorth et al., 1994). Pharmacological concentrations of glucagon also stimulate secretion of catecholamines and growth hormone, both of which have powerful lipolytic effects (Mitchell et al., 1969; Stallknecht et al., 1995), possibly as part of a generalized sympathetic nervous system discharge (Paschoalini and Migliorini, 1990). Glucagon was not found to have any lipolytic effects in clinical studies using glucagon concentrations ranging from 19 to 64 pM (Wu et al., 1990; Jensen et al., 1991; Gravholt et al., 2001; Xiao et al., 2011). In some clinical studies investigating the lipolytic effect of supra-physiological glucagon concentrations, the lipolytic effect of glucagon could be abolished by insulin (Samols et al., 1965; Goldfine et al., 1972; Liljenquist et al., 1974; Schade and Eaton, 1975; Schneider et al., 1981), and in rat adipocytes insulin is a potent inhibitor of lipolysis (Rodbell and Jones, 1966; Lefebvre and Luyckx, 1969; Prigge and Grande, 1971; Liljenquist et al., 1974; Gerich et al., 1976). A lipolytic effect of glucagon, if any, on human adipocytes may therefore only be physiologically relevant when insulin secretion is low. Supporting this, a 2-h infusion of 1 ng/kg × min glucagon (presumably resulting in physiologically relevant elevations) and somatostatin in insulin-deficient diabetic subjects caused a two to three-fold increase in FFA and glycerol plasma concentrations, compared to infusion of somatostatin alone. However, when insulin, somatostatin, and glucagon were infused together, glucagon had no lipolytic effect

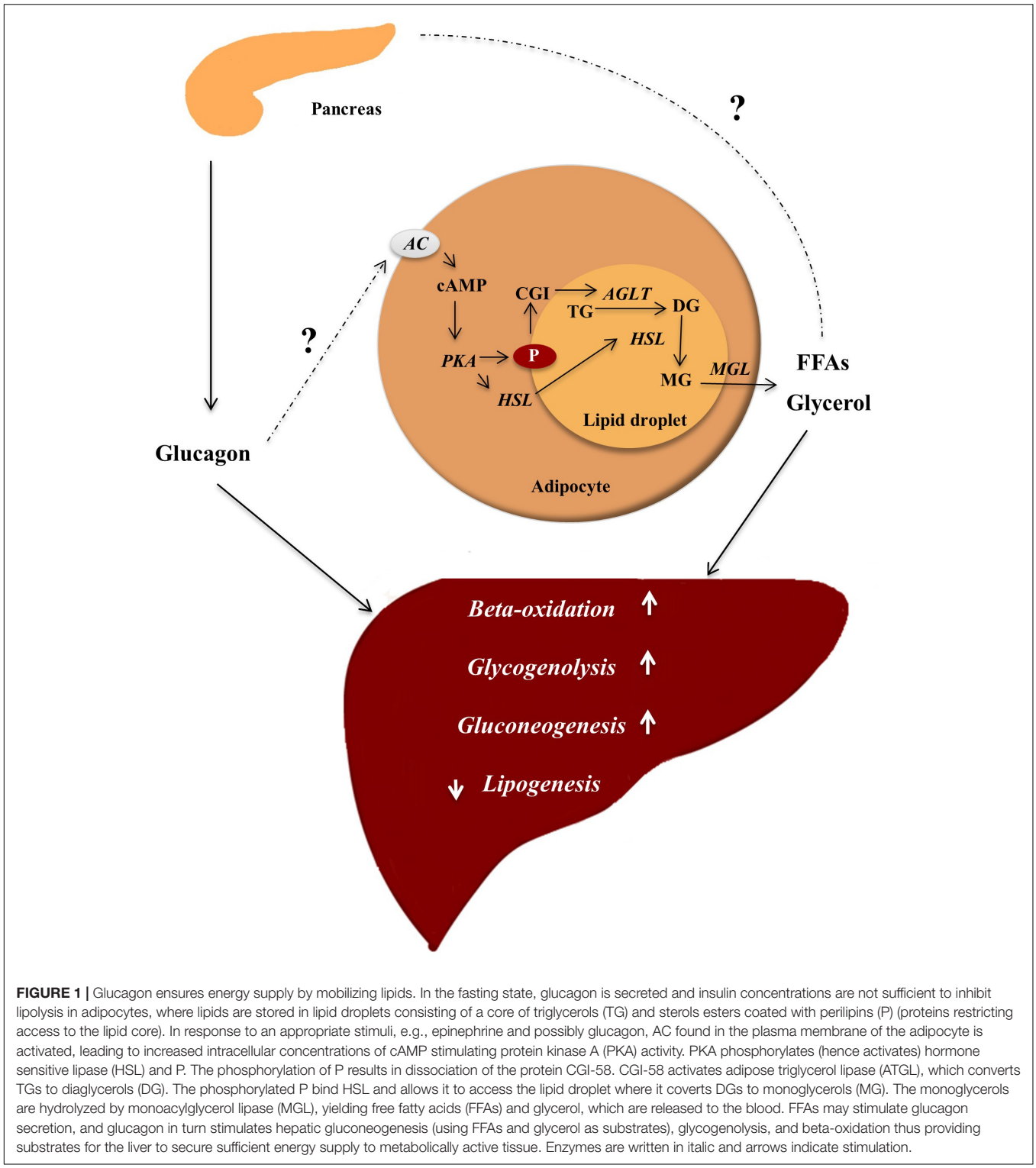

FIGURE 1 | Glucagon ensures energy supply by mobilizing lipids. In the fasting state, glucagon is secreted and insulin concentrations are not sufficient to inhibit lipolysis in adipocytes, where lipids are stored in lipid droplets consisting of a core of triglycerols (TG) and sterols esters coated with perilipins (P) (proteins restricting access to the lipid core). In response to an appropriate stimuli, e.g., epinephrine and possibly glucagon, AC found in the plasma membrane of the adipocyte is activated, leading to increased intracellular concentrations of cAMP stimulating protein kinase A (PKA) activity. PKA phosphorylates (hence activates) hormone sensitive lipase (HSL) and P. The phosphorylation of P results in dissociation of the protein CGI-58. CGI-58 activates adipose triglycerol lipase (ATGL), which converts TGs to diaglycerols (DG). The phosphorylated P bind HSL and allows it to access the lipid droplet where it coverts DGs to monoglycerols (MG). The monoglycerols are hydrolyzed by monoacylglycerol lipase (MGL), yielding free fatty acids (FFAs) and glycerol, which are released to the blood. FFAs may stimulate glucagon secretion, and glucagon in turn stimulates hepatic gluconeogenesis (using FFAs and glycerol as substrates), glycogenolysis, and beta-oxidation thus providing substrates for the liver to secure sufficient energy supply to metabolically active tissue. Enzymes are written in italic and arrows indicate stimulation.

(Gerich et al., 1976). Furthermore, infusion with saline only gave the same increase in FFA as compared to glucagon infusion. In another study glucagon was infused at 1.2 ng/kg × min (high but also relevant) together with somatostatin for 2 h, but there was no lipolytic effect of glucagon at insulin concentrations of 38 pM (Jensen et al., 1991). In contrast, a 2-h glucagon infusion at 1.3 ng/kg × min, during a mean insulin plasma concentration of 65 pM, increased the rate of appearance of labeled FFA and glycerol by 40 and 36%, respectively (Carlson et al., 1993). As glucagon receptors are expressed on beta

cells (Adriaenssens et al., 2016; Svendsen et al., 2018) and may stimulate insulin secretion through both GLP-1 and glucagon receptors (Svendsen et al., 2018) it may be speculated that intraislet regulation of insulin through glucagon may contribute to its effect on lipid metabolism.

It is important to note that FFA and glycerol in plasma are not only determined by release from adipocytes, but also by rate of uptake and re-esterification in other tissues. A lack of effect of glucagon on the free plasma pool of FFA and glycerol, does therefore not rule out that glucagon has a direct effect on lipid metabolism in adipocytes and hepatocytes (**Figure 1**).

GLUCAGON STIMULATES HEPATIC BETA-OXIDATION AND INHIBITS LIPOGENESIS

In hepatocytes, glucagon action increases the transcription factor cAMP responsive element binding (CREB) protein, which induces the transcription of carnitine acyl transferase 1 (CPT-1) (Longuet et al., 2008). CPT-1 enables catabolism of long-chain fatty acids by converting fatty acids to acyl-carnitines, which are transported into the mitochondria and subjected to beta-oxidation (Kim et al., 2000; Stephens et al., 2007). During beta-oxidation the fatty acids are degraded into acetate, which ultimately enters the citric acid cycle (DiMarco and Hoppel, 1975). Furthermore, through PKA-dependent phosphorylation, glucagon receptor signaling inactivates acetyl-CoA carboxylase, the enzyme catalyzing the formation of malonyl-CoA. Malonyl-CoA is the first intermediate in fatty acid synthesis and inhibits CPT-1 (i.e., inhibits beta-oxidation). By inhibiting the formation of malonyl-CoA, glucagon diverts FFAs to beta-oxidation rather than re-esterification into TGs (**Figure 2**). Periportal and perivenous hepatocytes receive different concentrations of substrates and oxygen and as a consequence periportal hepatocytes primarily mediate oxidative processes, including beta-oxidation, whereas perivenous hepatocytes preferentially mediate glucose uptake and lipogenesis (Jungermann, 1988; Guzman and Castro, 1989).

In hepatocytes, glucagon may bring about an energy-depleted state (increasing the AMP/ATP ratio) sufficient to activate AMP-activated kinase (Berglund et al., 2009), which phosphorylates acetyl-CoA carboxylase (Peng et al., 2012) and p38 mitogen-activated protein kinase, leading to transcriptional activation of peroxisome proliferator-activated receptor-α (PPARα) (Longuet et al., 2008). PPARα stimulates the transcription of genes involved in beta-oxidation including CPT-1, CPT-2, and acetyl-CoA oxidase (Patsouris et al., 2006), and the transcription of fibroblast growth factor 21, which is produced in the liver in response to glucagon (Xu et al., 2009; Cyphert et al., 2014). Glucagon also stimulates forkhead transcription factor A2 activity (FoxA2), which induces transcription of genes involved in beta-oxidation, such as CPT-1, very-, and medium- long-chain acyl-CoA dehydrogenase (Wolfrum and Stoffel, 2006; von Meyenn et al., 2013). Subsequent to activating its receptors on hepatocytes, insulin suppresses most of these pathways, and the metabolic state in the hepatocytes may therefore be determined by the insulin-glucagon ratio, rather than by the hormone concentrations *per se* (Parrilla et al., 1974). Insulin inhibits lipolysis in adipocytes and by reducing the amount of substrate (FFA and glycerol) reaching the liver may reduce (Perry et al., 2015) hepatic gluconeogenesis.

To investigate the physiological effects of glucagon in lipid metabolism, several studies have relied on glucagon receptor knockout ($Gcgr^{-/-}$) mice or animals treated with GRA. In the livers of $Gcgr^{-/-}$ mice there is an increase in glycolysis and a decrease in gluconeogenesis and citric acid cycle activity, which results in decreased acetyl-CoA oxidation and acetyl-CoA accumulation. The accumulation of acetyl-CoA in the cytosol of hepatocytes results in increased lipogenesis. Supporting this, genes involved in lipogenesis, e.g., ATP citrate lyase and fatty acid synthase, were found to be upregulated in livers of $Gcgr^{-/-}$ mice at both the mRNA and protein level (Longuet et al., 2008; Yang et al., 2011), while CPT-1 and -2 levels, and other enzymes necessary for beta-oxidation, were downregulated (Yang et al., 2011). Hepatic beta-oxidation is essential for the production of both glucose and ketones since it provides the substrates acetyl-CoA and acetate and mitochondrial energy supply (ATP/NADH) needed for gluconeogenesis (Staehr et al., 2003). The hepatic gene expression profile changes markedly in response to fasting, and major differences have been reported in expression levels of genes involved in lipid metabolism between the fed and fasted state (Longuet et al., 2008; Zhang et al., 2011). Following a prolonged fast (16 h), wild-type mice had an increased hepatic expression of genes involved in beta-oxidation, such as CPT-1, CPT-2, and acyl-CoA dehydrogenase, but this was not observed in $Gcgr^{-/-}$ mice, which displayed an impaired beta-oxidation in both the fasted and fed state (Longuet et al., 2008) and $Gcgr^{-/-}$ mice failed to change the hepatic energy state in response to fasting (Berglund et al., 2009). Furthermore, $Gcgr^{-/-}$ mice showed increased hepatic TG secretion and increased plasma concentrations of TG and FFA after a 16 h fasting period, but not after 5 h of fasting (Longuet et al., 2008). Others (Gelling et al., 2003) also found similar TG and FFA plasma concentrations in $Gcgr^{-/-}$ and wild-type mice after a short-term fast; they did, however, find increased plasma concentrations of LDL in $Gcgr^{-/-}$ mice. Glucagon thus seems to regulate hepatic metabolism in response to fasting by stimulating glucose-producing processes, including beta-oxidation. When challenged with a high fat diet (HFD) for 8 weeks, $Gcgr^{-/-}$ mice did not increase the amount of inguinal and epididymal fat, whereas the amount of both doubled in wild-type mice (Longuet et al., 2008). In line with this, others (Gelling et al., 2003) showed a decrease in white adipose tissue mass and an increase in lean body mass in $Gcgr^{-/-}$ compared to wild-type mice, without changes in bodyweight, food consumption, or energy expenditure and one group (Conarello et al., 2007) found that $Gcgr^{-/-}$ mice had lower amounts of white adipose tissue when fed both a HFD and a low fat diet compared to wild-type mice, and thus seemed to be resistant to diet-induced obesity. This could reflect an inability of $Gcgr^{-/-}$ mice to mobilize the hepatic lipid storage; instead adipocyte lipolysis (by catecholamines) maintain the energy supply to other metabolically active tissues.

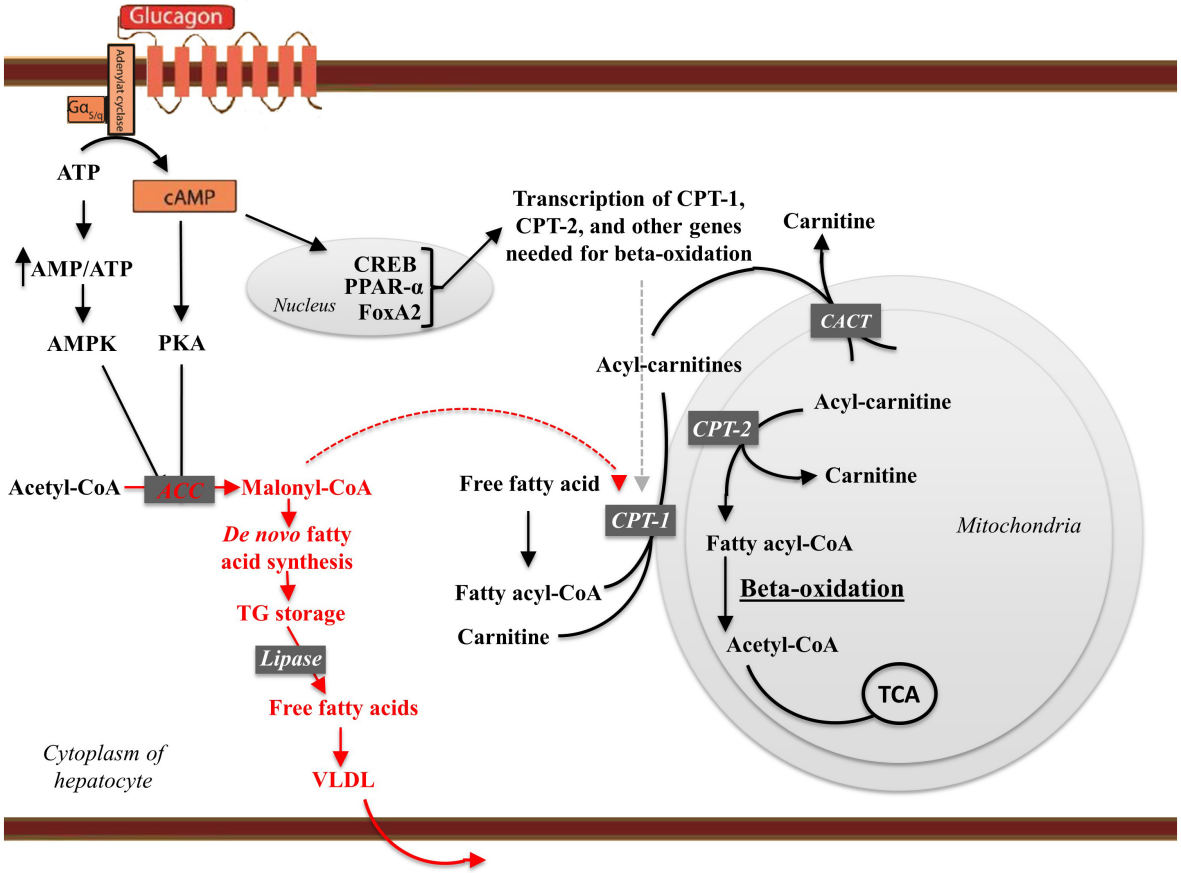

FIGURE 2 | The effects of glucagon receptor signaling on hepatic lipid metabolism. Glucagon activates its cognate receptor, a seven transmembrane receptor coupled to a Gs protein, resulting in AC activity and cAMP production. The increase in intracellular cAMP activates protein kinase A (PKA), which phosphorylates (hence inactivates) acetyl-CoA carboxylase (ACC). Glucagon thus inhibit malonyl-CoA formation and the subsequent *de novo* fatty acid synthesis. When formed, the fatty acids are, after re-esterification, stored as trigycerides in and released from the hepatocytes in the form of very-low density lipoprotein (VLDL). Thus, glucagon leads the free fatty acids toward beta-oxidation and decreases *de novo* fatty acid synthesis and VLDL release. cAMP accumulation in hepatocytes activates the cAMP responsible binding element (CREB) protein, which induces the transcription of carnitine acyl transferase-1 (CPT-1), and other genes needed for beta-oxidation. CPT-1 catalyzes the attachment of carnitine to fatty acyl-CoA, forming acyl-carnitine. The acyl-carnitines transverse the mitochondrial membrane mediated via the carnitine-acylcarnitine translocase (CACT). Once in the mitochondrial matrix, carnitine acyl transferase-2 (CPT-2) is responsible for transferring the acyl-group from the acyl-carnitine back to CoA. Carnitine leaves the mitochondria matrix through the carnitine-acylcarnitine translocase. During beta-oxidation, the fatty acid chains are degraded into acetate. Acetate reacts with CoA to yield acetyl-CoA, which reacts with oxaloacetate to form citrate that inhibits glycolysis through inhibition of pyruvate dehydrogenase and phosphofructokinase-1. Finally, citrate enters the citric acid cycle (TCA). Thus, glucagon increases fatty acid catabolism, inhibits glycolysis, and fuels the TCA cycle. By increasing AC activity glucagon increase the AMP/ATP ratio sufficient to activate AMP-activated kinase (AMPK), which phosphorylates ACC, leading to transcriptional activation of peroxisome proliferator-activated receptor-α (PPARα). PPARα stimulates the transcription of genes involved in beta-oxidation including CPT-1, CPT-2, and acetyl-CoA oxidase. Glucagon stimulates FoxA2 activity, which induces transcription of genes such as CPT-1, very-, and medium- long-chain acyl-CoA dehydrogenase. Enzymes and pathways inhibited by glucagon are shown in red, while enzymes and pathways stimulated by glucagon are shown in black.

IMPLICATIONS OF GLUCAGON RECEPTOR SIGNALING IN THE DEVELOPMENT OF STEATOSIS

Administration of GRAs has been associated with increased hepatic fat content (assessed as hepatic fat fraction measured by magnetic resonance imaging) and increased plasma concentrations of LDL (Guzman et al., 2017). Furthermore, subjects with endogenous glucagon deficiency (pancreatectomized subjects) (Dresler et al., 1991) and rats (Sloop et al., 2004) and diabetic (db/db) mice (Liang et al., 2004) treated with glucagon antisense oligonucleotide have increased hepatic fat. These data suggest that inhibition of glucagon receptor signaling results in hepatic lipid accumulation. In addition, $Gcgr^{-/-}$ mice may be prone to steatosis when challenged with a high fat diet (HFD) for 8 weeks (Longuet et al., 2008). However, a study involving a similar HFD diet for 12 weeks and mice with the same sex, gene modification, and background (C57BL/6J), showed that $Gcgr^{-/-}$ mice were protected from steatosis (Conarello et al., 2007). Of notice,

C57BL/6J mice do not consistently develop steatosis upon HFD feeding (Charlton et al., 2011), and this might have influenced the results. In rats, impaired glucagon action also associates with development of hepatic steatosis (Charbonneau et al., 2005a). Interestingly, HFD feeding has been reported to decrease glucagon receptor expression at the plasma membrane of rat hepatocytes (Charbonneau et al., 2005b, 2007). These data suggest that hepatic lipid accumulation may cause impaired glucagon receptor signaling, and that this (as demonstrated using GRAs) may contribute to and accelerate hepatic lipid accumulation.

Acute administration of 30 μg/kg glucagon decreased FFA and TG plasma concentrations and reduced hepatic TG content and secretion in mice (Longuet et al., 2008). Chronic hyperglucagonemia (injection of 10 μg glucagon every 8 h for 21 days) had hypolipidemic effects in rats, evident by a 70 and 38% decrease in plasma concentrations of TGs and phospholipids, respectively (Guettet et al., 1988). Consistent with this, glucagon inhibited synthesis and secretion of TGs in cultured hepatocytes (Longuet et al., 2008), in perfused rat livers (Penhos et al., 1966; Heimberg et al., 1969), and decreased the synthesis of hepatic VLDL in rats (Eaton, 1973). In humans, hyperglucagonemia (56 ± 20 pM), during a pancreatic clamp, reduced hepatic lipoprotein particle turnover (Xiao et al., 2011), and glucagon administration increased hepatic beta-oxidation in humans (Prip-Buus et al., 1990). In diet-induced obese (DIO) mice, a once-weekly treatment with 70 nmol/kg glucagon/GLP-1 receptor co-agonist resulted in loss of fat mass, which in the same study was also found, although less pronounced in GLP-1 receptor knockout mice, and improved hepatic lipid metabolism and steatosis within 4 weeks (Day et al., 2009). Another glucagon/GLP-1 co-agonist (1.9 μmol/kg daily for 14 days) decreased acetyl-CoA and malonyl-CoA concentrations and increased CPT-1 mRNA in the livers of DIO mice, whereas a selective GLP-1 receptor agonist had no effect (Pocai et al., 2009). Both of these dual agonists reduced hepatic steatosis, increased HSL activity in adipocytes, and improved dyslipidemia in DIO mice (Day et al., 2009; Pocai et al., 2009). Supporting these data, other glucagon/GLP-1 receptor co-agonists have been reported to lower plasma concentrations of TG and cholesterol (Clemmensen et al., 2014), decrease hepatic fat content (Henderson et al., 2016), and reduce adipose mass in rodent models of T2D and obesity (Evers et al., 2017; Zhou et al., 2017). Importantly, acute administration of 25 nmol/kg glucagon/GLP-1 co-agonist decreased plasma concentration of TGs, cholesterol, and LDL in DIO mice within 1 h, whereas liraglutide (a pure GLP-1 receptor agonist) administration had no effect (More et al., 2017). In addition, hepatic synthesis of VLDL and palmitate, and fatty acid esterification decreased, while beta-oxidation and LDL receptors expression increased upon co-agonist, but not liraglutide, administration (More et al., 2017). The inhibitory effect on hepatic lipogenesis and stimulatory effect on beta-oxidation therefore seems to be mediated by glucagon receptor signaling. Several clinical studies are currently investigating the potential treatment of obesity and T2D using glucagon/GLP-1 co-agonists (Capozzi et al., 2018).

REGULATION OF GLUCAGON SECRETION BY LIPIDS

FFAs are under certain circumstances insulin secretagogs (Boden and Carnell, 2003) but their ability to stimulate glucagon secretion remains debated (Gerich et al., 1974; Bollheimer et al., 2004; Gromada et al., 2007). Some clinical studies found a suppression of glucagon secretion at increased FFA concentrations (Madison et al., 1968; Edwards and Taylor, 1970; Luyckx and Lefebvre, 1970; Gerich et al., 1974) whereas isolated alpha cells were shown to secrete glucagon in response to FFA stimulation (Gross and Mialhe, 1986; Collins et al., 2008). In isolated rat pancreatic islets, palmitate stimulated glucagon secretion (Gremlich et al., 1997; Dumonteil et al., 2000). Others found palmitate to stimulate glucagon secretion in a glucose-dependent manner using isolated pancreatic islets; increasing at glucose concentrations of 2.8, 5.6, and 10 mM (Olofsson et al., 2004) but not at 16.7 mM (Bollheimer et al., 2004). Medium and long-chain fatty acids (>C5) have been reported to stimulate glucagon secretion by activation of FFA receptor G protein-coupled receptor 40 (GPR40) (Wang et al., 2011; Kristinsson et al., 2017) and GPR119 (Hansen et al., 2012; Li et al., 2018), both present in the pancreatic islets (Briscoe et al., 2003). FFAs may also function as metabolic substrate and stimulate alpha cell secretion through beta-oxidation (Kristinsson et al., 2017; Briant et al., 2018). FFAs decrease secretion of somatostatin (Gromada et al., 2001), and may lower the tonic inhibition of somatostatin on alpha cells (Gromada et al., 2007; Müller et al., 2017). A clinical study investigating the effects of ingestion of lipids on hormone secretion, found no change in glucagon secretion after intravenous or oral administration of a lipid emulsion (3 ml/kg) (Lindgren et al., 2011), neither did glucagon plasma concentrations change upon a 300 min lipid infusion raising FFA plasma concentrations from 0.4 to 0.8 mM (Staehr et al., 2003). No difference in glucagon secretion was observed between subjects consuming a HFD or a low-fat diet for 2 weeks (Raben et al., 2001). In contrast to this, ingestion of long–chain fatty acids (olive oil and C8 fatty acids) lead to increased plasma concentrations of glucagon 40 min after, whereas no increase was observed after ingestion of short-chain fatty acids (C4), however, glucose-dependent insulinotropic polypeptide (GIP) concentrations also increased upon ingestion of long-chain fatty acids and this may have caused an increase in glucagon secretion (Mandoe et al., 2015). Another study observed that a meal rich in mono-unsaturated fatty acids resulted in a larger glucagon response when compared to a control meal (Sloth et al., 2009). Others also observed an increase in glucagon concentrations upon fat-enriched meals (Radulescu et al., 2010; Niederwanger et al., 2014). The glucagon response observed upon a 90 min intraduodenal infusion of linoleic, oleic, and palmitic acids were significant lower than observed upon protein infusion (Ryan et al., 2013). Studies of ability of FFAs to stimulate glucagon secretion are complex, since FFAs are found in many forms and their stimulatory effect may vary (Radulescu et al., 2010) [as is the case for incretin secretion (Feltrin et al., 2004; Thomsen et al., 1999)]. Furthermore, the

increased glucagon concentrations reported in some studies may result from other proglucagon products (e.g., glicentin or oxyntomodulin), since measurements of plasma glucagon concentrations have been marred with problems regarding sensitivity and specificity (Wewer Albrechtsen et al., 2016), and further studies investigating the regulation of glucagon secretion by FFAs are needed.

CONCLUSION

Glucagon may, aside from its physiological actions on glucose and amino acid metabolism, also be important for lipid metabolism via effects on hepatic beta-oxidation and lipogenesis, and potentially increased lipolysis in adipocytes. A direct role of glucagon on adipocytes may be of importance in rodents, as glucagon stimulates lipolysis (Vaughan and Steinberg, 1963; Rodbell and Jones, 1966; Prigge and Grande, 1971; Manganiello and Vaughan, 1972; Lefebvre et al., 1973; Livingston et al., 1974), whereas in humans an adipocyte-dependent lipolysis of glucagon is more complex. In both rodents and humans, glucagon is a powerful regulator of hepatic lipid metabolism (Day et al., 2009; Xiao et al., 2011) as highlighted in studies using GRAs (Guzman et al., 2017). The clinical use of GRAs is further challenged by glucagon's role in amino acid metabolism, and blocking the glucagon receptor results in hyperaminoacidemia and eventually alpha cell hyperplasia (Holst et al., 2017b). Treatment of diabetes using the current GRAs may therefore not be feasible, however, one may speculate that targeted antagonism of glucagon signaling may circumvent these unwarranted side-effects. Currently glucagon receptor agonists, combined with GLP-1 and GIP receptor agonists, are investigated as possible therapeutic agents (Gu et al., 2011; Sadry and Drucker, 2013; Sanchez-Garrido et al., 2017; Capozzi et al., 2018). In preclinical studies, these agents improve steatosis and dyslipidemia, possibly as a consequence of regulation of hepatic lipid metabolism by glucagon agonism (Day et al., 2009).

Taken together, glucagon seems to play an important physiological role in the acute regulation of lipid metabolism but clearly further studies particularly in humans are warranted.

AUTHOR CONTRIBUTIONS

All authors wrote and approved the final edition of the manuscript.

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REVIEW
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Is the Brain a Key Player in Glucose Regulation and Development of Type 2 Diabetes?

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Ever since Claude Bernards discovery in the mid 19th-century that a lesion in the floor of the third ventricle in dogs led to altered systemic glucose levels, a role of the CNS in whole-body glucose regulation has been acknowledged. However, this finding was later overshadowed by the isolation of pancreatic hormones in the 20th century. Since then, the understanding of glucose homeostasis and pathology has primarily evolved around peripheral mechanism. Due to scientific advances over these last few decades, however, increasing attention has been given to the possibility of the brain as a key player in glucose regulation and the pathogenesis of metabolic disorders such as type 2 diabetes. Studies of animals have enabled detailed neuroanatomical mapping of CNS structures involved in glucose regulation and key neuronal circuits and intracellular pathways have been identified. Furthermore, the development of neuroimaging techniques has provided methods to measure changes of activity in specific CNS regions upon diverse metabolic challenges in humans. In this narrative review, we discuss the available evidence on the topic. We conclude that there is much evidence in favor of active CNS involvement in glucose homeostasis but the relative importance of central vs. peripheral mechanisms remains to be elucidated. An increased understanding of this field may lead to new CNS-focusing pharmacologic strategies in the treatment of type 2 diabetes.

Keywords: CNS, hypothalamus, glucose, regulation, fMRI, neuroimaging, neuroendocrine, autonomic nervous system

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INTRODUCTION

The global prevalence of diabetes in adults – approximately 90% consisting of type 2 diabetes – was estimated to 6.4% in 2010 and is predicted to increase to 7.7% in 2030 (Nolan et al., 2011). The macro- and microvascular complications that are associated with diabetes lead to increased morbidity and mortality and the economic burden posed by management of diabetes and its complications is substantial (Ng et al., 2014; Norhammar et al., 2016). Type 2 diabetes typically evolves gradually. An initial phase of insulin resistance with maintained normoglycemia is followed by a transitional phase of impaired fasting glucose and/or impaired glucose tolerance until manifest diabetes is established. While the pancreatic beta cells can compensate for the insulin resistance by increasing insulin secretion at first, they eventually fail to do so as the disease progresses, frequently necessitating exogenously administered insulin in advanced stages. Since the discovery of the pancreatic hormones insulin and glucagon, the prevailing understanding of type 2 diabetes development has circled around processes in the periphery, particularly in the pancreas.

Likewise, pharmacological targets in the treatment of type 2 diabetes have been largely limited to the peripheral domain. However, this "islet-centric" model has these last decades been challenged by mounting evidence in favor of a "brain-centric" model, according to which the brain is actively involved in systemic glucose regulation. Further advances in this area may change the way we look at metabolic disorders and may specifically result in new CNS-targeted strategies for the pharmacological management of type 2 diabetes. In this narrative review, we aim to present the current knowledge of the field. In the first section, we will provide a brief summary of findings from animal studies that have been extensively reviewed by other authors (Marty et al., 2007; Carey et al., 2013; Grayson et al., 2013; Mergenthaler et al., 2013; Roh et al., 2016; Tups et al., 2017; López-Gambero et al., 2019). This will be followed up by a more in-depth presentation of evidence from human studies where the implementation and advances of neuroimaging techniques has offered new and interesting insights.

EVIDENCE FROM ANIMAL STUDIES

In 1854, Claude Bernard reported that a lesion in the floor of the fourth ventricle in dogs altered glucose levels, thereby presenting the first evidence of the brain's role in glucose regulation (Bernard, 1855). In the 1960s two sets of neurons were identified in the CNS that responded to high and low values of glucose, respectively (Anand et al., 1964; Oomura et al., 1964, 1969, 1974). These neurons were subsequently termed glucose-excitatory (GE, responding to high levels of glucose) and glucose-inhibitory (GI, responding to low levels of glucose) (Routh et al., 2014). While present in the entire CNS, these neurons are especially numerous in several nuclei of the hypothalamus and the brainstem (López-Gambero et al., 2019).

The hypothalamus is located below the thalamus and above the pituitary gland and brain stem. It constitutes the floor of the third ventricle which contains cerebrospinal fluid (CSF). This anatomical position allows for access to nutrients and hormones. It consists of a network of interconnected nuclei among which the arcuate nucleus (ARC), ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), and the lateral hypothalamus (LH) are implicated in the regulation of glucose homeostasis. In the brainstem the nucleus of the solitary tract (NTS), area postrema (AP), dorsal motor nucleus of the vagus (DMNX) and the rostral ventrolateral medulla (RVLM) are key regions (López-Gambero et al., 2019). Glucose-excited neurons are primarily prevalent in the ARC, VMH, and PVN, whereas glucose-inhibited neurons are concentrated in the LH, AN, and PVN. In the brainstem, both of these types of neurons are represented in the areas mentioned above (NTS, AP, and DMNX) (Roh et al., 2016) (**Figure 1**).

Glucose injected into the third ventricle of rodents leads to a reduction of systemic glucose levels, principally by suppression of hepatic glucose production (HGP) (Lam et al., 2005a). The same has been demonstrated for long-chain fatty acids (Obici et al., 2002b; Lam et al., 2005b) and amino acids such as leucine (Su et al., 2012), proline (Arrieta-Cruz et al., 2013) and histidine (Kimura et al., 2013). Apart from being able to sense levels of circulating glucose, the brain also receives afferent nervous input via the brainstem related to glucose availability from taste buds in the oral cavity (Ahrén, 2000), from the intestinal mucosa (Ashley Blackshaw and Young, 2011) and from hepatoportal glucoreceptors (Delaere et al., 2010). This allows for rapid responses to ingested and circulating glucose such as observed in the cephalic insulin phase (Marty et al., 2007). Likewise, ingestion of lipids leads to release of CCK whereas amino acids stimulate release of CCK, GLP-1, and PYY. These gut hormones in turn activate vagal afferents projecting to the brainstem (Duca and Yue, 2014; Liu et al., 2016).

Not only neurons but also surrounding glial cells are believed to have an active role in the sensing of nutrients. It has been hypothesized that astrocytes may metabolize glucose and proline into lactate for subsequent translocation in the so-called astrocyte-neuron-lactate-shuttle to neurons to signal glucose depletion (Arrieta-Cruz et al., 2013). Likewise, oxidation of fatty acids may occur to a larger extent in astrocytes, generating ketones which after translocation to neurons may override the influence of glucose and fatty acid sensing (Bruce et al., 2017).

Similarly, central administration of hormones or targeting of their intracellular pathways in neurons have demonstrated that insulin (Obici et al., 2002a,c; Pocai et al., 2005), leptin (Schwartz et al., 1996; Kievit et al., 2006; Koch et al., 2010), glucagon (Mighiu et al., 2013; LaPierre et al., 2015), GLP-1 (Sandoval et al., 2008) and CCK (Zhu et al., 2012; Montgomery et al., 2013) reduces systemic glucose levels by CNS actions, whereas the effect of ghrelin seems to be the opposite (Scott et al., 2012; Wang et al., 2013; Stark et al., 2015; Lee et al., 2016). Perhaps most interesting is the potent CNS-mediated glucose lowering properties of the fibroblast growth factors FGF-1 (Scarlett et al., 2016) and FGF-19 (Fu et al., 2004; Morton et al., 2013; Marcelin et al., 2014). A single low dose of FGF-1 injected into the third ventricle induced sustained diabetic remission in mice with diet-induced obesity. The effect was rapid and sustained. Thus, fasting blood glucose concentration was reduced by 25% within 6 h, normalized after 7 days and remained in the normal range for the full 17 weeks studied without any observed hypoglycemia (Scarlett et al., 2016).

The transport of glucose across the blood–brain barrier (BBB) is primarily mediated by insulin-independent GLUT-1 and, particularly in conditions of CNS ischemia and glucose deprivation, by SGLT-1 (Patching, 2017). Interestingly though, insulin-mediated increase in CNS glucose uptake via upregulation of GLUT-1 in astrocytes has been described (García-Cáceres et al., 2016). Additionally, insulin-dependent GLUT4 is expressed in cells of the BBB, albeit at low levels (Patching, 2017). The existence of a more direct route, bypassing the BBB by fenestrated capillaries or via the CSF has been proposed and is supported by some work (Langlet et al., 2013; Routh et al., 2014). Fatty acids and amino acids have been demonstrated to cross the BBB (Smith, 2000; Rapoport et al., 2001; Smith and Nagura, 2001) by mechanisms that are not fully understood but may involve fatty acid transport proteins (FATPs) (Mitchell et al., 2011) and similar systems for amino acids (Smith, 2000). The levels of the large hormones insulin and leptin are considerably lower in the

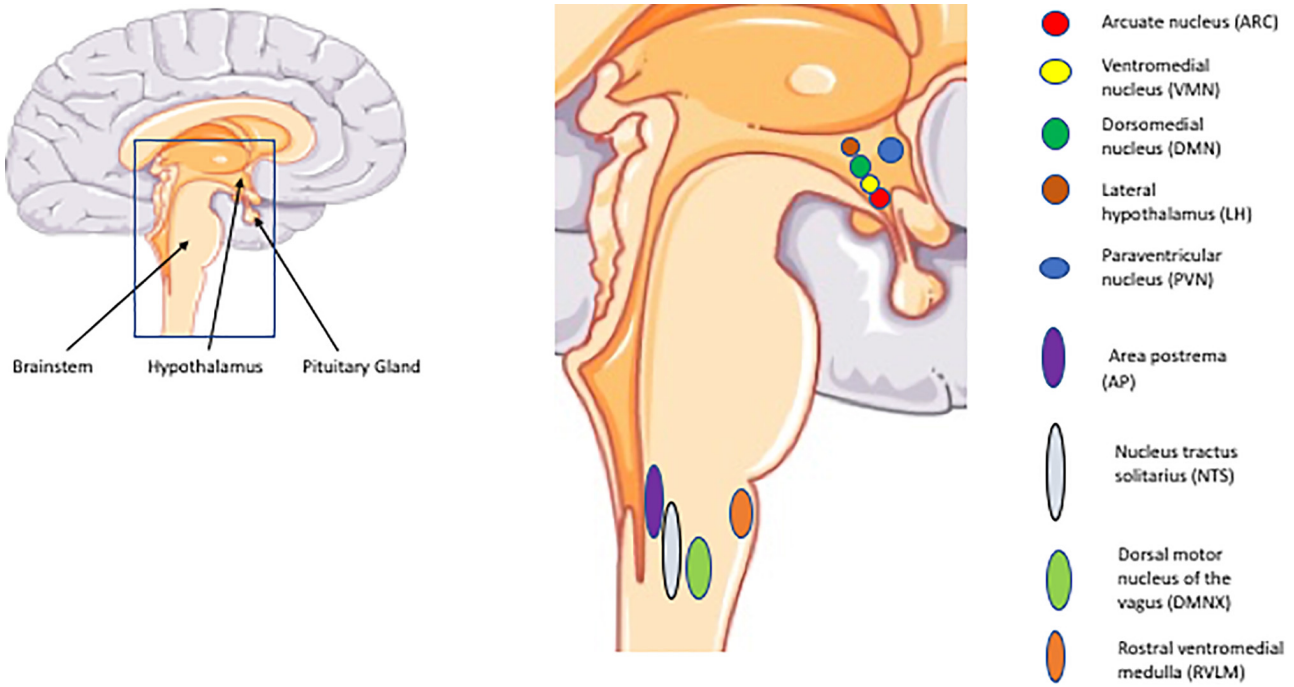

FIGURE 1 | Important CNS regions and nuclei implicated in glucose regulation. The illustrations of anatomical structures were retrieved from http://smart.servier.com and have thereafter been assembled and processed.

CFS than in the systemic circulation, suggesting the existence of saturable transporter proteins rather than simple diffusion (Banks et al., 1996; Gray and Barrett, 2018). While the exact mechanisms of CNS transport for these hormones remain elusive, hormone receptors seem to be involved (Banks et al., 1997; Bjørbaek et al., 1998; Boado et al., 1998; Hileman et al., 2002; Meijer et al., 2016). GLP-1 is synthesized within the CNS where it acts as transmitter (Katsurada et al., 2014; Katsurada and Yada, 2016), whereas the hypothesized intracerebral insulin synthesis is still a matter of some controversy (Gray and Barrett, 2018).

The intracellular mechanisms of nutrient and hormonal sensing in the CNS have been intensively investigated in animal studies. Glucose uptake into neurons occurs mainly via insulin-independent glucose transporter channels (GLUT1, 2, and 3) and this activates signaling pathways that differ for GE and GI neurons, involving activation of K_{ATP}-channels in the former and the signaling substance AMPK in the latter. In GE neurons, metabolism-independent sensing mechanisms involving SGLT1 and 3 and the sweet receptor T1R2/3 are also implicated (López-Gambero et al., 2019). The receptors of insulin (Chen et al., 2017), leptin (Mercer et al., 1996), glucagon (Abraham and Lam, 2016), GLP-1 (Katsurada and Yada, 2016), CCK (Pathak et al., 2018), and ghrelin (Cowley et al., 2003; Sun et al., 2007) have been identified in various regions of the CNS. Insulin signaling in neurons is dependent on activation of K_{ATP}-channels (Pocai et al., 2005) and is facilitated by activation of the IRS-PI3K- pathway (Niswender et al., 2003). Leptin also acts on this pathway by phosphorylation of IRS1 (Koch et al., 2010) in addition to the JAK2-STAT3 and WNT signaling pathways (Tups et al., 2017). Thus, leptin may sensitize neurons in the hypothalamus to the effects of insulin but may also exert central effects independent of insulin, as is supported by the fact that leptin can reverse hyperglycemia in mice with streptozotocin-induced type 1 diabetes (Yu et al., 2008).

There is much evidence that the central effects of insulin and leptin differ in obese compared to lean animals. Impairments in CNS transport, receptor expression and the downstream signaling cascades have been observed for both hormones in models of obesity (Van Heek et al., 1997; El-Haschimi et al., 2000; Kaiyala et al., 2000; Spanswick et al., 2000; Carvalheira et al., 2003; Asilmaz et al., 2004; Wilsey and Scarpace, 2004; Urayama and Banks, 2008). Hypothalamic inflammation has been proposed as a causative factor. Increased expression of inflammatory mediators such as TNF-alpha and increased endoplasmatic reticulum (ER) stress is a well-established feature of obesity (Hotamisligil et al., 1993; Ozcan et al., 2004). Cytokines and ER stress lead to activation of the intracellular JNK1 and IKKβ/NFkB pathways. This has been demonstrated to induce insulin resistance in peripheral tissues through serine phosphorylation of IRS-1. Obesity and over-nutrition is associated with hypothalamic inflammation, which may in turn result in insulin and leptin resistance via the mechanisms described above (Hosoi et al., 2008; Zhang et al., 2008; Milanski et al., 2009; Ozcan et al., 2009; Posey et al., 2009). Indeed, central inhibition of IKKβ has been observed to reduce food intake and increase insulin sensitivity (Zhang et al., 2008; Ozcan et al., 2009; Posey et al., 2009). Moreover, structural changes to

the hypothalamus and evidence of increased inflammation have been observed well before the onset of obesity (Zhang et al., 2008; Thaler et al., 2012). Free fatty acids may also activate these pathways as well as activate macrophages by binding to toll-like receptors (TLRs) on their cell surface (Hotamisligil, 2006). High fat feeding, selective intake of saturated fatty acids and ICV infusion of palmitate have been associated with hypothalamic inflammation independently of excess caloric intake (Milanski et al., 2009; Posey et al., 2009). Finally, switching the diet to more unsaturated fats was observed to reduce this inflammation as well as hypothalamic and systemic insulin resistance (Cintra et al., 2012).

The role of inflammation in obesity is still disputed and may be multi-faceted, as is demonstrated by the positive effects of the cytokines IL-6 and IL-10 on energy and glucose metabolism, which seem to be partly mediated by central mechanisms (Wallenius et al., 2002; Benrick et al., 2009; Gotoh et al., 2012; Timper et al., 2017). The expression and circulating levels of these cytokines are increased in obese humans (Kern et al., 2001; Vozarova et al., 2001; Esposito et al., 2003). IL-6 administered to mice intracerebroventricularly was recently shown to induce reduced food intake and improved glucose metabolism, the latter mediated by enhanced hepatic insulin action. Moreover, this was more marked in obese, leptin-resistant mice than in lean ones (Timper et al., 2017). Thus, the increased levels of these cytokines may actually counteract rather than add to the other detrimental metabolic effects of obesity. Of further interest, IL-6 secretion by myocytes increases considerably after physical exercise and leads to subsequent increase in circulating levels of IL-10, secreted by immune cells (Steensberg et al., 2003; Pedersen and Febbraio, 2008). IL-6 and IL-10 have been shown to mediate positive metabolic effects of exercise through inhibition of IKKβ and ER stress in the hypothalamus (Ropelle et al., 2010).

In order to exert its proposed influence on peripheral glucose metabolism, the CNS input from nutrients, hormones and other neurons has to result in an efferent response that is conveyed to peripheral target organs. The known routes for this response is the autonomic nervous system (ANS) – further divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PASY) – and the hypothalamic-pituitary axis (**Figure 2**).

The DMNX of the brainstem contains the soma of preganglionic parasympathetic nerve fibers and can thus transmit PASY signals to target organs like the liver (Yi et al., 2010) (reducing hepatic glucose production and increasing glycogen synthesis) and the pancreas (Thorens, 2014) (increasing insulin secretion and glucagon secretion). Neurons in the RVLM connect with the SNS to increase hepatic glucose production (HGP) (Yi et al., 2010), decrease insulin secretion and increase glucagon secretion (Thorens, 2014), promote glucose uptake in skeletal muscles (Seoane-Collazo et al., 2015) and lipolysis in adipocytes (Bartness et al., 2005). Neurons in the PVN engage in the hypothalamic-pituitary-adrenal axis by secreting corticotrophin releasing hormone (CRH) in the bloodstream for downstream release of ACTH in the pituitary and ultimately secretion of glucocorticoids from the adrenal cortex. TRH, somatostatin,

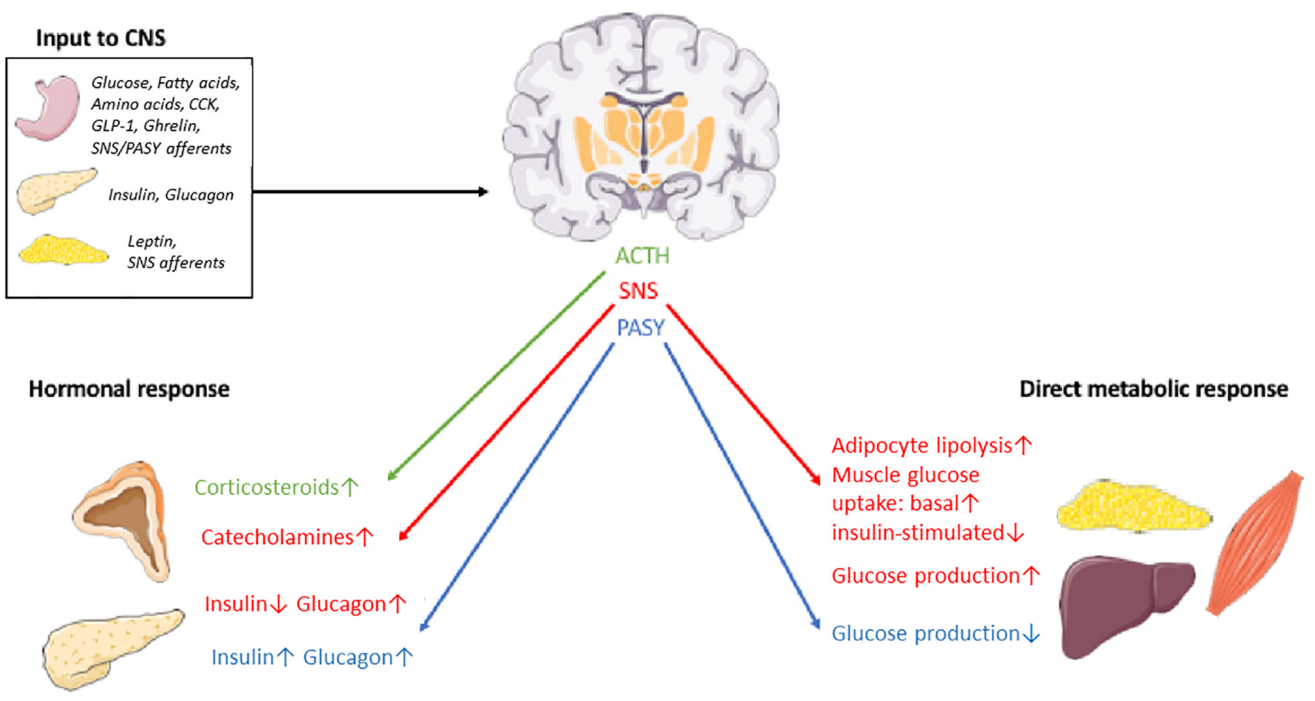

FIGURE 2 | Schematic overview of some organs, nutrients, hormones and effector pathways involved in the postulated CNS-coordinated glucose regulation. SNS, sympathetic nervous system. PASY, parasympathetic nervous system. The illustrations of anatomical structures were retrieved from http://smart.servier.com and have thereafter been assembled and processed.

vasopressin and oxytocin is also synthesized in the PVN and may also contribute to long-term regulation of energy and glucose metabolism (Roh et al., 2016; López-Gambero et al., 2019). Finally, growth hormone (GH) release from the pituitary is stimulated by growth hormone-releasing hormone (GHRH) and inhibited by somatostatin, both released from the hypothalamus. The increase in GH levels observed during hypoglycemia seem to be dependent on alpha-adrenergic dependent somatostatin inhibition and GHRH stimulation (Lim and Khoo, 2000). GH raises glucose levels in the periphery by essentially counteracting insulin. This effect is, however, opposed by the insulin-like action of IGF-1, release of which from the liver is stimulated by GH (Clemmons, 2012). Nevertheless, type 2 diabetes is a distinct clinical consequence of GH excess in acromegaly (Hannon et al., 2017), attesting to a net hyperglycemic effect of the growth hormone axis. A subset of neurons in the ARC express GHRH and seem to be involved in the regulation of GH secretion in a complex interplay with POMC-, NPY- and somatostatin-expressing neurons (Steyn et al., 2016). Indeed, POMC and NPY/AgRP neurons are implicated in CNS-mediated glucose and energy regulation, acting in opposite directions (Tups et al., 2017). Thus, the hypothalamus-pituitary-growth hormone axis may provide an additional effector pathway from the brain to the periphery that influences systemic glucose homeostasis. By binding to the receptor GHSR on GHRH neurons, ghrelin may interfere with this pathway along with a direct effect on NPY/AgRP neurons (Steyn et al., 2016).

Evidence of CNS involvement in glucose homeostasis also comes from a study in which mice that moved from a warm to a cold environment had a coordinated reduction in both insulin secretion and insulin sensitivity, despite the absence of hypoglycemia. This was reversed within 4 h of returning to room temperature and within 30 min of administration of the alpha-adrenergic blocker phentolamine. According to the authors, the results imply a key role of the brain in rapidly coordinating insulin secretion and insulin sensitivity via the SNS (Morton et al., 2017). The control of body temperature is coordinated in the hypothalamus (Morrison, 2016) and crosstalk between this neuronal circuitry and those that are supposedly involved in glucose regulation would not be surprising.

EVIDENCE FROM STUDIES IN HUMANS

Clinical Observations in CNS and Metabolic Disorders

There is an intriguing epidemiological correlation between features of the metabolic syndrome and various diseases affecting the CNS of humans that gives indirect support to the brain's involvement in peripheral glucose regulation. Depression is twice as common in patients with type 2 diabetes (Moulton et al., 2015). A correlation between depression and insulin resistance that was attenuated when adjusting for bodyweight and other confounders was reported in a meta-analysis from 2013 (Kan et al., 2013). In a longitudinal study, depression in middle-aged women was associated with increased insulin resistance and increased incidence of diabetes type 2, both of which were explained by increased central adiposity (Everson-Rose et al., 2004). Other longitudinal studies have highlighted an increased risk in patients with depression to develop the metabolic syndrome and/or its components (Marazziti et al., 2014).

An increased prevalence of the metabolic syndrome has also been reported repeatedly in studies of patients with psychotic illness including schizophrenia (Newcomer, 2007). While this may be partly due to the well-known side effects of antipsychotics, a meta-analysis from 2016 reported that first-episode psychosis was associated with increased insulin resistance and impaired glucose tolerance (Perry et al., 2016). Similar associations have been observed for anxiety and bipolar disorder (Czepielewski et al., 2013; Smith et al., 2013) while studies addressing the connection between work-related stress and development of diabetes type 2 have been inconsistent (Cosgrove et al., 2012; Krajnak, 2014). Patients with type 2 diabetes also have an increased risk of developing dementia, particularly vascular dementia and Alzheimer's disease and it has been hypothesized that insulin resistance in the brain contributes to the development of Alzheimer's disease (Li et al., 2015; Chatterjee and Mudher, 2018).

Naturally, epidemiological observations such as these do not prove causation, still leaving us with the question: does CNS diseases cause metabolic disorders or vice versa? In some cases, there is probably a bidirectional causality, e.g., for Alzheimer's disease and type 2 diabetes (Shinohara and Sato, 2017). However, one plausible causal mechanism is activation of the stress system (primarily the ANS and the hypothalamic-pituitary-adrenal axis) in psychiatric and neurodegenerative diseases leading to metabolic consequences down the line. As outlined previously, these two systems constitute efferent pathways by which the brain may exert its putative influence on whole-body glucose homeostasis. Over-activation of either or both of these systems have been postulated to contribute to the development of metabolic disorders.

Disturbances of the Autonomic Nervous System in CNS and Metabolic Disorders

As previously described, the ANS consists of the SNS and the PASY. It has a range of biologic effects including influencing glucose homeostasis in the periphery. Activation of the SNS raises circulating glucose levels by inhibiting insulin release from beta cells, stimulating release of glucagon from alpha cells, increasing release of epinephrine from the adrenal glands (augmenting and prolonging the effect of nervous activity) and increasing hepatic glucose production. In contrast, PASY activation lowers glucose levels by stimulating the secretion of insulin from beta cells and suppressing hepatic glucose production, while similarly increasing glucagon levels (Roh et al., 2016). While intravenous use of adrenalin more or less replicates the effects of SNS activity, blocking the cholinergic receptors of the PASY with atropin was reported to induce an unexpected rise in insulin sensitivity in one study (Svensson et al., 2011). This is possibly explained by differential effects of nicotinic and muscarinic receptors on peripheral insulin sensitivity. By and large though, chronic over-activation of SNS and/or under-activation of PASY

would in theory raise blood glucose levels and thereby possibly contribute to the development of type 2 diabetes and other metabolic disorders.

Studies employing biochemical measurements of catecholamines as markers of SNS activity in subjects with insulin resistance, obesity and the metabolic syndrome have been inconsistent (Kjeldsen et al., 1983; Troisi et al., 1991; Tuck et al., 1992; Young et al., 1992; Tataranni et al., 1997; Lee et al., 2001; De Pergola et al., 2008; Surwit et al., 2010; Dai et al., 2012; Reimann et al., 2017). Those using measurement of sympathetic reactivity as reflected by levels of catecholamines in serum or urine after provocation by mental stress, cold stress or food intake have found some correlation to obesity (Schwartz et al., 1987), future development of obesity (Flaa et al., 2008b) and insulin resistance (Flaa et al., 2008a).

By using microneurography, muscle sympathetic nerve activity (MSNA) can be measured as pulse synchronous nerve bursts in resting muscles and constitute a surrogate measure for sympathetic activity in the whole body (Parati and Esler, 2012). Some studies using this method have demonstrated increased SNS in subjects with the metabolic syndrome compared to those without (Huggett et al., 2004; Grassi et al., 2009).

Heart rate variability can be computed by analyzing long-term or short-term ECG monitoring and used as a marker of sympathetic and parasympathetic activity and to reflect the balance of the two. The PASY and the SNS activity influence the heart rate variability in partially differential frequency bands. PASY activity is affected by the respiratory cycle and is detectable up to 1 Hz while SNS activity is largely dependent on input from baroreceptors, mediating a slower response up to 0.15 Hz. Therefore, the high frequency (HF) band of 0,15–0,40 Hz corresponds well to PASY activity while the low frequency (LF) parameter of 0.04–0.15 Hz is influenced by both SNS and PASY activity (Malik et al., 1996). There are several other indices of HRV (Kleiger et al., 2005). The ratio of LF/HF has been used to represent balance between SNS and PASY activity, although this has been criticized as being somewhat simplistic (Perini and Veicsteinas, 2003). Moreover, it has been pointed out that increased heart rate as such contributes to unfavorable HRV profiles, and that this must be taken into consideration when interpreting results from HRV studies (Monfredi et al., 2014). Henceforth, the term "low HRV," although somewhat imprecise, will be used to describe an HRV profile indicative of relative over-activation of the SNS.

Several studies have shown a lower HRV in subjects with T2DM but whether HRV is a consequence of glycemic dysregulation or a causative mechanism behind the development of diabetes type 2 is not clear (Benichou et al., 2018). One study reported that reduced variability in the LF range was associated with increased risk of developing type 2 diabetes (Carnethon et al., 2003) while another study only found a correlation between baseline heart rate (and no other HRV indices) and the incidence of type 2 diabetes (Carnethon et al., 2006).

It is generally accepted that increasing insulin resistance precedes the development of overt type 2 diabetes. First degree relatives to patients with type 2 diabetes have a markedly increased risk of developing type 2 diabetes and are more insulin resistant than subjects with no family history of type 2 diabetes (Eriksson et al., 1989; Harrison et al., 2003). One study aimed at studying the association between insulin resistance and HRV in first-degree relatives of patients with diabetes type 2 compared with controls. While discovering no differences in insulin resistance in the groups, BMI and the LF/HF ratio were significantly and independently associated with insulin resistance (Svensson et al., 2016). Several other studies have found a correlation between measures of insulin resistance, glycemic status and HRV (Reims et al., 2004; Perciaccante et al., 2006; Stein et al., 2007; Thiyagarajan et al., 2012; Charles et al., 2013; Jarczok et al., 2013; Li et al., 2013; Cherkas et al., 2015; Indumathy et al., 2015; Meyer et al., 2016). In other studies, no correlation has been observed (Rannelli et al., 2017) or a differential correlation between men and women has been reported (Flanagan et al., 1999; Rannelli et al., 2017).

Multiple studies have also found an association between obesity and impaired HRV. BMI in itself has been significantly related to HRV in some studies (Molfino et al., 2009; Alrefaie, 2014; Hillebrand et al., 2015; Indumathy et al., 2015; Johncy et al., 2015) but not in others (Antelmi et al., 2004). Some studies suggest a stronger correlation between HRV and other anthropometric indices such as visceral adipose tissue, waist-hip ratio, waist circumference and body fat percentage: arguably markers that better reflect metabolically detrimental adiposity (Lindmark et al., 2005; Kimura et al., 2006; Sztajzel et al., 2009; Poliakova et al., 2012; Windham et al., 2012; Yi et al., 2013; Chintala et al., 2015; Indumathy et al., 2015; Yoo et al., 2016; Rastović et al., 2017; Yadav et al., 2017). Several studies have shown an improvement of HRV following weight-loss after surgery or by caloric restriction (Karason et al., 1999; Akehi et al., 2001; Ito et al., 2001; Nault et al., 2007; de Jonge et al., 2010; Sjoberg et al., 2011; Lips et al., 2013; Maser et al., 2013; Pontiroli et al., 2013; Casellini et al., 2016).

In a systematic review from 2014 on the correlation between HRV and the metabolic syndrome, the authors concluded that heart rate variability was generally reduced in females with the metabolic syndrome compared to those without while results in men were inconsistent (Stuckey et al., 2014). Since then, some studies have demonstrated a correlation between the metabolic syndrome and indices of low HRV (Ma et al., 2017; Carvalho et al., 2018) and one study has supported the differential correlations with regards to sex mentioned above (Stuckey et al., 2015). However, some studies suggest that the link between the metabolic syndrome and autonomic dysregulation as measured by HRV is fully explained by insulin resistance (Balcioğlu et al., 2016; Saito et al., 2017) and while low HRV was reported to predict the development of the metabolic syndrome in one study (Wulsin et al., 2016), another study demonstrated that this correlation was only significant for development of hyperglycemia and high blood pressure, whereas the risk of developing obesity and dyslipidemia were not significantly correlated to low HRV (Wulsin et al., 2015).

There is some support of ANS disturbances in various CNS disorders. Low employment status and high psychosocial stress have been associated with low HRV (Hemingway et al., 2005; Brosschot et al., 2007). An impaired HRV has also been reported

in anxiety disorder (Chalmers et al., 2014), bipolar disorder (Faurholt-Jepsen et al., 2017), depression (Kemp et al., 2010), schizophrenia (Montaquila et al., 2015), and dementia (da Silva et al., 2018) although it has been suggested that this correlation may be confounded by the effects of medication (Sgoifo et al., 2015). Evidence of ANS and HPA dysregulation in CNS and metabolic disorders are summarized in **Table 1**.

The well-documented propensity of beta-blockers to cause insulin resistance and impair glycemic control in patients with diabetes (Lithell, 1991) and the fact that physical exercise improves insulin sensitivity in spite of increased SNS activity (Motahari-Tabari et al., 2014; Fisher et al., 2015) attest to the complicated relation between ANS disturbances and metabolic disorders. A troublesome aspect of studies utilizing measurement of HRV as methods is the plethora of indices available and the inconsistent manner in which these are used in different studies. This complicates comparison and somewhat limits the validity of the combined results.

Disturbances of the Hypothalamus-Pituitary-Adrenal Axis in CNS and Metabolic Disorders

An excess of glucocorticoids, such as seen in Cushing's disease or pharmacological glucocorticoid treatment, typically leads to insulin resistance and obesity as well as other features of the metabolic syndrome (Liu et al., 2014; Pivonello et al., 2016). This raises the question if elevated levels of glucocorticoids are also a feature in the "normal" pathophysiology of metabolic diseases. Glucocorticoids are produced and secreted by the adrenal cortex. This is regulated by the hypothalamus-pituitary-adrenal (HPA) axis in which hypothalamic release of CRH stimulates pituitary release of ACTH. This in turn stimulates glucocorticoid

TABLE 1 | Summary of documented disturbances of the autonomic nervous system and the hypothalamus-pituitary-adrenal axis associated with CNS and metabolic disorders.

Disorder/condition	ANS disturbances	HPA disturbances	References
Psychosocial stress	↓LF, ↓HF associated with low employment grade; ↓RMSSD associated with psychosocial stress	Inconsistent findings	Hemingway et al., 2005; Brosschot et al., 2007; Joseph and Golden, 2017
Depression	↓HF, ↑LF/HF	↑Cortisol, ACTH; Flatted or blunted diurnal cortisol curve; ↑Cortisol awakening response in youths and adolescents; Improvement after treatment with SSRI; ↓ Cortisol reactivity	Kemp et al., 2010; Stetler and Miller, 2011; Joseph and Golden, 2017; Zorn et al., 2017
Anxiety disorder	↓HF	↓ Cortisol reactivity in females with anxiety disorder; ↑Cortisol reactivity in males with social anxiety disorder	Chalmers et al., 2014; Zorn et al., 2017
Bipolar disorder	↓LF	↑Cortisol and ↑ACTH, basal and after dexamethasone test	Belvederi Murri et al., 2016; Faurholt-Jepsen et al., 2017
Schizophrenia	↓HF; several studies in favor of HRV profiles indicative of normal SNS but reduced PASY	↓ Cortisol reactivity	Montaquila et al., 2015; Zorn et al., 2017
Dementia	↓RMSSD	↑Cortisol in Alzheimer's	Notarianni, 2017;
Type 2 diabetes	↓RMSSD, ↓LF, ↓HF	Flatter diurnal cortisol curve; ↓Cortisol awakening response (Joseph and Golden, 2017)	Joseph and Golden, 2017; Benichou et al., 2018; da Silva et al., 2018
Insulin resistance	Cold pressor test and mental stress test predicative of future IR; ↓RMSSD; ↓HF;↑LF/HF; ↓↑LF	–	Reims et al., 2004; Perciaccante et al., 2006; Flaa et al., 2008a; Thiyagarajan et al., 2012; Charles et al., 2013; Jarczok et al., 2013; Li et al., 2013; Indumathy et al., 2015; Meyer et al., 2016; Svensson et al., 2016
Obesity	↓HF; ↑LF; ↓LF ↑LF/HF; ↓RMSSD	↑Cortisol reactivity; ↑Expression of 11βHSD1 ↑Hair cortisol levels	Molfino et al., 2009; Alrefaie, 2014; Wester et al., 2014; Hillebrand et al., 2015; Incollingo Rodriguez et al., 2015; Indumathy et al., 2015; Johncy et al., 2015; Jackson et al., 2017
Metabolic syndrome	↓LF; ↓HF; ↑LF/HF (females);↓RMSSD	↓Urinary free cortisol levels (females); ↑Hair cortisol levels	Abraham et al., 2013; Stuckey et al., 2014, 2015; Kuehl et al., 2015; Ma et al., 2017; Carvalho et al., 2018

Only major results presented for reasons of clarity. HF, power in the high frequency range of HRV (>0,15 Hz), mostly affected by the parasympathetic nervous system (PASY); LF, power in the low frequency range (0,04–0,15), affected by activity in both PASY and the sympathetic nervous system (SNS); LF/HF, reflects the balance between SNS and PASY, high values reflecting relative SNS over-activity, due to either low PASY or high SNS activity; RMSSD, Root mean square of successive RR interval differences, mostly affected by PASY.

TABLE 2 | Summary of some important studies on CNS effects of nutrients and hormones on systemic glucose metabolism in humans.

Nutrient/hormone	Major findings	References
Glucose	↓ARC activity on fMRI directly after glucose ingestion predicted subsequent insulin levels; Hypoglycemia induced changes in hypothalamus activity prior to rise in CRH in healthy subjects; ↓Brain responses to hypoglycemia associated with attenuated levels of CRH in T1D with HU; CNS glucose uptake correlates negatively with insulin sensitivity	Dunn et al., 2007; Page et al., 2009; Osada et al., 2017; Boersma et al., 2018; Hwang et al., 2018
Insulin	Intranasal insulin leads to ↑insulin sensitivity and ↓HGP associated with changes in hypothalamic activity on fMRI and ↑PASY outflow; different CNS response to intranasal insulin and hyperinsulinemia in obese vs. lean subjects	Tschritter et al., 2006; Stingl et al., 2010; Heni et al., 2012, 2014b, 2017
GLP-1	Extra-pancreatic ↓HGP; GLP-1 receptors found in the CNS; fMRI-signs of altered CNS activity after administration	Prigeon et al., 2003; Alvarez et al., 2005; De Silva et al., 2011; van Bloemendaal et al., 2015; Farr et al., 2016a,b; Ten Kulve et al., 2016; Coveleskie et al., 2017

ARC, arcuate nucleus; fMRI, functional magnetic resonance imaging; CRH, counter-regulatory hormones; T1D, type 1 diabetes; HU, hypoglycemic unawareness; HGP, hepatic glucose production; PASY, parasympathetic nervous system.

production and secretion. Feedback mechanisms occur at several levels. In the periphery, particularly in the liver and in adipose tissue, 11βHSD1 converts inactive cortisone to active cortisol whereas 11βHSD2 inactivates cortisol in a reverse manner, collectively providing an additional regulatory mechanism of glucocorticoid activity.

Circulating glucocorticoids act in several ways to disrupt normal glucose homeostasis. Glucocorticoids may cause beta cell dysfunction by influencing the uptake and metabolism of glucose in beta cells and may also attenuate the effects of GLP-1. In the periphery, glucocorticoids counteract insulin action by inducing insulin resistance through reduction of insulin downstream signaling substrates as well as indirectly by increasing lipolysis and proteolysis. Glucocorticoids also increase hepatic glucose production by activating enzymes involved in gluconeogenesis. Moreover, glucocorticoids also cause obesity and consequently insulin resistance by promoting differentiation and proliferation of adipocytes. Since glucocorticoid receptors are more abundant on visceral adipocytes, this favors visceral obesity (Di Dalmazi et al., 2012). Finally, excess glucocorticoids may lead to general weight gain (Berthon et al., 2014), probably at least partially by stimulating appetite through actions in the CNS, as is supported by a recent study (Serfling et al., 2019).

There is a multitude of ways to assess activity in the HPA-axis. These include plasma levels of cortisol, salivary levels of cortisol, 24 h urinary excretion of cortisol (UFC) and hair cortisol concentration (HCC). Activity in the HPA axis is highly variable and influenced by a range of factors including circadian rhythmicity, exercise, stress, and food-intake. Therefore, not only absolute concentrations of but also the diurnal curve of levels and dynamic changes in cortisol levels, as measured by cortisol reactivity to stress and dexamethasone suppression test, are interesting markers.

A few reviews on the topic have been published (Abraham et al., 2013; Incollingo Rodriguez et al., 2015) and will not be covered in detail here. From these and from original studies we can conclude that connections between obesity or the metabolic syndrome and basal levels of cortisol as measured in plasma, saliva, or urine are either inconsistent or disparate with regards to sex (Abraham et al., 2013; Incollingo Rodriguez et al., 2015). HCC is arguably a more reliable indicator of long-term levels of cortisol (Russell et al., 2015), in analogy with HbA1c, and studies using this outcome are more consistently in favor of such connections (Wester et al., 2014; Kuehl et al., 2015; Jackson et al., 2017). Moreover, consistently elevated cortisol reactivity tests in obese subjects as well as tendencies toward a flatter cortisol curve during day-time in obese subjects and toward impaired dexamethasone suppression tests in subjects with abdominal obesity was reported in one systematic review (Incollingo Rodriguez et al., 2015). Studies of HPA dysregulation in CNS disorder show variable results that are summarized in **Table 1** (Stetler and Miller, 2011; Belvederi Murri et al., 2016; Joseph and Golden, 2017; Notarianni, 2017; Zorn et al., 2017).

In summary, long-term elevations, disturbances in the diurnal pattern, abnormal reactivity to stress and impaired feedback regulation of cortisol may have a small but limited impact on the development of obesity and metabolic disorder. Finally, there is substantial evidence that the expression of 11βHSD1 is upregulated in the adipose tissue of obese humans and animals. Thus, local dysregulation of glucocorticoids just as HPA dysregulation may contribute to the development of obesity and the metabolic syndrome (Incollingo Rodriguez et al., 2015).

Central Effects of Glucose and Insulin on Systemic Glucose Metabolism: What Have We Learned From Neuroimaging Studies of Humans?

The methods employed in animal research previously discussed in this review are for obvious reasons not directly transferable to studies involving human subjects. The development and refinement of neuroimaging techniques has, however, offered an indirect way to explore changes in brain activity induced by and associated with metabolic changes. The neuroimaging techniques at hand (described in **Box 1**) have been extensively used to study CNS control of feeding and appetite which has been reviewed comprehensively elsewhere (De Silva et al., 2012; Zanchi et al., 2017). While PET is still used to a limited degree,

BOX 1 | Neuroimaging techniques.

Magnetoencephalography (MEG)

This technique measures magnetic fields produced by electrical currents in the brain just as EEG measures electric fields. It is limited by the fact that cortical but not subcortical regions (including the hypothalamus) can be evaluated and has been largely replaced by other techniques to study the impact of metabolic input on brain activity.

Functional Magnetic Resonance Imaging (fMRI)

Blood oxygen level dependent (BOLD) signal is based on the fact that deoxygenated hemoglobin is paramagnetic whereas oxygenated hemoglobin is not, resulting in a higher signal in T2 weighted MRIs in situations with increased blood flow. Cerebral blood flow can also be measured by arterial spin labeling (ASL) where the water of incoming blood in for instance carotid arteries are labeled magnetically which changes the T1 signaling of water in the region that is examined.

The resolution in time and space is better than PET. Neuronal activity is not measured directly but via cerebral blood flow as a surrogate marker. By measuring spontaneous fluctuations in resting state brain activity, functional connectivity can be assessed and utilized to divide the brain into small functional units.

Positron Emission Tomography (PET)

This method measures gamma-rays that are emitted by unstable isotopes. Molecules that are labeled with positron-emitting isotopes are injected into the circulation. Fludeoxyglucose (18F) or FDG is widely used as a measure of glucose-uptake into tissues including the brain. To allow for neuroanatomic correlation of the signals, the method is combined with either CT or MRI and thus the space resolution does not differ from fMRI. However, the half-life of FDG is 110 min and it takes considerable time to generate a strong enough signal for the analysis. Hence, the time resolution is markedly limited compared to other methods.

Single-Photon Emission Computed Tomography (SPECT)

Similar to PET, this technique utilizes radioactive tracer material, but these emit gamma rays directly instead of positrons, limiting the spatial resolution as compared to PET.

fMRI has largely replaced other techniques as the method of choice for investigative purposes. Studies may use imaging of the whole brain or, more commonly, specific regions of interest (ROI) such as the hypothalamus. The small size and the location of the hypothalamus makes it difficult to image. In early studies, the hypothalamus was divided by a mid-sagittal slice resulting in the upper anterior, upper posterior, lower anterior, and lower posterior hypothalamus (Matsuda et al., 1999; Smeets et al., 2005). This division is too rough to allow for differential analysis of individual nuclei. However, a recent study utilized areal parcellation of the hypothalamus with a resolution of 1.25 mm × 1.25 mm and resting-state functional connectivity to generate 10 focii corresponding to different nuclei based on a previous histological study, demonstrating that such analysis of individual nuclei is indeed feasible (Osada et al., 2017).

It is not within the scope of this review to fully discuss the findings from the numerous studies aimed at exploring functional changes on brain activity as related to appetite or feeding control. However, a brief summary is warranted given the intimate relationship between energy intake and glucose regulation. A decrease in neural activity following glucose and meal intake has been reported in the hypothalamus and elsewhere (Matsuda et al., 1999; Tataranni et al., 1999; Gautier et al., 2000; Liu et al., 2000; Smeets et al., 2005, 2007; Flanagan et al., 2012; Page et al., 2013) that differs in obesity and type 2 diabetes according to some studies (Matsuda et al., 1999; Gautier et al., 2000; Vidarsdottir et al., 2007). Moreover, studies employing task-based fMRI protocols have demonstrated activation of several CNS regions (in particular the orbitofrontal cortex, amygdala, hippocampus and insula) upon presenting the subject with visual food cues (LaBar et al., 2001; Killgore et al., 2003; St-Onge et al., 2005; Porubská et al., 2006; Führer et al., 2008) which was attenuated in the fed state in some studies (Baldo and Kelley, 2007; De Silva et al., 2011) and different in obesity or insulin resistance in others (Killgore and Yurgelun-Todd, 2005; Rothemund et al., 2007; Stoeckel et al., 2008; Martin et al., 2010; Wallner-Liebmann et al., 2010; Dimitropoulos et al., 2012; Heni et al., 2014a; Alsaadi and Van Vugt, 2015). Caloric restriction normalized the hypothalamic response to glucose challenge in patients with type 2 diabetes in one study (Teeuwisse et al., 2012). Finally, appetite and feeding patterns in the CNS have been examined with respect to pharmacological administration and circulating levels of implicated hormones (Zanchi et al., 2017).

Collectively it seems that there are relatively well-characterized brain responses that are triggered by ingested and circulating levels of nutrients and hormones. Whether these brain responses have an actual effect on peripheral glucose metabolism has not, however, been investigated to a similar extent. The existence of the cephalic phase of insulin secretion as well as the observation that visual food-cues leads to lower postprandial glucose levels without affecting food-intake, insulin levels or other neuroendocrine parameters (Brede et al., 2017) is suggestive of such a response, the exact effector mechanisms of which remains elusive.

In the previously discussed study by Osada et al. (2017), 12 healthy subjects, both male and female, underwent fMRIs on two separates days following ingestion of 75 g of glucose solution or an equal volume of water. The hypothalamus was the region of interest and the imaging was analyzed by area parcellation, enabling analysis of individual nuclei. Thereby, the authors could report that the decrease in ARC activity between 0 and 10 min after the glucose ingestion significantly predicted insulin levels at 10 min. Thus, a causative link between the two events was plausible, according to the authors.

Hypoglycemia, on the other hand, was shown to induce changes in hypothalamic activity prior to the rise in counter-regulatory hormones (glucagon, cortisol, adrenaline, or noradrenaline) in healthy subjects (Page et al., 2009). The effect of hypoglycemia on CNS activation has been intensively studied in the setting of type 1 diabetes with unawareness. These patients have been shown to have a blunted rise in cerebral blood flow in hypoglycemia (Dunn et al., 2007; Mangia et al., 2012; Hwang et al., 2018) associated with a corresponding attenuation of counter-regulatory hormonal response (Mangia et al., 2012; Hwang et al., 2018).

While the intracerebroventricular administration of nutrients and hormones performed in animal studies is not an option in studies of humans, intranasal treatment of insulin preferentially targets the CNS, and thus provides an alternative for studying CNS effects isolated from systemic effects (Born et al., 2002). Intriguingly, intranasal insulin treatment has been demonstrated to lead to weight loss (Hallschmid et al., 2004), suppressed lipolysis (Iwen et al., 2014), positive cognitive results (Benedict et al., 2004; Hallschmid et al., 2008) and rapid reduction of glucose levels without any impact on C-peptide or insulin levels (Hallschmid et al., 2012). Heni et al. (2012, 2014b, 2017) have investigated the mechanisms behind this in a series of experiments where they showed an improvement of insulin sensitivity that was correlated with changes in hypothalamic blood flow on fMRI. An increase of HRV in the HF band was seen after intranasal insulin in one study, suggestive of augmented PASY outflow activity as a possible mediator between the CNS and the periphery (Heni et al., 2014b). In conflict with these studies, Ott et al. (2015) found no central effect of intranasal insulin aspart that could be discerned from its spillover effect in the periphery. Heni et al. (2018) recently reported yet unpublished results showing increased C-peptide levels 10 min after intranasal insulin administration during a hyperglycemic clamp in subjects with hypothalamic insulin sensitivity (as evidenced by a decrease of hypothalamic blood flow following administration) compared to those without. No reduction of C-peptide levels occurred in either group after administration, speaking against a substantial spill-over effect of intranasal insulin in this experiment (Heni et al., 2018).

Dash et al. (2015a) reported an inhibition of HPG 180 min after treatment with intranasal insulin, much later than what was seen in the studies by Heni et al. (2012, 2014b, 2017, 2018) According to the authors, this delayed response could be explained by a CNS-mediated effect on gene expression of proteins involved in hepatic gluconeogenesis rather than a more direct effect (Dash et al., 2015b). Similar kinetics of central insulin effects have been reported in dogs (Ramnanan et al., 2011) and rodents (Obici et al., 2002c). Moreover, the potent K_{ATP} channel opener diazoxide induced a 30% reduction of HGP after 6 h in humans in the settings of a pancreatic clamp, excluding the influence of pancreatic hormones and suggesting extra-pancreatic effects (Kishore et al., 2011). As previously discussed, intracellular insulin signaling in the hypothalamus is dependent on functional K_{ATP}-channels and the brain may therefore be the extra-pancreatic site where diazoxide exerts its antidiabetic effects. In rats, ICV administration of diazoxide did indeed cause a reduction of HGP explained by downregulation of genes involved in gluconeogenesis (Kishore et al., 2011). Finally, human subjects with type 2 diabetes compared to controls did not exhibit suppression of HGP following diazoxide treatment in another study (Esterson et al., 2016), perhaps because of resistance to its extra-pancreatic effects.

There are several other findings suggesting the existence of CNS insulin resistance. A different response to intranasal insulin in obese vs. lean subjects has been reported in many studies (Hallschmid et al., 2004; Tschritter et al., 2006; Stingl et al., 2010; Heni et al., 2014b, 2017). In a recent study by our research team, whole-body ^{18}FDG-PET was performed on patients with varying degrees of insulin sensitivity during a hyperinsulinemic-euglycemic clamp. Interestingly the *M*-value, an index of insulin sensitivity, correlated positively with uptake in muscles, adipose tissue and the liver and correlated negatively with glucose uptake in the brain (Boersma et al., 2018). This is corroborated by yet unpublished results by Rebelos et al. (2018). In their study of ^{18}FDG-PET examinations in 151 subjects, the glucose uptake in the brain was correlated positively with BMI and negatively with insulin sensitivity. Of further interest, glucose uptake in the brain correlated positively with MRI signs of inflammation in the hypothalamus and amygdala (Rebelos et al., 2018), which is in consistency with another study (Thaler et al., 2012). Thus, hypothalamic inflammation may exist in obese humans in accordance with previously discussed findings in animal studies and may cause hypothalamic insulin resistance by the same proposed cellular mechanisms. Finally, Hirvonen et al. (2011) reported that brain glucose metabolism as assessed by ^{18}FDG-PET increased in subjects with impaired glucose tolerance during a hyperinsulinemic-euglycemic clamp but not in healthy subjects, in whom the effect of insulin on brain glucose metabolism appeared to be saturated at fasting levels of insulin. Although this may suggest that insulin resistant subjects need more insulin to have a maximal effect on brain glucose metabolism, the fact that there was no difference in brain glucose metabolism at baseline between the two groups argues against the concept of such an insulin resistance in the CNS as a feature of systemic insulin resistance (Hirvonen et al., 2011).

Aside from the potential role of hypothalamic inflammation, the observation that the CSF:plasma ratio of insulin is inversely related to BMI (Kern et al., 2006) supports deficient transport of insulin as a cause for the putative hypothalamic insulin resistance. **Table 2** summarizes some important studies of CNS effects of nutrients and hormones on systemic glucose metabolism in humans.

Central Effects of Other Hormones, Bariatric Surgery and Pharmacological Agents on Systemic Glucose Metabolism

Leptin seems to regulate energy homeostasis in humans by central mechanisms (Rosenbaum et al., 2008; Grosshans et al., 2012) and to have an impact on whole-body glucose regulation in humans, as witnessed by its antidiabetic effects on patients with congenital leptin deficiency, congenital lipodystrophy, HIV-associated lipodystrophy and uncontrolled diabetes type 1 (Moon et al., 2013; Ramos-Lobo and Donato, 2017). However, it is not clear to which extent glucose regulation is carried out by peripheral vs. central mechanisms and no large effects have been demonstrated on the ANS (Eikelis et al., 2003; Machleidt et al., 2013) or the HPA-axis (Licinio et al., 1997; Sienkiewicz et al., 2011; Khan et al., 2012) that would grant indirect support to a contribution of central mechanisms. Oxytocin has demonstrated positive effects on various indices of glucose metabolism in several studies when given as a nasal spray (Ott et al., 2013; Lawson et al., 2015; Thienel et al., 2016), whereas the evidence

from intravenous use is inconsistent (Lawson, 2017). Whether this is due to different central vs. peripheral effects on glucose metabolism and the preferential targeting of CNS by intranasal administration has not been illuminated.

The dopaminergic system has also been suggested to influence glucose homeostasis. The dopamine agonist bromocriptine-QR has been associated with an absolute reduction of HbA1c of 0,69% and is approved by the FDA for treatment of type 2 diabetes in adults (Garber et al., 2013). There are several possible mechanisms, probably working in concert. Firstly, dopamine receptors (D2R) are expressed in beta cells of the pancreas and activation of these have been shown to inhibit insulin secretion and cause an anti-proliferative effect (Rubi et al., 2005; Ustione et al., 2013). How this would lead to an improvement of glycemic indices is, however, challenging to comprehend. Secondly, activation of D2R in the pituitary gland reduces secretion of prolactin (excess of which is associated with hyperphagia and lipid accumulation in adipose tissue) and growth hormone (Woodside, 2007; Ben-Jonathan and Hugo, 2015). Thirdly, D2R-receptors are ubiquitous in the CNS where dopamine agonists have been shown to suppress levels of noradrenalin and serotonin in the hypothalamus (Luo et al., 1998), possibly resulting in reduced outflowing activity of the SNS from the brainstem to the periphery down the line. The dopaminergic system and D2R is also implicated in reward and appetite regulation (Baik, 2013). Interestingly, impairment of the dopaminergic system is a feature of Parkinson's disease as well as an effect of antipsychotic medication and may partly explain the association of metabolic disorders with these conditions (Lopez Vicchi et al., 2016).

The propensity of bariatric surgery to improve glycemic control in patients is well-recognized and may offer insights regarding the development and pathogenesis of type 2 diabetes. Superiority of surgery to pharmacological approaches has been consistently documented in a vast number of randomized controlled trials (Koliaki et al., 2017). According to guidelines endorsed by several international diabetes organizations, so-labeled metabolic surgery in the treatment of patients with type 2 diabetes and BMI $>$ 30 is advocated when lifestyle and pharmacological approaches has proved insufficient (Rubino et al., 2017). While still a matter of some controversy, there is substantial evidence indicating that the improvement of glucose indices seen after surgery is not fully explained by weight loss alone. The existence of weight independent mechanisms is suggested by the observed rapid improvement of glucose metabolism, occurring only days after surgery (Pories et al., 1995). Moreover, rates of diabetes remission differ depending on surgical technique, those bypassing the foregut, such as Roux-en-Y gastric bypass and biliopancreatic diversion being more efficacious in this regard than those who simply restricts the volume of the ventricle, such as gastric banding and gastric sleeve gastrectomy (Kodama et al., 2018).

A number of neuroimaging studies have demonstrated a profound impact of bariatric surgery on structural and functional features of the CNS. Obesity is associated with radiological signs of brain volume reduction and two studies have demonstrated normalization of these signs after bariatric surgery (Tuulari et al., 2016; Zhang et al., 2016). The enhanced response in reward regions to visual food-cues before surgery (and consistently demonstrated in obesity) have been reported to resolve after surgery (Scholtz et al., 2014; Frank et al., 2016). Two studies have shown an increase in these responses post-surgery following administration of octreotide (Goldstone et al., 2016) (an inhibitor of pancreatic hormones) and a GLP-1 receptor antagonist (Ten Kulve et al., 2017), respectively, suggesting altered secretion of gut hormones as a mediator. The role of GLP-1 in mediating satiety via the CNS is further supported by other neuroimaging studies (De Silva et al., 2011; van Bloemendaal et al., 2015; Farr et al., 2016a,b; Ten Kulve et al., 2016; Coveleskie et al., 2017). GLP-1 receptors are expressed in the brainstem and hypothalamus of humans (Alvarez et al., 2005) and some animal studies suggest that the effects of GLP-1 on reduction of food intake are partly if not primarily mediated by CNS actions (Kanoski et al., 2011; Secher et al., 2014). An effect on glucose metabolism independent of food intake has also been observed in animals (Sandoval et al., 2008) and in humans where a suppression of HGP without any measurable impact on pancreatic hormone levels has been reported (Prigeon et al., 2003).

The postprandial secretion of GLP-1 is augmented after bariatric surgery (Hutch and Sandoval, 2017). This is also true for oxyntomodulin (Laferrère et al., 2010), another gut hormone that has demonstrably induced weight loss in humans and rodents by reducing food intake and increasing energy expenditure (Dakin et al., 2001; Cohen et al., 2003; Baggio et al., 2004; Wynne et al., 2005, 2006). It appears to have a dual mechanism, acting on both GLP-1 and glucagon receptors (GCR) (Pocai, 2012). In rodents, a superior effect on weight loss was observed compared to GLP-1RA treatment alone at similar antihyperglycemic effects (Kosinski et al., 2012) making combined targeting of GLP1R and GCR attractive targets for future treatment of obesity as well as type 2 diabetes. Indeed, the combined agonist SAR425899 was recently evaluated in a phase 2 trial, reporting a significant weight loss and HbA1c reduction compared to placebo (Tillner et al., 2019). The concept of targeting glucagon receptors in the treatment of type 2 diabetes might be challenging to accept at first. Hyperglycemia or attenuation of the hypoglycemic effects of GLP-1R action would be a warranted concern. However, the improvement of glycemic indices reported in the study implies that the GLP-1R mediated effects on glucose homeostasis overrides those mediated by GCR. Intriguingly, glucagon injected into the CNS of rodents unexpectedly lowers systemic glucose levels by inhibition of HGP (Mighiu et al., 2013; LaPierre et al., 2015). Furthermore, intravenous infusion of glucagon leads to a transient hyperglycemia in rats, and this transiency is abolished when simultaneously blocking hypothalamic glucagon signaling (Mighiu et al., 2013). Thus, glucagon may actually have delayed CNS-mediated hypoglycemic effects opposing the acute hyperglycemic peripheral effects. The net effect of this could furthermore contribute to rather than impede the observed glycemic improvement of SAR425899.

The dual GIP/GLP-1 receptor agonists LY3298176 have been developed and recently demonstrated superiority to single GLP-1 RA treatment in terms of HbA1c reduction and weight loss (Frias et al., 2018). While considerably less is known about the CNS

effects of GIP compared to GLP-1 and glucagon, dual GIP/GLP-1 RA treatment has shown positive effects on animal models of Alzheimer's disease and Parkinson's disease (Hölscher, 2018).

Circulating bile acids also increases after bariatric surgery (Jahansouz et al., 2016) and appear to have positive metabolic effects on their own accord (Sinclair et al., 2018). Bile acids have been demonstrated to cross the blood–brain-barrier where the expression of one of its receptors GPBAR1 has been reported (Keitel et al., 2010). Binding to FXR, another receptor, leads to positive metabolic effects in several organs and increases expression of FGF 15/19 from the foregut (Pournaras et al., 2012). As described previously, members of the FGF family have been shown to exert long-lasting and pronounced antidiabetic effects that appears to be mediated largely by central mechanisms (Fu et al., 2004; Morton et al., 2013; Marcelin et al., 2014; Scarlett et al., 2016). The targeting of bile acid signaling and in particular FGF signaling thus constitute promising future strategies in the treatment of type 2 diabetes.

There are a handful of drugs approved for treatment of obesity that primarily target the CNS. Among these, both lorcaserin and topiramate-phentermine have shown positive effects on glycemic indices. Lorcaserin, a 5HT-2c agonist was found non-inferior to several glucose-lowering drugs in terms of HbA1c reduction (0.55% for lorcaserin) and proportion of patients reaching target HbA1c in a recent meta-analysis (Neff et al., 2017). The authors conclude that lorcaserin should be considered as a second line add-on treatment in patients with type 2 diabetes and BMI > 27. A recently published RCT reported a more moderate reduction of HbA1c of 0.33% for patients with type 2 diabetes compared to placebo (Bohula et al., 2018). The combination of the amphetamine-like drug phentermine and the antiepileptic drug topiramate approved for treatment of obesity has likewise demonstrated a reduction in HbA1c of 4.4 mmol/mol compared to placebo in one study (Garvey et al., 2014a) and reduced progression to diabetes in patients with prediabetes and the metabolic syndrome was reported in another study (Garvey et al., 2014b). Although the antidiabetic effects of these CNS acting antiobesity drugs are relatively small-scale and probably a consequence of weight loss, an independent effect on glucose metabolism cannot be excluded at this stage.

Recently, the growth differentiating factor 15 (GDF15) has emerged as a satiety signal and potential target for treating obesity and metabolic disorders. It is secreted by adipocytes and many other cell types in response to cell injury and inflammation (Tsai et al., 2016). GDF15 has been demonstrated to induce weight loss and reduce insulin resistance in mice and primates by binding to its receptor GFRAL, the expression of which has not been demonstrated anywhere but the AP and the NTS in the brainstem (Mullican et al., 2017). Peripheral administration of GDF15 in mice induced neuronal activity in the AP and NTS and the anorexic effects were reversed by ablation of these regions (Tsai et al., 2014). Thus, existing evidence from animal studies indicate the brainstem as the target organ for GDF15. In humans, increased circulating levels of GDF15 has been observed as soon as 1 week after bariatric surgery and levels correlate linearly with insulin sensitivity both before and after surgery (Kleinert et al., 2019). Additionally, mitochondrial stress in mouse skeletal muscle cells increases the expression of GDF15 (Chung et al., 2017) and increased circulating levels in humans after physical exercise has been reported (Kleinert et al., 2018). As previously discussed, IL-6 and IL-10 also increase after exercise in humans and have been shown to mediate positive metabolic effects via the CNS in animals (Pedersen and Febbraio, 2008; Ropelle et al., 2010).

Physical exercise has been associated with altered brain fMRI responses to food-cues, favoring low-calorie foods over high calorie-foods (Killgore et al., 2013; Crabtree et al., 2014). While other studies demonstrating changes in brain fMRI responses to exercise are abundant, these have primarily focused on brain regions and processes involved in memory and learning, that are implicated in the pathogenesis of neurodegenerative disorders and dementia. To our knowledge, there are no neuroimaging studies that address the possible link between functional brain alterations and the well-recognized positive metabolic effects associated with physical exercise.

CONCLUSION

Taken together, evidence from animal and human studies demonstrates that the brain detects levels of circulating nutrients and hormones and consequently organizes an outward response that contributes to the regulation of whole-body glucose homeostasis. However, there are major knowledge gaps about the exact nature of this response and its relative importance compared to peripheral processes. As we have seen, animal studies have provided an anatomical map of CNS glucose regulation and have identified important neurons and neural circuits involved. Additionally, the CNS sensing of key nutrients and hormones has been characterized in detail and the intracellular signaling pathways have been outlined for most of them.

Studies of humans entail inherent methodological challenges compared to animal studies that may explain the inconsistent findings in some areas. There are some intriguing epidemiological relationships between CNS and metabolic disorders and dysregulation of the ANS or HPA axis have both been proposed as the mediators of this connection. Attempts to demonstrate this have yielded somewhat discordant findings, however.

The extra-pancreatic effects of diazoxide and GLP-1 may very well be extra-cerebral as well and the favorable effect of intranasal oxytocin on glucose metabolism is interesting but not necessarily secondary to actions in the CNS. The use of somatostatin and octreotide in some studies to inhibit pancreatic hormone secretion may possibly induce CNS effects on their own accord and may thereby affect the results. The use of intranasal preparations of insulin arguably has a preferential CNS effect but dose-dependent spillover into the systemic circulation has been demonstrated in some studies that would make interpretation of results precarious.

Neuroimaging techniques have a large potential for further exploration of the interplay between CNS and systemic glucose regulation and have been used more extensively for investigation

of appetite and feeding control with special regards to obesity. In these studies, resting-state or task-based neuroimaging is usually performed before and after some sort of metabolic intervention (i.e., OGTT or intranasal insulin) and the imaging is then compared for differences before vs. after the intervention and in between different subjects (i.e., lean vs. obese). Arguably, studies aimed at investigating acute CNS effects on glucose regulation must adopt a more sophisticated study design. Ideally, neuroimaging would be performed before vs. after some sort of metabolic intervention with concomitant measurements of hormonal activity and nutrients. If a detectable change in the activity of a brain region were to occur after the metabolic intervention but before a change in hormonal activity and nutrient levels, a causative link between the two events could be argued, in congruency with the results from Page et al. (2009) and Osada et al. (2017). A prerequisite for such a study design is that the utilized neuroimaging modality has a small enough time resolution to allow for such delicate temporal analysis, a description that fits fMRI but not PET. A matter of additional complexity is that the temporal relation between the CNS response to metabolic interventions and the putative peripheral effects is largely unknown and may differ between different stimuli. For instance, the response to hypoglycemia seems to be fast, occurring within 60 min in one study (Page et al., 2009), whereas the CNS-mediated insulin effects are not evident until after 180 min according to some studies (Obici et al., 2002c; Ramnanan et al., 2011; Dash et al., 2015a). Furthermore, the CNS response to shifts in nutrient availability and hormones may be conveyed to target organs by routes other than the ANS and the HPA-axis and alternative hormonal or neuronal pathways should at least be considered and evaluated in the future.

Other than in the work by Osada et al. (2017), the spatial resolution of fMRI protocols that were utilized in the reviewed studies have not permitted image analysis of separate nuclei of the hypothalamus but have either measured blood flow in the hypothalamus as a whole or in more crudely defined subdivisions of it. Since there is evidence that the relative proportion of GE and GI neurons varies in different nuclei (Mergenthaler et al., 2013), accurate subdivision of the hypothalamus may be imperative to produce reliable and relevant results. The mild inconsistencies between different neuroimaging studies is quite possibly a consequence of suboptimal resolution. This is best illustrated by the work of Heni et al. (2014b, 2017), in which one study examining the effect of intranasal insulin showed an increase of hypothalamic CBF whereas a decrease after administration was seen in another study. It is also important to underscore that the use of the surrogate markers CBF in fMRI and ^{18}FDG uptake in PET may not correctly reflect changes in neuronal activity. The glucoCEST technique in MRI uses measurement of the proportion of phosphorylated and unphosphorylated 2-deoxy-D-glucose (2DG) and could thereby provide a better assessment of glucose metabolism than ^{18}FDG-PET, while also embodying all the advantages with the fMRI modality. Likewise, MR spectroscopy provides a non-invasive method of measuring metabolite concentration in tissues and has for example been used to study hypothalamic glucose levels after hypoglycemic preconditioning (Seaquist et al., 2017). Nevertheless, neurons may exert either stimulatory or inhibitory downstream effects which are not discernable by methods limited to measurement of general neuronal activity, be that by proxy of cerebral blood flow or glucose metabolism.

In one study by Page et al. (2009), small reductions in systemic glucose levels induced increases in hypothalamic CBF before counter-regulatory hormones had risen. This raises the question: does this pattern differ in patients with glycemic dysregulation compared to normal subjects? The "brain-centric" model of development of type 2 diabetes stipulates that the hypothalamus regulates systemic glucose levels around a set-point that is skewed upwards as the disease progresses (Deem et al., 2017). Accordingly, glucose levels below this set-point would lead to processes aiming at raising glucose levels. Most likely, these processes would consist of the same counter-regulatory response that is mounted upon hypoglycemia in healthy individuals. It follows that the hypothalamic and counter-regulatory hormonal response would be activated at higher glucose levels in subjects with prediabetes compared to normal subjects. If this could be demonstrated in a hypoglycemic clamp during concomitant functional neuroimaging it would indeed lend strong support to the concepts of the "brain-centric" model. To our knowledge, this has not been demonstrated to date. Conversely, obese subjects exhibit different fMRI-responses to visual food cues during hyperglycemia than normal weight subjects according to one study (Belfort-DeAguiar et al., 2018). Whether the hypothalamic response to hyperglycemia differs in subjects with prediabetes compared to normal subjects and if such a possible difference corresponds to differences in hormonal activity remains to be investigated.

Since the brain is more or less dependent on glucose as a fuel substrate, its postulated ability to influence systemic glucose values or at least to correct hypoglycemia makes perfect sense from a teleological standpoint. As a consequence of the counter-regulatory response to hypoglycemia, systemic glucose levels are maintained at the expense of glucose uptake and utilization in insulin-sensitive organs such as muscles, adipose tissue and liver. Thus, the insulin-independent brain essentially prioritizes its own need for fuel over that of the rest of the body by mobilizing this counter-regulatory response. Expanding on this and on the "brain-centric" model, type 2 diabetes may be considered a condition in which the brain is "spoiled" by ever-increasing glucose levels and maintains those in a "selfish" fashion at the expense of the rest of the body.

In an elegant study published recently by Rodriguez-Diaz et al. (2018), pancreatic islets from three different species (humans, rhesus monkeys, and C57BL/6 mice) were transplanted to mice with streptozotocin-induced type 1 diabetes after which the glucose levels of the transplanted mice adapted to the normal ranges of the donors (varying considerably in between species). Thus, pancreatic islets were sufficient to regulate glucose levels on their own accord and this could not be overpowered by possible extra-pancreatic glucose regulation mechanisms. These findings indeed challenge the "brain-centric" model, but

the transferability of these results from rodent models with experimental type 1 diabetes to humans with type 2 diabetes is a matter of reasonable doubt. Additionally, there may be brain-islet communication pathways that are species-specific and lost upon xenotransplantation.

Overall, there is robust evidence in favor of brain involvement in the regulation of glucose metabolism. Further investigations are needed to characterize the neurocircuits involved and the cross-talk with peripheral tissues. The use of refined neuroimaging methods is a promising way to test hypotheses derived from animal studies on human subjects. Such research will deepen our understanding of the pathogenesis of type 2 diabetes and potentially open up avenues for novel pharmacological approaches.

AUTHOR CONTRIBUTIONS

ML did most of the literature search. ML and JE conceived and wrote the manuscript. All authors contributed to literature search, reviewed and approved the final version.

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Correlation Between Serum miR-154-5p and Osteocalcin in Males and Postmenopausal Females of Type 2 Diabetes With Different Urinary Albumin Creatinine Ratios

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Purpose: To investigate the serum levels of miR-154-5p, osteocalcin (OC), and other clinical parameters in male and post-menopausal female type 2 diabetes mellitus (T2DM) patients with different urinary albumin creatinine ratio (UACR) levels and to discuss the relationship between miR-154-5p and glycolipid metabolism, bone metabolism, and different urinary albumin excretion rate in T2DM.

Methods: Seven hundred thirty-eight T2DM patients were categorized into six groups, including 374 men and 364 post-menopausal women who were sub-divided into three groups based on albumin excretion that involved normal albuminuria, microalbuminuria, and large amount of albuminuria (138, 127, 109, 135, 125, and 104 cases, UACR<30, 30–300, and >300 mg/g, M1, M2, M3, F1, F2, and F3). Measurement of circulating miR-154-5p, OC, and other biochemical indicators were performed by real-time PCR, ELISA, and chemiluminescence assays in T2DM patients and in 141 M0 and 139 F0 control subjects.

Results: There are few differences appeared between groups. Comparing with men, women had higher age, waist-to-hip ratio (WHR), adiponectin (ADPN), connective tissue growth factor (CTGF), UACR, procollagen type 1 N-terminal propeptide (P1NP), β-C-terminal telopeptide of type I collagen (β-CTx), OC, and miR-154-5p, but lower FPG, HOMA-IR, and HbA1c. T2DM patients with albuminuria (micro or macro) had lower bone turnover markers (P1NP, β-CTx, and OC) and adiponectin, but higher HbA1c, CTGF, and miR-154-5p. In addition, after regression analysis, UACR was positively correlated with CTGF, HbA1c, and miR-154-5p, and negatively correlated with ADPN and bone turnover markers (P1NP, β-CTx, and OC). However, OC showed a positive correlation with ADPN and other bone turnover markers (P1NP and β-CTx), but negative correlation with CTGF, UACR, and miR-154-5p in all three groups.

Conclusion: These findings suggested that increased serum levels of miR-154-5p and decreased OC levels may influence osteogenesis and proteinuria in T2DM and may identify novel targets for diagnosis and treatment of diabetic kidney disease and osteoporosis.

Keywords: type 2 diabetes mellitus, miR-154-5p, osteocalcin, diabetic kidney disease, diabetic osteoporosis

HIGHLIGHTS

- Serum miR-154-5p was elevated in post-menopausal female T2DM patients and increased successively with UACR.
- Serum miR-154-5p was found to positively correlate with UACR and negatively correlate with OC.
- OC and UACR were associated with glycolipid metabolism, bone metabolism, and urinary albumin.

INTRODUCTION

Type 2 diabetes mellitus (T2DM), recognized as a global epidemic by the World Health Organization, is a long-term metabolic disorder characterized by high blood glucose, insulin resistance, and relative lack of insulin. One of the chronic complications of T2DM is diabetic kidney disease (DKD) that causes glomerular fibrosis, persistent proteinuria, and eventually, end-stage renal disease (ESRD) (1, 2). Another chronic complication of T2DM is diabetic osteoporosis (DOP). Clinical evidences indicate that the risk of low bone mass, osteoporosis, and fracture increases in T2DM, especially in elder men and post-menopausal women (3–7). The pathogenesis of these diseases has not been fully elucidated and is considered related to renal dysfunction and abnormal bone metabolism caused by glucolipid metabolism-related disorders (1–7).

The balance between osteoblasts and osteoclasts activities determines bone mass and osteocytes orchestrate these communications in reaction to endocrine and mechanical stimuli. Osteocalcin (OC), procollagen type 1 N-terminal propeptide (P1NP), and alkaline phosphatase (ALP) are the markers of bone formation, while β-C-terminal telopeptide of type I collagen (β-CTx) is the characteristic biochemical indicator of bone resorption. Moreover, 25(OH) Vitamin D3 [25(OH) VD3] and parathyroid hormone (PTH) promote absorption of calcium and phosphorus and increase blood calcium (Ca) and phosphorus (P) concentration thereby promoting bone calcification (8, 9). Previous studies have reported that these bone metabolic indicators may be involved in the crosstalk between bone, islet, and adipose tissues (8, 10–13) and may be associated with early renal damage in DKD (14, 15). However, these findings remain controversial and inconclusive.

MiR-154, a recently discovered microRNA with anti-cancer effects, is involved in pathophysiology of various disorders (16–20). It has been reported that miR-154-5p, a mature serum sequence of miR-154 (21–23), regulates osteogenic and differentiation of mesenchymal stem cells derived from adipose (24) and may be involved in renal fibrosis (25–27). These data suggest a potential association between miR-154-5p and pathology of osteogenic differentiation and urinary protein increasing in chronic kidney diseases.

We investigated the relationship between serum levels of miR-154-5p and other clinical parameters such as glucolipid metabolism and bone metabolism in male and post-menopausal female T2DM patients with different UACR levels.

MATERIALS AND METHODS

Subjects

We conducted a cross-sectional cohort study of 738 T2DM patients initially diagnosed and treated at the Department of Endocrinology of the First Hospital Affiliated of China Medical University from August 2017 to December 2018. The normal control groups (141 and 139 cases for M0 and F0, respectively) were from the Health Examination Center of the First Hospital Affiliated to China Medical University. This study was approved by the Ethics Committee of China Medical University and all participants signed informed consent.

Inclusion criteria included: men diagnosed with T2DM based on ADA standard (26) and post-menopausal women diagnosed with both T2DM and spontaneous menstruation for more than 1 year. Exclusion criteria included: (1) diabetic hyperosmolar coma, diabetic ketoacidosis or other acute diabetic complications in the last 3 months; (2) cerebrovascular diseases, liver function damage, infectious diseases, hemolysis induced by related diseases, or a history of malignant tumor; (3) recent stress, such as infection, surgery, trauma, or special physical conditions, such as pregnancy and lactation; (4) patients using angiotensin-converting enzyme inhibitors (ACEI) or angiotensin-receptor blockers (ARB) to affect the excretion rate of urinary albumin; (5) history of fracture within 1 year, osteoporosis, thyroid, parathyroid diseases, or other endocrine diseases; (6) use of agents that may affect bone metabolism such as thiazolidinediones, vitamin K, warfarin, vitamin D, calcium supplement, bisphosphonates, estrogen, and hormones, and agents that may lower lipid levels.

The subjects included 374 males and 364 post-menopausal females with T2DM. All the subjects were divided into six groups among males and post-menopausal females: (1) normal albuminuria male group (138 cases, UACR<30 mg/g, M1), (2) microalbuminuria male group (127 cases, UACR 30–300 mg/g, M2), (3) large amount of albuminuria male group (109 cases, UACR>300 mg/g, M3); (4) normal albuminuria post-menopausal female group (135 cases, UACR<30 mg/g, F1), (5) microalbuminuria post-menopausal female group (125 cases, UACR 30–300 mg/g, F2), and (6) large amount of albuminuria post-menopausal female group (104 cases, UACR>300 mg/g,

F3) based on urinary albumin excretion rate (UACR) (27). Meanwhile, the age-matched normal control male and post-menopausal female groups (141 and 139 cases for M0 and F0, respectively) were included from physical examination center during the same period.

Fasting blood samples were collected from T2DM patients using standard venipuncture (28) including non-hemolized samples with an absorbance<0.2 at 414 nm (29). Two samples of 5 ml venous blood were collected without anticoagulant and kept at room temperature for 30 min. The first sample was centrifuged for 15 min (1,000 × g, 4°C) for enzyme-linked immunosorbent assay (ELISA) and the second sample was centrifuged for 5 min (2,000 × g, 4°C) for real-time PCR assay as we conducted in the previous research (30). The serum samples were collected, sealed, and stored at −80°C till further use.

Measurements

All volunteers were asked about age, course of T2DM and menstrual history. The height (H), weight (W), waist circumference, hip circumference, and blood pressure (SBP, DBP) were monitored according to a standard protocol (31). Body mass index (BMI) was calculated as H $(m)^2$/W (kg) while waist hip rate (WHR) was calculated as waist circumference (cm)/hip circumference (cm). Fasting blood samples by standard venipuncture and moderate morning urine were collected from all subjects. Double centrifugation with a Beckman J-6M Induction Drive Centrifuge (Beckman Coulter, Inc., Brea, CA, USA) was used to separate the serum. All urine, serum, and plasma samples were stored at −80°C until final analysis.

Sandwich ELISA was used to measure adiponectin (ADPN, product number: CSB-E07270h; detection range: 1.56 −100 ng/ml; CUSABIO, Wuhan, P.R.China) and connective tissue growth factor (CTGF, product number: CSB-E07875h; detection range: 3.75–120 pg/ml; CUSABIO, Wuhan, P.R.China) in the serum samples according to the manufacturers' instructions. Inter- and intra-assay coefficients of variation was between <8 and <10%, respectively.

TABLE 1 | Levels of serum miR-154-5p and clinical characteristic in the studied groups.

	Male	Post-menopausal female	*T/Z*	*P*-values
N	515	503	–	–
Age (years)	53.84 ± 10.20	63.84 ± 10.60*	−15.3464	<0.0001
Course (months)	87 (0–96)	86 (0–97)	−0.3363	0.7366
BMI (kg/m^2)	24.78 ± 2.84	24.56 ± 2.61	1.2755	0.2024
WHR	0.92 ± 0.06	0.93 ± 0.06*	−2.0238	0.0433
FPG (mmol/L)	8.68 ± 2.95	7.66 ± 2.58*	5.8227	<0.0001
FINS (mIU/L)	11.87 ± 6.69	11.83 ± 6.42	0.1056	0.9159
FCP (pmol/L)	7.99 ± 2.41	8.27 ± 2.49	−1.7958	0.0728
ISI	−3.84 ± 1.38	−3.79 ± 1.27	−0.5588	0.5765
HOMA-IR	4.69 ± 3.55	4.14 ± 2.99*	2.6658	0.0078
HbA1c (%)	8.1 (5.8–9.5)	7.5 (4.9–8.5)*	−6.9392	<0.0001
HDL-C (mmol/L)	1.07 ± 0.25	1.08 ± 0.26	−0.9609	0.3368
LDL-C (mmol/L)	3.24 ± 0.94	3.20 ± 0.89	0.6683	0.5041
TC (mmol/L)	5.00 ± 1.21	4.99 ± 1.24	0.2341	0.8149
TG (mmol/L)	2.23 ± 1.00	2.12 ± 0.94	1.7688	0.0772
ADPN (mg/L)	25.57 ± 6.44	32.39 ± 6.36*	−16.9782	<0.0001
25(OH)VD3(pg/ml)	7.95 (6.65–8.82)	7.93 (6.77–9.10)	−1.1214	0.2621
PTH (pg/ml)	34.18 (30.83–37.54)	33.78 (30.33–36.64)	−1.1481	0.2509
P1NP (pg/ml)	33.43 ± 10.35	48.61 ± 14.88*	−18.9326	<0.0001
β-CTx (pg/ml)	384.24 ± 77.92	525.02 ± 130.74*	−20.9261	<0.0001
Ca (mmol/L)	2.23 ± 0.15	2.23 ± 0.15	−0.0251	0.9799
P (mmol/L)	1.16 ± 0.20	1.18 ± 0.19	−1.7010	0.0892
ALP (U/L)	70.94 ± 20.72	69.55 ± 19.37	1.1068	0.2686
OC (pg/ml)	14.31 ± 5.59	20.20 ± 8.76*	−12.8062	<0.0001
CTGF (pg/ml)	246.16 ± 96.31	297.55 ± 134.78*	−7.0125	<0.0001
UACR (mg/g)	17.22 (13.84–116.73)	19.15 (15.90–116.95)*	−3.9030	0.0001
miR-154-5p	0.49 (0.35–0.68)	0.75 (0.58–0.95)*	−13.4884	<0.0001

*Data are expressed as the mean ± SD or the median (interquartile range). BMI, body mass index; WHR, waist hip rate; FPG, fasting plasma glucose; FINS, fasting insulin; FCP, fasting c-peptide; ISI, insulin sensitive index (ISI = –ln[FPG × FINS]); HOMA-IR, homeostatic model assessment of insulin resistance (HOMA-IR = FPG × FINS/22.5); HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; ADPN, adiponectin; PTH, parathyroid hormone; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; Ca, calcium; P, phosphorus; ALP, alkaline phosphatase; OC, osteocalcin; CTGF, connective tissue growth factor; UACR, urinary albumin excretion rate. Post-menopausal female vs. male, *P < 0.05.*

Glucose oxidase examined by double-antibody radioimmunoassay were used to measure fasting plasma glucose (FPG), fasting plasma insulin (FINS), and fasting c-peptide (FCP) levels. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin resistance as follows: FINS (mU/L) × FPG (mmol/L)/22.5 and insulin sensitive index (ISI = −ln[FPG × FINS]). Glycosylated hemoglobin (HbA1c) was detected using an automated HbA1c analyzer (Bio-Rad, Hercules, CA, USA).

An automated biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA) was used to measure urinary albumin, urinary creatinine (uCr), total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), Ca, P, PTH, 25(OH)VD3, ALP, OC, P1NP, as well as β-CTx in the Laboratory Medicine, Nephrology Laboratory, Central Laboratory and the Laboratory of Endocrine and Metabolism of the First Hospital of China Medical University. UACR (urinary albumin/creatinine ratio) was calculated to estimate the levels of urinary proteins (32, 33).

MiRNA Isolation and Quantitative PCR Analysis

MiRNA was extracted from 400 μL serum samples using a miRcute miRNA isolation kit (DP501, Beijing Tiangen) (34). Before extraction, the *C. elegans* synthetic miRNA- cel-miR-39-mimic (Qiagen, Hilden, Germany) was added to each serum sample to correct for extraction errors. Strand of cDNA modified by tail reverse transcription was synthesized using a miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China) (35). Real-time PCR detection of miR-154-5p in 3.0 μL cDNA template was performed using a miRcute miRNA qPCR Detection Kit (FP401, SYBR Green, Tiangen, Beijing, China) on Takara Thermal Cycler Dice Real Time System. PCR amplification was performed using the following conditions: initial denaturation at 94°C for 2 min, 45 cycles denaturation at 95°C for 5 s, annealing and extension at 60°C for 40 s. Absolute quantification of miR-154-5p in each sample was calculated using the 2∧ (-ΔCt) method (26, 36).

Statistical Analysis

IBM SPSS Statistics (V.22.0, IBM Corp., Armonk, NY, USA) was used for data analysis. The normality of each group was judged according to the previous literature and normality test. All values were expressed as the mean ± SD for normally distributed values and as median (interquartile range) for non-parametric values. Difference between UACR groups was analyzed by one-way ANOVA and sex groups by Student's *t*-test for normally distributed values and Kruskal-Wallis *H*-test for non-parametric values. The least-significant difference *t*-test or Mann-Whitney *U*-test were used for pairwise comparison of differences among multiple groups. After logarithmic transformation was used for non-parametric values, clinical parameters related to OC and Ln UACR (Natural logarithmic UACR) were analyzed using Pearson correlation and multiple linear regression analysis. When there was collinearity, ridge regression analysis was performed to determine the association between the clinical parameters.

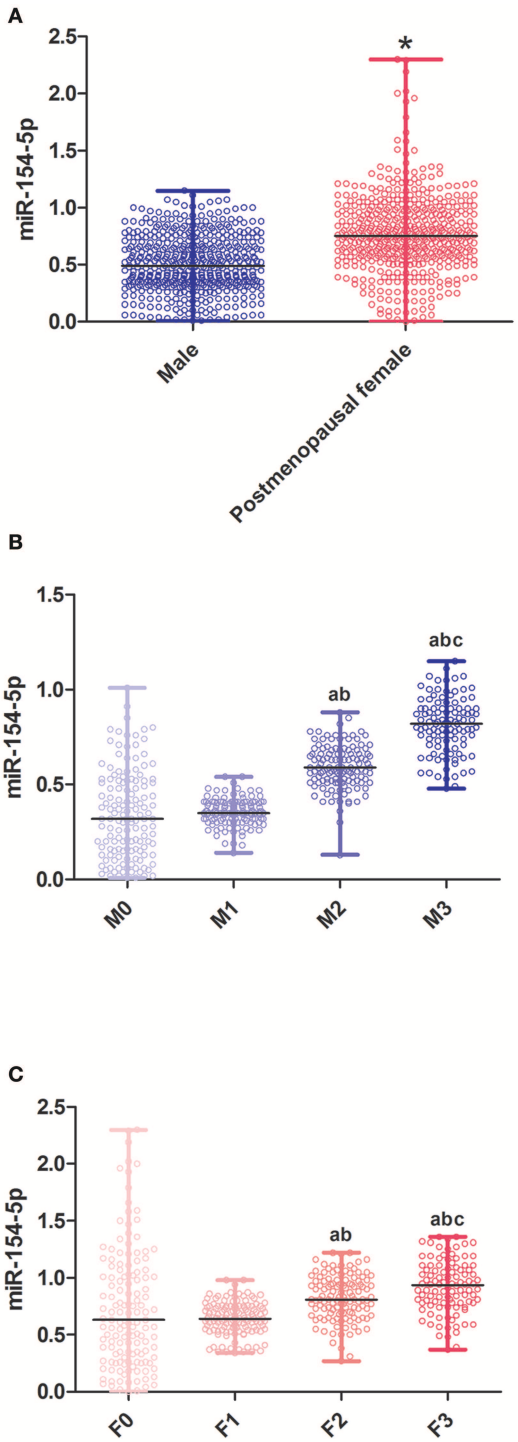

FIGURE 1 | Relative expression of miR-154-5p in different sex and UACR groups. **(A)** Between genders, *post-menopausal female vs. Male, $P < 0.05$; **(B)** different UACR in male patients, a F1-3 vs. F0, $P < 0.05$, b F3, F2 vs. F1, $P < 0.05$, c F3 vs. F2, $P < 0.05$; and **(C)** different UACR in post-menopausal female patients, a M1-3 vs. M0, $P < 0.05$, b M3, M2 vs. M1, $P < 0.05$, c M3 vs. M2, $P < 0.05$. Data were expressed by median (quartile). M0, the normal control male group; M1-3, the normal albuminuria, microalbuminuria, and large amount of albuminuria male groups; F0, the normal control post-menopausal female group; F1-3, the normal albuminuria, microalbuminuria, and large amount of albuminuria post-menopausal female groups; UACR, urinary albumin excretion rate.

All *P*-values were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Serum miR-154-5p Levels and Clinical Characteristic in the Male and Post-menopausal Female Groups

1018 volunteers participated in our study, included 515 males and 503 post-menopausal females. We observed significant differences in age and levels of WHR, FPG, HOMA-IR, HbA1c, ADPN, P1NP, β-CTx, OC, CTGF, UACR, and miR-154-5p ($P < 0.05$, **Table 1** and **Figure 1A**) between the two groups. Compared to the male group, age, WHR, ADPN, P1NP, β-CTx, OC, CTGF, UACR, and miR-154-5p were elevated and FPG, HOMA-IR, and HbA1c were significantly reduced in the post-menopausal female group ($P < 0.05$). However, no significant difference was observed in other parameters between different genders ($P > 0.05$).

Serum miR-154-5p Levels and Clinical Characteristic in Male T2DM Groups With Different UACR

Table 2 and **Figure 1B** illustrates the changes in serum biological markers in male T2DM patients with different UACR. Compared to the M0 group, course, FPG, FINS, FCP and HOMA-IR in T2DM patients were significantly increased and the ISI levels were significantly decreased ($P < 0.05$). The levels of HbA1c, CTGF, UACR, and miR-154-5p increased successively in the M1-3 groups while ADPN, P1NP, β-CTx, and OC decreased significantly ($P < 0.05$).

TABLE 2 | Levels of serum miR-154-5p and clinical characteristic in male T2DM groups with different UACR.

	M0	M1	M2	M3	F/χ^2	*P*-values
N	141	138	127	109	–	–
Age (years)	54.09 ± 10.79	54.05 ± 10.69	52.17 ± 9.82	55.19 ± 9.02[c]	1.8258	0.1414
Course (months)	0 (0–0)	91 (84–99)[a]	92 (83–97)[a]	93 (85–100)[a]	314.2062	<0.0001
BMI (kg/m^2)	25.00 ± 2.95	24.97 ± 2.97	24.44 ± 2.74	24.66 ± 2.62	1.1527	0.3273
WHR	0.92 ± 0.06	0.92 ± 0.06	0.91 ± 0.07	0.93 ± 0.07[c]	1.8038	0.1455
FPG (mmol/L)	5.61 ± 1.53	9.69 ± 2.48[a]	10.01 ± 2.56[a]	9.81 ± 2.47[a]	117.6565	<0.0001
FINS (mIU/L)	10.44 ± 3.14	12.40 ± 7.30[a]	11.92 ± 7.38	13.00 ± 8.06[a]	3.5073	0.0153
FCP (pmol/L)	6.81 ± 1.85	8.32 ± 2.34[a]	8.24 ± 2.44[a]	8.83 ± 2.58[a]	18.5787	<0.0001
ISI	−2.25 ± 0.51	−4.39 ± 1.23[a]	−4.45 ± 0.98[a]	−4.50 ± 1.08[a]	170.7250	<0.0001
HOMA-IR	2.56 ± 0.89	5.45 ± 3.75[a]	5.34 ± 3.80[a]	5.72 ± 4.03[a]	26.8978	<0.0001
HbA1c (%)	5.5 (5.2–5.8)	8.4 (7.7–9.3)[a]	9.0 (8.0–10.1)[ab]	9.7 (8.5–11.0)[abc]	300.5268	<0.0001
HDL-C (mmol/L)	1.08 ± 0.26	1.08 ± 0.26	1.06 ± 0.23	1.05 ± 0.25	0.2803	0.8396
LDL-C (mmol/L)	3.36 ± 0.92	3.38 ± 0.91	3.09 ± 0.90[ab]	3.08 ± 1.02[ab]	3.9443	0.0084
TC (mmol/L)	4.99 ± 1.12	4.98 ± 1.12	5.09 ± 1.33	4.95 ± 1.30	0.3258	0.8067
TG (mmol/L)	2.24 ± 1.04	2.25 ± 1.04	2.16 ± 0.90	2.26 ± 1.02	0.2463	0.8640
ADPN (mg/L)	31.19 ± 2.74	27.46 ± 3.28[a]	23.57 ± 4.73[ab]	18.27 ± 6.60[abc]	190.9144	<0.0001
25(OH)VD3(pg/ml)	7.96 (6.75–8.83)	7.97 (6.75–8.82)	7.81 (6.60–8.78)	7.89 (6.42–8.89)	0.1863	0.9798
PTH (pg/ml)	34.79 (31.08–38.03)	34.81 (31.11–38.03)	33.73 (30.82–36.69)	33.13 (30.16–36.63)	3.8989	0.2726
P1NP (pg/ml)	37.43 ± 9.89	37.27 ± 9.91	31.59 ± 8.91[ab]	25.54 ± 7.68[abc]	45.0235	<0.0001
β-CTx (pg/ml)	410.46 ± 84.08	411.49 ± 84.50	376.31 ± 56.84[ab]	325.04 ± 38.08[abc]	39.6529	<0.0001
Ca (mmol/L)	2.23 ± 0.15	2.23 ± 0.15	2.23 ± 0.14	2.24 ± 0.16	0.0910	0.9650
P (mmol/L)	1.16 ± 0.19	1.16 ± 0.19	1.16 ± 0.20	1.18 ± 0.20	0.3396	0.7967
ALP (U/L)	70.89 ± 22.46	70.29 ± 22.16	72.39 ± 18.02	70.16 ± 19.59	0.3037	0.8227
OC (pg/ml)	18.01 ± 6.77	15.73 ± 5.05[a]	12.24 ± 3.03[ab]	10.14 ± 1.84[abc]	69.2499	<0.0001
CTGF (pg/ml)	159.49 ± 32.80	209.83 ± 42.95[a]	254.70 ± 52.03[ab]	394.31 ± 59.38[abc]	551.4301	<0.0001
UACR (mg/g)	14.10 (12.64–15.47)	14.11 (12.58–15.48)	106.31 (93.14–114.59)[ab]	1062.24 (524.06–1530.10)[abc]	400.8755	<0.0001
miR-154-5p	0.32 (0.15–0.52)	0.35 (0.32–0.40)	0.59 (0.52–0.66)[ab]	0.82 (0.72–0.90)[abc]	315.5174	<0.0001

Data are expressed as the mean ± SD or the median (interquartile range). BMI, body mass index; WHR, waist hip rate; FPG, fasting plasma glucose; FINS, fasting insulin; FCP, fasting c-peptide; ISI, insulin sensitive index (ISI = –ln[FPG × FINS]); HOMA-IR, homeostatic model assessment of insulin resistance (HOMA-IR = FPG × FINS/22.5); HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; ADPN, adiponectin; PTH, parathyroid hormone; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; Ca, calcium; P, phosphorus; ALP, alkaline phosphatase; OC, osteocalcin; CTGF, connective tissue growth factor; UACR, urinary albumin excretion rate. M0, the normal control male group; M1-3, the normal albuminuria, microalbuminuria, and large amount of albuminuria male groups.

[a] *M1-3 vs. M0, P < 0.05.*

[b] *M3, M2 vs. M1, P < 0.05.*

[c] *M3 vs. M2, P < 0.05.*

Serum miR-154-5p Levels and Clinical Characteristic in Post-menopausal Female T2DM Groups With Different UACR

Compared to the F0 group, course, FPG, FINS, FCP and HOMA-IR in T2DM patients were significantly increased and the ISI levels were significantly decreased ($P < 0.05$) in the T2DM female group. Moreover, HbA1c, CTGF, UACR, and miR-154-5p levels in the post-menopausal female T2DM patients increased successively in the F1-3 groups while the levels of ADPN, P1NP, β-CTx, and OC were reduced ($P < 0.05$, **Table 3** and **Figure 1C**).

Correlation Between OC, UACR, and Other Clinical Parameters in Male and Post-menopausal Female T2DM Patients

Table 4 and **Figures 2–4** shows the correlation analysis in all the groups of T2DM patients. Ln UACR was found to be positively correlated with CTGF, Ln HbA1c and Ln miR-154-5p and negatively correlated with ADPN, P1NP, β-CTx, and OC ($P < 0.05$). In contrast, OC showed a positive correlation with ADPN, P1NP, and β-CTx and negative correlation with Ln HbA1c, CTGF, Ln UACR, and Ln miR-154-5p ($P < 0.05$).

Moreover, Ln miR-154-5p was positively correlated with Ln HbA1c, CTGF, and Ln UACR and negatively correlated with ADPN and OC ($P < 0.05$). However, Ln miR-154-5p was negatively correlated with P1NP and β-CTx of single gender groups ($P < 0.05$), while no significant correlation was observed among two genders ($P > 0.05$).

Ridge Regression Analysis of OC, UACR Levels, and Clinical Parameters in T2DM Patients

Multiple regression analysis was conducted by taking the indicators significantly related to Ln HbA1c, ADPN, P1NP, β-CTx, CTGF, OC, Ln UACR, and Ln miR-154-5p as independent

TABLE 3 | Levels of serum miR-154-5p and clinical characteristic in post-menopausal female T2DM groups with different UACR.

	F0	F1	F2	F3	F/χ^2	*P*-values
N	139	135	125	104	–	–
Age (years)	64.68 ± 10.53	64.71 ± 10.56	63.46 ± 9.96	62.05 ± 11.34	1.6426	0.1786
Course (months)	0 (0–0)	91 (83–100)[a]	93 (84–101)[a]	93 (86–100)[a]	308.8528	<0.0001
BMI (kg/m^2)	24.35 ± 2.60	24.43 ± 2.59	24.78 ± 2.62	24.77 ± 2.63	0.9131	0.4344
WHR	0.93 ± 0.06	0.93 ± 0.06	0.92 ± 0.06	0.93 ± 0.06	0.8408	0.4720
FPG (mmol/L)	5.31 ± 1.66	8.53 ± 2.29[a]	8.52 ± 2.11[a]	8.65 ± 2.52[a]	77.5714	<0.0001
FINS (mIU/L)	9.61 ± 2.82	12.04 ± 7.20[a]	13.21 ± 7.05[a]	12.86 ± 7.29[a]	8.8203	<0.0001
FCP (pmol/L)	7.29 ± 2.03	8.79 ± 2.59[a]	8.72 ± 2.55[a]	8.38 ± 2.50[a]	11.2033	<0.0001
ISI	−2.24 ± 0.46	−4.29 ± 1.01[a]	−4.50 ± 0.78[a]	−4.39 ± 1.00[a]	224.4831	<0.0001
HOMA-IR	2.26 ± 0.93	4.56 ± 3.01[a]	5.10 ± 3.30[a]	4.95 ± 3.28[a]	30.5453	<0.0001
HbA1c (%)	4.6 (4.4–4.8)	7.5 (7.0–7.9)[a]	8.2 (7.3–8.7)[ab]	9.0 (8.1–10.0)[abc]	82.9865	<0.0001
HDL-C (mmol/L)	1.10 ± 0.25	1.10 ± 0.25	1.09 ± 0.27	1.03 ± 0.26[ab]	355.2069	<0.0001
LDL-C (mmol/L)	3.13 ± 0.88	3.12 ± 0.89	3.24 ± 0.89	3.35 ± 0.91	1.6360	0.1801
TC (mmol/L)	5.04 ± 1.31	5.04 ± 1.30	4.88 ± 1.19	4.97 ± 1.11	0.4628	0.7084
TG (mmol/L)	2.18 ± 0.92	2.19 ± 0.93	2.03 ± 0.94	2.04 ± 1.00	1.0249	0.3812
ADPN (mg/L)	38.73 ± 3.71	34.23 ± 2.67[a]	30.03 ± 3.32[ab]	24.36 ± 5.09[abc]	324.7018	<0.0001
25(OH)VD3(pg/ml)	8.02 (6.66–9.12)	7.93 (6.66–9.02)	7.48 (6.48–9.17)	8.10 (7.22–9.02)	3.3691	0.3381
PTH (pg/ml)	33.81 (30.51–37.12)	33.78 (30.51–37.24)	33.57 (30.10–35.97)	33.84 (30.25–36.96)	1.1314	0.7695
P1NP (pg/ml)	52.62 ± 15.85	52.27 ± 15.58	46.25 ± 13.04[ab]	41.34 ± 11.08[abc]	16.8790	<0.0001
β-CTx (pg/ml)	571.07 ± 123.12	571.88 ± 123.39	515.35 ± 107.75[ab]	414.29 ± 104.81[abc]	46.6004	<0.0001
Ca (mmol/L)	2.23 ± 0.15	2.23 ± 0.15	2.23 ± 0.15	2.23 ± 0.14	0.0340	0.9916
P (mmol/L)	1.19 ± 0.19	1.20 ± 0.18	1.16 ± 0.18	1.18 ± 0.21	0.7094	0.5467
ALP (U/L)	69.74 ± 20.03	69.51 ± 20.16	68.11 ± 18.29	71.10 ± 18.86	0.4561	0.7131
OC (pg/ml)	26.55 ± 9.03	21.26 ± 8.07[a]	17.89 ± 5.90[ab]	13.10 ± 5.01[abc]	72.1206	<0.0001
CTGF (pg/ml)	168.82 ± 37.79	223.89 ± 79.06[a]	380.54 ± 72.94[ab]	465.46 ± 76.01[abc]	496.1420	<0.0001
UACR (mg/g)	16.08 (14.34–17.75)	16.10 (14.43–17.75)	101.82 (90.85–111.36)[ab]	1246.65 (656.44–1723.61)[abc]	407.8570	<0.0001
miR-154-5p	0.63 (0.30–1.05)	0.64 (0.56–0.75)	0.81 (0.69–0.97)[ab]	0.94 (0.78–1.09)[abc]	82.9865	<0.0001

Data are expressed as the mean ± SD or the median (interquartile range). BMI, body mass index; WHR, waist hip rate; FPG, fasting plasma glucose; FINS, fasting insulin; FCP, fasting c-peptide; ISI, insulin sensitive index (ISI = –ln[FPG × FINS]); HOMA-IR, homeostatic model assessment of insulin resistance (HOMA-IR = FPG × FINS/22.5); HbA1c, glycated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; ADPN, adiponectin; PTH, parathyroid hormone; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; Ca, calcium; P, phosphorus; ALP, alkaline phosphatase; OC, osteocalcin; CTGF, connective tissue growth factor; UACR, urinary albumin excretion rate. F0, the normal control post-menopausal female group; F1-3, the normal albuminuria, microalbuminuria, and large amount of albuminuria post-menopausal female groups.

[a] *F1-3 vs. F0, P < 0.05.*

[b] *F3, F2 vs. F1, P < 0.05.*

[c] *F3 vs. F2, P < 0.05.*

TABLE 4 | Correlation between OC, UACR, Ln miR-154-5p, and other clinical parameters in male and post-menopausal female T2DM patients.

		All			Male			Post-menopausal female		
		Ln miR-154-5p	OC	Ln UACR	Ln miR-154-5p	OC	Ln UACR	Ln miR-154-5p	OC	Ln UACR
OC	*r*	−0.2070	1.0000	−0.4090	−0.5050	1.0000	−0.5090	−0.4870	1.0000	−0.4460
	P	<0.0001	–	<0.0001	<0.0001	–	<0.0001	<0.0001	–	<0.0001
ADPN	*r*	−0.1200	0.4590	−0.5500	−0.4790	0.3560	−0.5890	−0.3810	0.3610	−0.7200
	P	0.0011	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CTGF	*r*	0.5790	−0.2680	0.7030	0.6430	−0.4330	0.7330	0.4490	−0.4290	0.7360
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
P1NP	*r*	0.0069	0.4620	−0.3000	−0.4440	0.3710	−0.4650	−0.1980	0.3230	−0.3140
	P	0.8521	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
β-CTx	*r*	0.0344	0.4520	−0.3640	−0.4180	0.3380	−0.4470	−0.2150	0.3140	−0.4810
	P	0.3508	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ln UACR	*r*	0.5860	−0.4090	1.0000	0.7870	−0.5090	1.0000	0.4560	−0.4460	1.0000
	P	<0.0001	<0.0001	–	<0.0001	<0.0001	–	<0.0001	<0.0001	–
Ln miR-154-5p	*r*	1.0000	−0.2070	0.5860	1.0000	−0.5050	0.7870	1.0000	−0.4870	0.4560
	P	–	<0.0001	<0.0001	–	<0.0001	<0.0001	–	<0.0001	<0.0001
Ln HbA1c	*r*	0.0570	−0.2280	0.3370	0.2270	−0.1330	0.2530	0.1670	−0.1740	0.5090
	P	0.1220	<0.0001	<0.0001	<0.0001	0.0101	<0.0001	0.0014	0.0009	<0.0001

HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin; UACR, urinary albumin excretion rate.

variables and Ln UACR, OC as dependent variables. Exploratory multiple regression analysis showed that there was a significant correlation between the respective variables. As common linear regression analysis revealed severe colinearity (the colinearity diagnosis found that the maximum conditional index>30, variance expansion factor>10, variance component>0.5), we adopted a ridge regression analysis to address this problem.

The ridge trace shown in **Figure 5** (x1–x7 = OC, ADPN, P1NP, β-CTx, Ln HbA1c, Ln miR-154-5p, and CTGF, respectively) with Ln UACR as the dependent variable in all, men and post-menopausal women T2DM patients indicated that when the ridge parameter was set at $k = 0.4$, standardized regression coefficients of each independent variable tended to be stable. Results showed that Ln UACR was affected by OC, ADPN, P1NP, β-CTx, CTGF, Ln HbA1c, and Ln miR-154-5p in all the three groups ($P < 0.05$, **Table 5**). Combined with the regression coefficients, we concluded that an increase in CTGF, Ln HbA1c, and Ln miR-154-5p resulted in an increase in Ln UACR, while an increase in ADPN, P1NP, β-CTx, and OC resulted in a decrease in Ln UACR.

The ridge trace was performed with Ln UACR, ADPN, P1NP, β-CTx, Ln HbA1c, Ln miR-154-5p, and CTGF as independent variables and OC as a dependent variable. Parameter estimation of the ridge regression was performed with $k = 0.4$ ($k = 0.6$ in **Figure 5F**). Using these settings, we found that OC was affected by ADPN, P1NP, β-CTx, CTGF, Ln UACR, and Ln miR-154-5p ($P < 0.05$, **Table 5**), but was not associated with Ln HbA1c ($P > 0.05$). Combined with the regression coefficients, it can be concluded that ADPN, P1NP, and β-CTx were positively correlated with OC while CTGF, Ln UACR and Ln miR-154-5p were negatively correlative with OC.

DISCUSSION

Mir-154 is a recently discovered microRNA with protective effect on cancer as demonstrated by two miRNA sequences (37, 38), miR-154-5p and miR-154-3p (39–41). Only a few studies have reported the role of miR-154-3p in cancer, and the exact target mRNA and molecular function of miR-154-3p remain unexplored (42, 43). The other mature sequence in human serum, miR-154-5p, is localized to the chromosome 14q32 microRNA with multiple binding elements and is involved in several diseases related to fibrosis (25, 26, 41, 44, 45). As chronic renal diseases can lead to proteinuria through renal fibrosis (27), miR-154-5p may affect the production of urinary protein.

A similar fundamental study documented that overexpression of miR-154-5p inhibited osteogenic differentiation of adipose-derived mesenchymal stem cells by suppressing expression of osteogenic marker gene and matrix mineralization. In contrast, inhibition of endogenous miR-154-5p promoted osteogenic differentiation (24).These findings indicate a negative regulatory role of miR-154-5p on osteogenic differentiation and warrant further investigation.

Based on the previous studies, we propose the hypothesis that serum miR-154-5p levels may be related to the mechanism of fibrosis in DKD and osteogenic differentiation of DOP in T2DM patients.

UACR is recommended as one of the basic classification standard of DKD by American Diabetes Association (ADA) and Kidney Disease: Improving Global Outcomes (KDIGO) (46, 47). Serum OC is a marker of bone formation and regeneration as well as an important indicator of bone conversion in DOP, which has been observed to correlate with microRNAs in post-menopausal females (8, 48, 49). To test

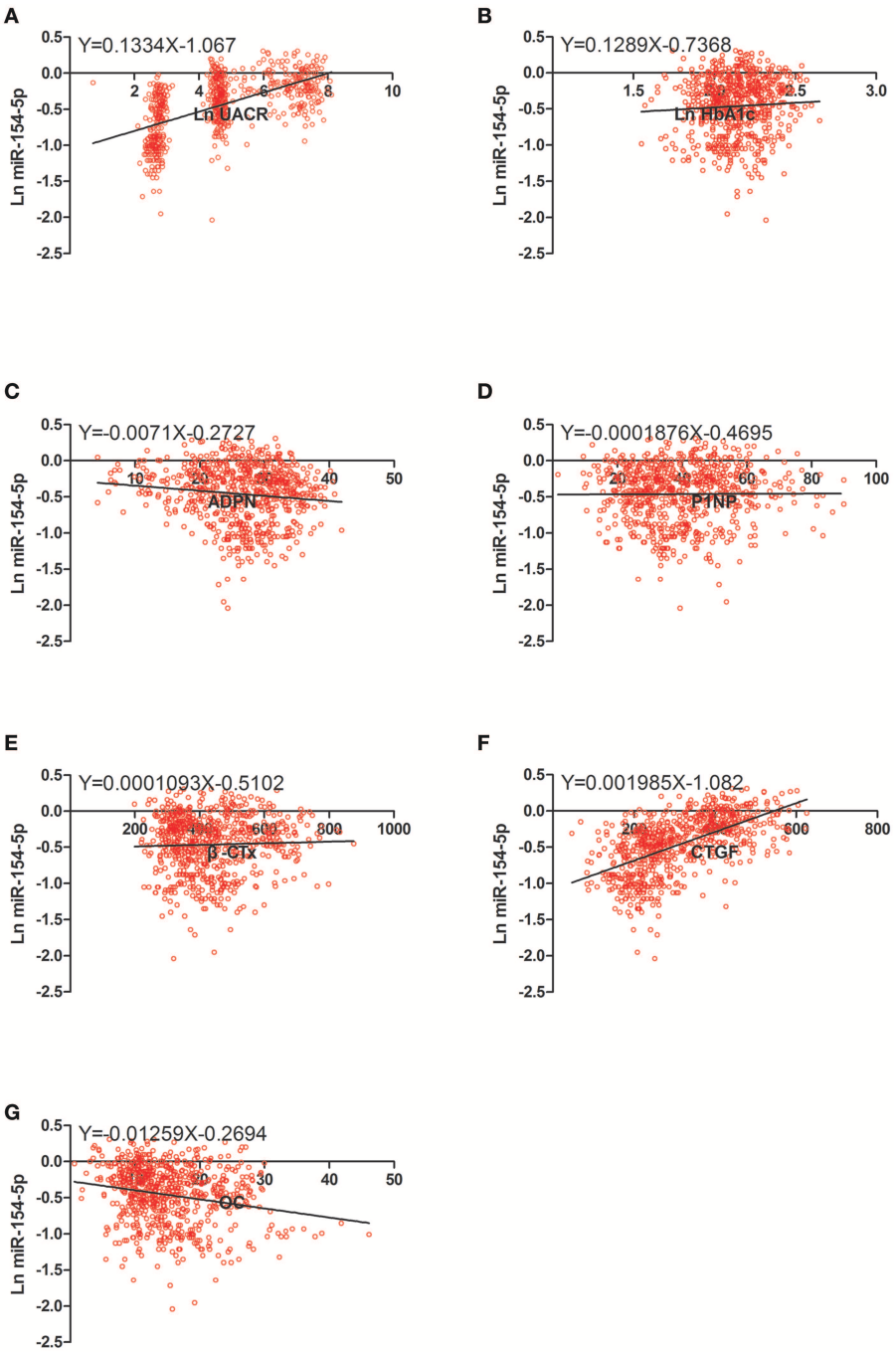

FIGURE 2 | Scatter dot plots between Ln miR-154-5p and **(A)** Ln UACR, **(B)** Ln HbA1c, **(C)** ADPN, **(D)** P1NP, **(E)** β-CTx, **(F)** CTGF, **(G)** OC. UACR, urinary albumin excretion rate; HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin.

our hypothesis, we investigated the expression levels of serum miR-154-5p and OC in men and post-menopausal women T2DM patients with different UACR, and analyzed correlation between these parameters. To clarify the effect of miR-154-5p in T2DM patients, we performed a cross-sectional cohort study and detected serum miR-154-5p and other glycolipid metabolic pathways, bone metabolism, urine albumin, and other biochemical indicators.

We observed significant differences in the levels of miR-154-5p, OC, and UACR between male and post-menopausal female patients groups and between the normal control groups and the T2DM groups with different UACR levels. In groups

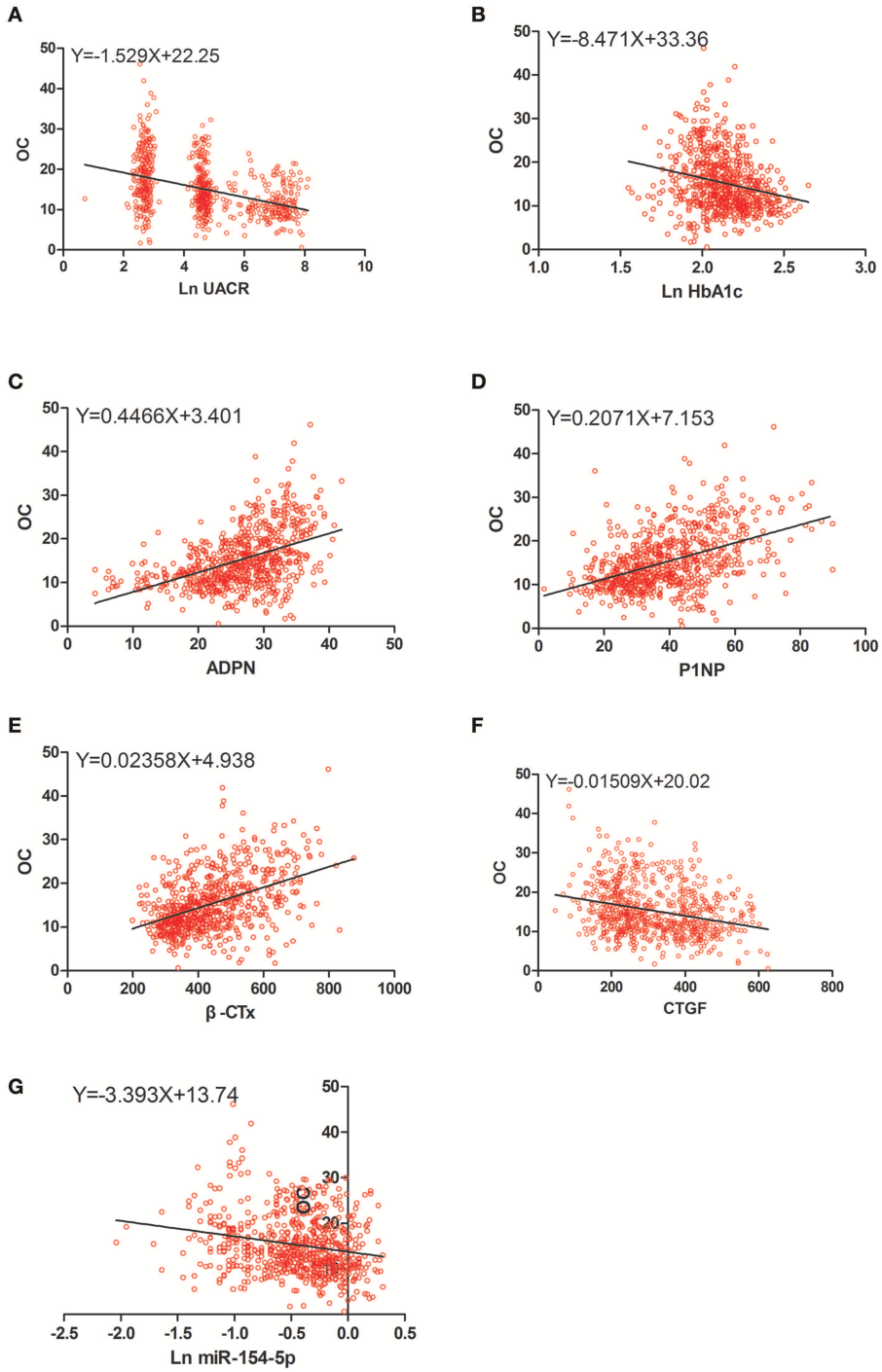

FIGURE 3 | Scatter dot plots between OC and **(A)** Ln UACR, **(B)** Ln HbA1c, **(C)** ADPN, **(D)** P1NP, **(E)** β-CTx, **(F)** CTGF, **(G)** Ln miR-154-5p. UACR, urinary albumin excretion rate; HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin.

of single-sex T2DM patients, miR-154-5p increased, while OC decreased successively with UACR. Serum miR-154-5p was positively correlated with UACR and negatively correlated with OC. Collectively, our data suggest that circulating miR-154-5p may be involved in the pathological process of glycolipid metabolism, bone metabolism, and proteinuria.

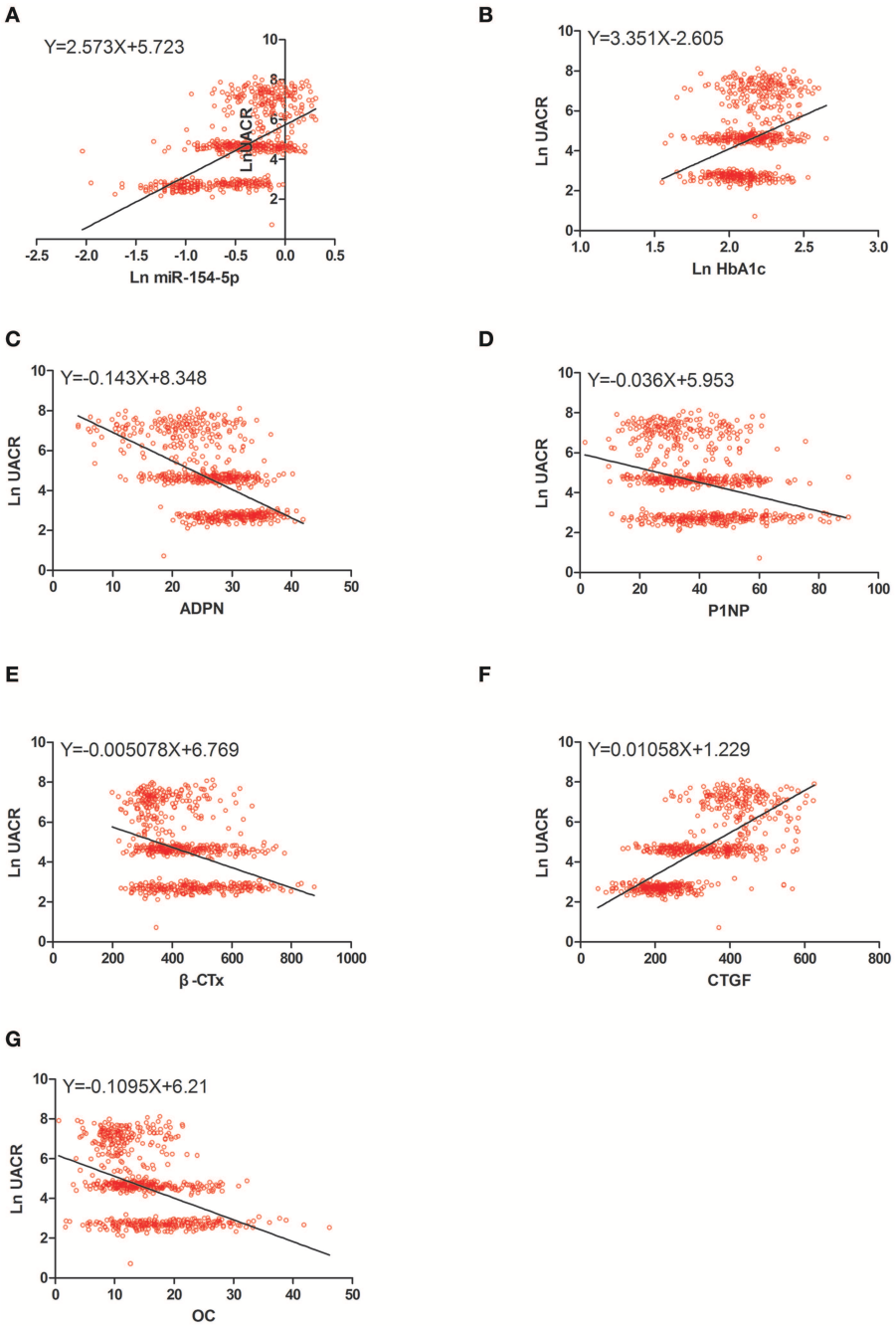

FIGURE 4 | Scatter dot plots between Ln UACR and **(A)** Ln miR-154-5p, **(B)** Ln HbA1c, **(C)** ADPN, **(D)** P1NP, **(E)** β-CTx, **(F)** CTGF, **(G)** OC. UACR, urinary albumin excretion rate; HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin.

There are two types of cells responsible for bone homeostasis, osteoblasts for secreting new bone, and osteoclasts for breaking bone down. The close cooperation between these cells determines the structure of bones in addition to supplying adequate of calcium. P1NP and ALP are markers of bone formation and β-CTx, a segment of collagen degradation excreted by the kidney during bone remodeling, is a marker of bone resorption. β-CTx is also one of the important clinical parameters utilized in the diagnosis and treatment of osteoporosis (9). As a sign of bone formation and regeneration, serum OC has been proven to be closely related to the above two processes in previous studies (8). 25 (OH) VD3 can promote the absorption of calcium and phosphorus in the intestinal tract and the re-absorption of calcium by renal tubules. PTH

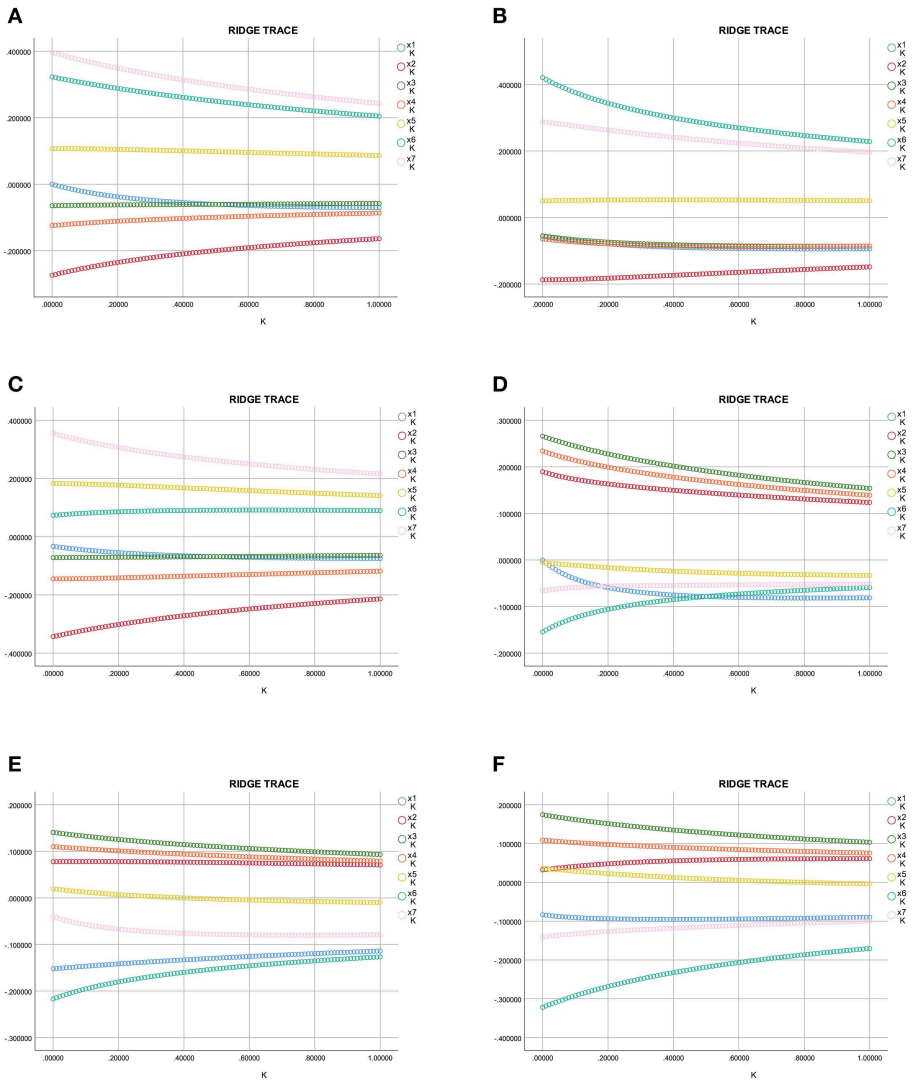

FIGURE 5 | Ridge trace curve of the association between Ln UACR, OC, and the clinical parameters. **(A–C)** y = Ln UACR as dependent variables in all **(A)**, male **(B)**, and post-menopausal female **(C)** patients, and x1–x7 = OC, ADPN, P1NP, β-CTx, Ln HbA1c, Ln miR-154-5p, and CTGF as the independent variables, respectively; **(D–F)** y = OC as dependent variables in all **(D)**, male **(E)**, and post-menopausal female **(F)** patients, and x1–x7 = Ln UACR, ADPN, P1NP, β-CTx, Ln HbA1c, Ln miR-154-5p, and CTGF as the independent variables, respectively. UACR, urinary albumin excretion rate; HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin.

can strengthen osteolysis, enhance reabsorption of calcium by renal tubules, mobilize calcium into the blood, and increase blood calcium. All of these can increase the concentration of Ca and P in the blood and promote bone calcification (8, 9). In addition, all these biomarkers can affect the whole process of bone metabolism.

To further confirm the correlation between miR-154-5p and bone metabolism, we detected the serum levels of the bone metabolism related indicators (P1NP, β-CTx, 25[OH] VD3, PTH, ALP, Ca, and P) and found that P1NP and β-CTx were significantly increased in post-menopausal female compared to the male group. However, no significant difference was observed in the other bone metabolism factors (25[OH] VD3, PTH, ALP, Ca, and P) between the two groups. Compared to the normal control group, P1NP and β-CTx decreased successively with UACR in T2DM men and post-menopausal women. Pearson correlation analysis showed that miR-154-5p was significantly negatively correlated with P1NP and β-CTx in males and post-menopausal females with T2DM suggesting that miR-154-5p may be involved in the metabolic process of bone formation and absorption.

Our results also showed that serum miR-154-5p and HbA1c increased with the rise of UACR. Serum miR-154-5p and HbA1c were positively correlated with UACR indicating that miR-154-5p may be associated with chronic elevated blood glucose levels, thus involved in the pathological changes in DKD. However, the

pathological mechanisms of the changes in urine protein caused by miR-154-5p remain unknown. CTGF is thought to regulate the progression of fibrosis in almost all diseases especially in renal tissues (50). Our data showed that CTGF increased with UACR and was positively correlated with UACR, providing supplementary evidence for the relationship between urinary protein changes and renal fibrosis. Further, we measured the levels of serum fibrosis factor CTGF and used correlation analysis to examine a possible relationship between miR-154-5p and CTGF. It was found that Ln miR-154-5p positively correlated with CTGF indicating that miR-154-5p is involved in the pathological process of renal injury by regulating fibrosis factors and causes the continuous increase of urinary protein.

Our study also found that OC, P1NP, and β-CTx were significantly reduced in male and post-menopausal female patients with different UACR, but there was no significant correlation with other bone metabolism indicators, consistent with previous literature that early chronic kidney disease can cause abnormal bone mineral metabolism and appearance of abnormal bone metabolic markers such as OC, P1NP, and β-CTx (14, 15). In addition, the levels of ADPN were significantly reduced and UACR was found to be negatively correlated with ADPN, an insulin–sensitizing hormone and blood lipid regulator, but not significantly correlated with other blood lipid indicators revealing a relationship between UACR and lipid metabolism, or potential associations with early metabolic processes such as insulin resistance and coronary atherosclerosis.

Our study showed that OC, P1NP, and β-CTx were significantly increased in post-menopausal women presenting a high bone transition state. OC positively correlated with P1NP and β-CTx adding to the evidence that OC is closely related to the osteogenesis process of bone formation and resorption. OC, encoded by bone gamma-carboxyglutamic acid-containing protein (BGLAP) gene in humans, is a specific protein solely secreted by osteoblasts in the general circulatory system during osteogenesis (51). As a hormone, OC plays a role in the metabolic regulation, bone mineralization, calcium ion homeostasis, is pro- osteoblastic, and involved in bone-building process.

OC can also cause islet beta cells to release more insulin and direct fat cells to release the hormone adiponectin thus increasing sensitivity to insulin (8). Clinical trials have reported that low OC levels were associated with metabolic syndrome, glucose, and lipid metabolism (10–13) suggesting that osteocalcin may be involved in the interactive relationship between bone and islet, adipose tissues. Studies investigating the relationship between OC and glucolipid metabolism have yielded conflicting data and the underlying mechanisms remain unclear (11, 52–56). Similar observations in our research have showed OC positively correlated with ADPN and negatively correlated with HbA1c, but no significant differences were observed between OC and other glucose and lipid metabolism indicators (FPG, FINS, FCP, ISI, HOMA-IR, and blood lipids) which is consistent with the clinical evidence and our previous study (57, 58). The relationship between OC, blood glucose and insulin may be interrupted by no limit of hypoglycemic drugs, and unmeasured undercarboxylated OC (ucOC), the active form of OC (59). In addition, different race selection, experimental design, index measurement, and analysis may account for these differences.

We adopted men and post-menopausal women as the research volunteers to avoid the effect of estrogen on metabolism, resulting in the age-mismatched phenomenon in different genders. Age, WHR, ADPN, P1NP, β-CTx, OC, CTGF, UACR, and mir-154-5p were significantly increased in post-menopausal women, while FPG, HOMA-IR and HbA1c decreased compared with the male group. However, according to the results of correlation analysis, glucolipid, and bone metabolic factors had no significant correlation with age, thus excluding significant differences resulting from age differences, revealing that changes in metabolic factors are caused by DOP rather than senile osteoporosis.

However, there are several limitations of this study: (1) as a cross-sectional cohort study, this study only indicates a correlation between miR-154-5p, OC, and UACR. There is a

TABLE 5 | Ridge regression analysis of OC, UACR, and clinical parameters in male and post-menopausal female T2DM patients.

	All				Male				Post-menopausal female			
	OC		Ln UACR		OC		Ln UACR		OC		Ln UACR	
	β	*P*	*β*	*P*	*β*	*P*	*β*	*P*	*β*	*P*	*β*	*P*
Constant	7.2693	0.0004	4.0592	<0.0001	9.3882	0.0000	5.7971	<0.0001	12.0612	0.0004	3.4068	<0.0001
Ln HbA1c	−0.8903	0.2675	0.9988	<0.0001	−0.0027	0.9970	0.4879	0.0067	0.2707	0.8442	2.0205	<0.0001
ADPN	0.1455	<0.0001	−0.0545	<0.0001	0.0541	0.0166	−0.0490	<0.0001	0.0799	0.0237	−0.0865	<0.0001
P1NP	0.0903	<0.0001	−0.0073	0.0000	0.0490	0.0004	−0.0141	0.0000	0.0630	0.0000	−0.0084	0.0006
β-CTx	0.0093	<0.0001	−0.0014	<0.0001	0.0056	0.0033	−0.0020	0.0000	0.0048	0.0019	−0.0018	<0.0001
CTGF	−0.0031	0.0107	0.0047	<0.0001	−0.0036	0.0169	0.0045	<0.0001	−0.0065	0.0000	0.0038	<0.0001
Ln UACR	−0.2810	0.0002	–	–	−0.3328	0.0000	–	–	−0.4001	0.0001	–	–
OC	–	–	−0.0148	0.0002	–	–	−0.0358	0.0000	–	–	−0.0155	0.0014
Ln miR-154-5p	−1.3982	0.0001	1.1479	<0.0001	−1.7380	<0.0001	1.3029	<0.0001	−5.1221	<0.0001	0.5278	0.0000

HbA1c, glycated hemoglobin A1c; ADPN, adiponectin; P1NP, procollagen type 1 N-terminal propeptide; β-CTx, β-C-terminal telopeptide of type I collagen; CTGF, connective tissue growth factor; OC, osteocalcin; UACR, urinary albumin excretion rate.

need for a follow-up and long-term observational trials and survival analyses to validate these observations. (2) Subjects with pre-diabetes were not included and the quality control of hypoglycemic drugs was not conducted. (3) We used ridge regression to solve the problem of collinearity in multiple regression which means a modified analysis of least squares estimation by abandoning the unbiasedness of least squares. The regression coefficient is obtained at the cost of partial information loss and accuracy reduction, however, the fitting of pathological data is stronger than that of least squares.

In summary, our study speculates that serum miR-154-5p levels is positively correlated with HbA1c, UACR, and CTGF and negatively correlated with ADPN, OC, P1NP, and β-CTx. These findings indicate that miR-154-5p may participate in the pathological process of glucolipid, bone metabolism, and renal fibrosis, which may further contribute to novel target development for DKD and DOP. However, the specific mechanisms of miR-154-5p remain elusive and still present a challenge to medical research field.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University. All procedures were performed in accordance with the ethical standards as mentioned in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

STATEMENT OF INFORMED CONSENT

Informed consent was obtained from all participants included in the study.

AUTHOR CONTRIBUTIONS

HR and QW designed the experiments and wrote the manuscript. HR, XM, YS, MY, and JH carried out the experiments and collected the cases and data. HR analyzed the experimental results.

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The Impact of Oxidative Stress on Adipose Tissue Energy Balance

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Overnutrition and sedentary activity reinforce the growing trend of worldwide obesity, insulin resistance, and type 2 diabetes. However, we have limited insight into how food intake generates sophisticated metabolic perturbations associated with obesity. Accumulation of mitochondrial oxidative stress contributes to the metabolic changes in obesity, but the mechanisms and significance are unclear. In white adipose tissue (WAT), mitochondrial oxidative stress, and the generation of reactive oxygen species (ROS) impact the endocrine and metabolic function of fat cells. The central role of mitochondria in nutrient handling suggests pharmacological targeting of pathological oxidative stress likely improves the metabolic profile of obesity. This review will summarize the critical pathogenic mechanisms of obesity-driven oxidative stress in WAT.

Keywords: adipocyte, metabolism and obesity, oxidative stress, mitochondria, mitochondrial disorders

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White adipose tissue (WAT) is an endocrine organ that stores energy in the form of lipids and secretes hormones essential for insulin sensitivity and energy homeostasis. The fat cell interprets nutritional, hormonal, and sympathetic tone in the tissue microenvironment to store and liberate fuels until whole-body energy demands necessitate fatty acid liberation. Like other cells, mitochondria in adipocytes assimilate signals that reflect the energy status of the cell and produce the majority of ATP from macronutrients through cellular respiration and oxidative phosphorylation (OXPHOS).

Obesity engenders nutrient stress that stifles mitochondrial capacity to sustain ATP levels in response to energy demands (Bournat and Brown, 2010; Liesa and Shirihai, 2013). Elevated mitochondrial substrate load consequently increases electron transport chain (ETC) activity and reactive oxygen species (ROS) production. Obese individuals exhibit higher levels of oxidative stress in WAT, including elevated ROS levels and decreased antioxidant activity coupled with alterations in adipokines required for insulin sensitivity (Furukawa et al., 2004). Moreover, oxidative stress associates with intra-abdominal obesity and insulin resistance (Furukawa et al., 2004; Frohnert et al., 2011). These results indicate the oxidizing environment in WAT of obese individuals likely impacts fat cell function and energy balance. Numerous questions remain, including how gradients of ROS inside the cell impact signaling cascades and gene regulation.

NUTRIENT IMBALANCE PROVOKES MITOCHONDRIAL ROS

Oxidative stress represents a disturbance in the equilibrium of ROS production and antioxidant defenses (**Figure 1**). At the molecular level, ROS mainly emerge from the mitochondrial ETC (Starkov, 2008; Murphy, 2009). Electron transfer through the ETC generates superoxide anions as byproducts, with complex I and III representing primary sources of ROS. Under certain conditions, complex II and other cellular ROS sources can contribute to the overall pool. Superoxide is the primary ROS species that reacts with Fe-containing proteins to generate H_2O_2. H_2O_2 accumulation in the cell contributes directly to the metabolic imbalance linking excessive nutrient stress and

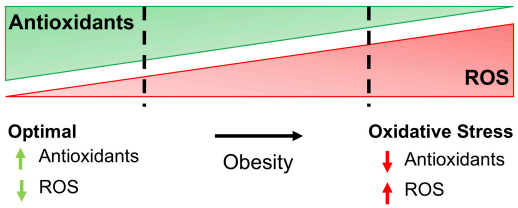

FIGURE 1 | The balance of antioxidants and ROS determine oxidative stress. For most cells, optimal redox conditions are achieved when higher levels of antioxidants are present to quench reactive oxygen species (ROS), maintaining ROS at low levels. Obesity and comorbidities increase ROS and decrease antioxidants in adipose, leading to oxidative stress and further complications of obesity, including insulin resistance and diabetes.

insulin resistance (Anderson et al., 2009; Akl et al., 2017; Fazakerley et al., 2018). However, ROS also encompass a diverse range of chemical entities, including nitric oxide, peroxynitrite, hypochlorous acid, singlet oxygen, and the hydroxyl radical. Consequently, the broad biological impacts of ROS derive from multiple cell and tissue microenvironments that divide physiological and pathological effects.

The conditions that favor mitochondrial superoxide production include reduction of electron carrier pools associated with the mitochondrial respiratory chain (NADH, flavins, ubiquinone), a high proton motive force, and elevated oxygen consumption within the mitochondria (Murphy, 2009). Overnutrition supplies excess electrons to the respiratory chain, while lack of physical activity and low ATP demand favors a high proton motive force with a low respiration rate, leading to mitochondrial superoxide formation and oxidative stress. By contrast, in mitochondria actively making ATP, superoxide production is low because the electron carriers are relatively oxidized, the proton motive force is small, and the respiration rate is high.

Prolonged oxidative stress directly impacts metabolism, including the activity of enzymes involved in the TCA cycle and the ETC (Quijano et al., 2016). The TCA cycle enzyme aconitase catalyzes the interconversion of citrate and isocitrate to regulate the availability of intermediates for lipid synthesis and ATP production. Citrate is the last common metabolite on the pathways for oxidation of acetyl-CoA and its export for fatty acid synthesis in the cytoplasm. Superoxide inhibits aconitase (Hausladen and Fridovich, 1994; Gardner et al., 1995), leading to diversion of acetyl-CoA away from OXPHOS and toward fat storage (Armstrong et al., 2004; Lushchak et al., 2014). This feedback loop may be part of an antioxidant defense mechanism that adapts prolonged mitochondrial superoxide production (Scandroglio et al., 2014). Acetyl-CoA diversion may slow delivery of electron carriers such as NADH to the respiratory chain, thereby decreasing ROS production (Armstrong et al., 2004).

Oxidative stress also impacts pyruvate dehydrogenase kinase 2 (PDK2) inhibition of the pyruvate dehydrogenase complex (PDC) (Hurd et al., 2012) and fat metabolism. ROS oxidize critical cysteine residues, disabling PDK2, and supporting acetyl-CoA synthesis from glucose-derived pyruvate. Therefore, elevated mitochondrial superoxide and H_2O_2 couples PDC activity with aconitase interruption to divert citrate from the TCA cycle to the cytoplasm as triglycerides during overnutrition. These studies suggest persistent nutrient stress impairs the physiological behavior of crucial metabolic enzymes needed for balanced ATP generation and consumption.

ANTIOXIDANT RESPONSE TO MITOCHONDRIAL ROS REGULATES ADIPOSE TISSUE FUNCTION

A variety of peroxidases, including catalase, glutathione peroxidases, and peroxiredoxins (Prdxs) that control the levels of H_2O_2 in the cell and protect against ROS-induced damage by catalyzing the reduction of H_2O_2 into water. Along these lines, overexpression of catalase (Anderson et al., 2009; Barbosa et al., 2013; Lee et al., 2017; Paglialunga et al., 2017) or other methods that block H_2O_2 generation (Anderson et al., 2009; Boden et al., 2012) preserve insulin sensitivity in cell models and rodents fed high-fat diet.

The mitochondrial antioxidant peroxiredoxin 3 (Prdx3) responds to oxidative stress and scavenges H_2O_2. Levels of Prdx3 are decreased in obese humans and mice, potentially contributing to oxidative stress intolerance (Huh et al., 2012). Whole-body deletion of *Prdx3* in mice causes obesity and increased expression of lipogenic genes in adipocytes, while decreasing expression of lipolytic genes. As a result, hypertrophic adipocytes exclusively accumulate excess lipids and cannot enable appropriate energy balance control. In addition to altering the balance of lipogenesis and lipolysis, Prdx3-deficient adipocytes exhibited increased superoxide production, decreased mitochondrial potential, and altered adipokine expression, including decreased adiponectin.

Okuno et al. (2018) created "Fat ROS-augmented" (AKO) and "Fat ROS-eliminated" (aP2-dTg) mice to address the question of how ROS affect WAT function. AKO mice leverage adipocyte-specific ablation of glutamate-cysteine ligase (*Gclc*) to disable the rate-limiting step in glutathione synthesis and increase ROS generation. AKO mice fed high fat/high sucrose (HF/HS) diet for 6 weeks had smaller adipocytes and decreased expression of lipogenic genes, including *Acly*, *Scd1*, *Fasn*, *Acaca*, and *Srebf1*. Insulin sensitivity was also reduced. Conversely, mice expressing rat catalase and human SOD1 under the aP2 promoter had the opposite phenotype. These mice (aP2-dTg) showed reduced H_2O_2 in subcutaneous and gonadal WAT. Feeding a HF/HS diet yielded beneficial subcutaneous and gonadal WAT expansion mirrored by increased expression of lipogenic genes (*Acly*, *Scd1*, *Fasn*, and *Acaca*) and insulin sensitivity.

While these data argue that increasing mitochondrial antioxidants protects against oxidative stress in WAT, genetic alteration of other mitochondrial antioxidants reveal different phenotypes. Manganese superoxide dismutase (MnSOD) is an important mitochondrial antioxidant that detoxifies superoxides (Holley et al., 2011). Adipocyte-specific knockout of MnSOD protected against diet-induced WAT expansion and weight gain (Han et al., 2016). Mechanistically, MnSOD knockout in

adipocytes triggered an adaptive stress response that activated mitochondrial biogenesis and enhanced mitochondrial fatty acid oxidation, thereby preventing diet-induced obesity and insulin resistance. Increased ROS levels correlated with Uncoupling Protein 1 (UCP1) activation in subcutaneous WAT and higher energy expenditure (Han et al., 2016). These disparate features of mice that lack the *Prdx3* and *MnSOD* genes coupled with therapeutic shortcomings of antioxidant therapies in human clinical trials (Fusco et al., 2007; Bjelakovic et al., 2013, 2014) suggest a more complex interaction of metabolism and redox balance in WAT.

MITOCHONDRIAL REDOX REACTIONS GENERATE DIVERGENT INPUTS FOR CELLULAR SIGNALING

The homeostatic systems that regulate oxidative stress in the lean state are largely repressed in obesity due to the accumulation of oxidized biomolecules within WAT. Excessive ROS irreversibly damages DNA, lipids, and proteins with adverse effects on cellular functions. Increased oxidative stress can alter proteins and lipids through direct and indirect pathways that culminate in oxidation of side chains and lipid-protein adduction (Grimsrud et al., 2008; Davies, 2016).

Reactive oxygen species oxidation of lipids ultimately generates lipid aldehydes that modify DNA, proteins, RNA, and other lipid species (Esterbauer et al., 1991; Uchida, 2003). Increased markers of lipid peroxidation, including thiobarbituric acid reactive substances (TBARS) and 8-epi-prostaglandin-F2α (8-epi-PGF2α) are observed in individuals with higher BMI and waist circumference (Furukawa et al., 2004). Oxidized lipids and proteins preferentially accumulate in visceral depots compared to subcutaneous depots of obese mice (Long et al., 2013; Hauck et al., 2018, 2019) and humans (Frohnert et al., 2011), suggesting ROS modifications correlate with conditions associated with type 2 diabetes, including central fat accrual.

Highly reactive hydroxyl radicals (·OH) can be generated when excess H_2O_2 reacts with ferrous iron. Unlike H_2O_2, ·OH cannot undergo detoxification. Instead, ·OH removes electrons from neighboring lipids, proteins, and nucleic acids. Lipid aldehydes are highly electrophilic and prone to irreversible nucleophilic attack by the side chains of lysine (Lys), histidine (His), and cysteine (Cys) residues of proteins, resulting in a covalent lipid-protein adduct termed protein carbonylation (Schaur, 2003; Curtis et al., 2012). Furthermore, Lys, His, and Cys residues often cluster within active sites of enzymes or critical structural motifs, so their stable modification by lipids generally leads to inhibition or deactivation of protein function. However, recent work challenges the notion that ROS-driven modifications broadly degrade fat cell function. Brown adipose tissue (BAT) contains elevated levels of mitochondrial superoxide, mitochondrial H_2O_2, and oxidized lipids that correlate with acute activation of thermogenesis (Chouchani et al., 2016a,b). Mitochondrial ROS in BAT can converge on UCP1 C253 inducing cysteine sulfenylation (-SOH) (Chouchani et al., 2016a). Interestingly, UCP1 C253A does not disable thermogenic responses in brown adipocytes but desensitizes the protein to adrenergic activation of uncoupled respiration. Further exploration of physiological ROS signaling outputs and modifications may show how redox status in adipocytes contributes to energy balance.

Polyunsaturated fatty acids (PUFAs) are abundant in WAT and particularly sensitive to lipid peroxidation. One major consequence of lipid peroxidation is mitochondrial membrane damage (Kowaltowski and Vercesi, 1999). Also, peroxidation of PUFAs results in the release of diffusible reactive lipid aldehydes. Among the wide variety of reactive lipids formed through this mechanism, 4-hydroxy-non-enal (4-HNE) derived from oxidation of n6 fatty acids and 4-hydroxy-hexenal (4-HHE) from n3 fatty acid oxidation are the most widely studied in the context of adipose biology. The WAT of obese mice showed decreased metabolism of 4-HNE, while stress response proteins, including glutathione-S-transferase M1, glutathione peroxidase 1, and Prdx (Grimsrud et al., 2007) were carbonylated. Lipid peroxidation end products can also inhibit insulin signaling as 4-HNE de-stabilizes IRS adapter proteins and insulin receptor β (Demozay et al., 2008; Frohnert and Bernlohr, 2013).

Lipid peroxidation products also damage the function of transcription factors that contain zinc-finger motifs, histones, and other nuclear proteins of visceral fat cells isolated from obese mice (Hauck et al., 2018). The lipid peroxidation of transcriptional regulatory proteins presents a consolidated mechanism for retrograde ROS signaling from mitochondria to the nucleus. Although mitochondria are the most significant source of ROS, the discovery of lipid-protein adducts in the nucleus of adipocytes suggests either a different pool of ROS contributes to lipid peroxidation or a mechanism exists to sequester and shuttle reactive aldehydes to specific subcellular localizations (Hauck et al., 2018). As with ROS, the timing of protein carbonylation may be important for beneficial or pathologic effects. Acute carbonylation of substrates after exercise are potentially beneficial, while chronic accumulation of carbonylated proteins in the muscle and WAT of obese and sedentary individuals may be pathological and contribute to comorbidities of obesity (Frohnert and Bernlohr, 2013).

Additionally, ROS seem to be important in the cellular aspects of adipocyte differentiation. Numerous studies demonstrate that mitochondrial biogenesis increases during adipocyte differentiation (Wilson-Fritch et al., 2003; Lu et al., 2010; Zhang et al., 2013). Dramatic expansion of mitochondrial content enables higher metabolic rates to overcome the energetic demands of differentiation. Induction of differentiation correlates with superoxide generation from complex III, conversion of superoxide to H_2O_2, and activation of transcriptional machinery necessary for adipogenesis (Tormos et al., 2011). Mechanistically, ROS production in differentiating cells coincides with increased C/EBPβ binding to DNA and accelerated mitotic clonal expansion (Kim J.W. et al., 2007; Lee et al., 2009). However, obesity-mediated ROS induction also restricts mitochondrial biogenesis and adipocyte differentiation. Higher accumulation of 4-HNE adducts occurs in cultured differentiating preadipocytes from insulin-resistant compared to insulin-sensitive individuals. In this manner, treatment of

primary subcutaneous preadipocytes from obese individuals with pathological levels of 4-HNE decreased markers associated with insulin sensitivity and mature fat cells (Dasuri et al., 2013; Elrayess et al., 2017). Other studies demonstrate that treatment with antioxidants decreases differentiation (Tormos et al., 2011) and disrupts UCP1-dependent thermogenic responses (Ro et al., 2014; Chouchani et al., 2016a). Divergent *in vitro* and *in vivo* findings illustrate existing challenges in defining the specifics of ROS signaling and its connectivity to metabolic diseases.

OXIDATIVE STRESS CONTRIBUTES TO THE COMORBIDITIES OF METABOLIC DISEASES

Nutrient overload has been linked to the development of insulin resistance. In a pioneering study, healthy men fed ∼6000 kcal/day for 1 week exhibited WAT insulin resistance and oxidative stress in addition to protein oxidation and carbonylation (Boden et al., 2015). One carbonylated protein of importance was GLUT4, whose carbonylation likely impairs insulin-stimulated glucose uptake. Of note, systemic oxidative stress and insulin resistance did not coincide with inflammatory cytokines in plasma nor ER stress in WAT. These findings provide a causal link between oxidative stress and insulin resistance in humans.

Mitochondrial metabolism is often altered in inherited diseases, such as inborn errors of metabolism (IEMs) that impinge upon ROS generation. Inhibition of OXPHOS increases ROS generation due to a backlog of electrons in the various complexes, resulting in electron leak, ROS generation, and production of H_2O_2. In IEMs affecting the ETC or other pathways of ATP generation, increased oxidative stress is often observed, while the exact mechanisms for increased ROS production are unknown. It is hypothesized that mutations affecting the formation of the protein complexes in the ETC or mutations that modify their assembly increase ROS generation by facilitating electron leak (Olsen et al., 2015). Additionally, accumulation of toxic intermediates, often observed in IEMs, can increase the ROS generation by further decreasing OXPHOS activity, as in the case of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. MCAD deficiency reflects the accumulation of medium-chain fatty acid derivatives, including cis-4-decenoic acid, octanoate, and decanoate, with these metabolites altering levels of antioxidants and increasing markers of oxidative stress (Schuck et al., 2007, 2009). Intriguingly, IEMs display metabolic reprograming with a switch to glycolysis for both ATP production and muted ROS generation (Olsen et al., 2015). Specifically, in myoclonic epilepsy with ragged red fibers (MERRF), increased intracellular H_2O_2 levels correspond with increased AMPK phosphorylation and expression of GLUT1, hexokinase II, and lactate dehydrogenase. These results, as well as increased lactic acid production, all point to increased glycolysis (De la Mata et al., 2012; Wu and Wei, 2012). In multiple acyl-CoA dehydrogenase deficiency (MADD), mutations in *ETFa*, *ETFb*, or *ETFDH*, lead to decreased ATP production with an accumulation of organic acids, including glutaric acid as well as acyl-carnitines. A subset of these patients is riboflavin responsive (RR-MADD) with high dose riboflavin alleviating some symptoms. Similar to MERRF, many RR-MADD patients exhibit increased oxidative stress (Cornelius et al., 2013, 2014). This defect may be due to defective electron transfer and increased electron leak from the misfolded ETFDH protein and decreased binding of CoQ10 (Cornelius et al., 2013). Treatment with CoQ10, but not riboflavin, decreased ROS levels (Cornelius et al., 2013). Analysis of mitochondrial function from RR-MADD fibroblasts showed increased mitochondrial fragmentation and reduced β-oxidation, while supplementation with the antioxidant CoQ10 decreased fragmentation and mitophagy (Cornelius et al., 2014). While obesity and IEMs are distinct disorders, both conditions impinge on energy balance in WAT. Even though these disorders have very different manifestations, oxidative stress plays an important role in both and may be a therapeutic target. For example, CoQ10 is often given as a broad-spectrum treatment to individuals with IEMs, and while its effectiveness is debated, the anti-inflammatory effects may be beneficial in reducing oxidative stress and the pathogenesis of the disease (Cornelius et al., 2013; Acosta et al., 2016; Zhai et al., 2017).

LEVERAGING REDOX BALANCE TO IMPROVE INSULIN SENSITIVITY

Mitochondria represent control centers of many metabolic pathways. Interventions that enhance adipocyte mitochondrial function may also improve whole-body insulin sensitivity. Mitigation of mitochondrial ROS production and oxidative stress may be a possible therapeutic target in type 2 diabetes and IEMs because some mitochondrial-targeted antioxidants and other small molecule drugs improve metabolic profiles in mouse models (Feillet-Coudray et al., 2014; Fouret et al., 2015; Rivera-Barahona et al., 2017) and human studies (Escribano-Lopez et al., 2018).

Thiazolidinediones (TZDs) are PPARγ agonists used for treating type 2 diabetes (Kelly et al., 1999; King, 2000; Khan et al., 2002; Goldberg et al., 2005; Deeg et al., 2007). TZDs, such as rosiglitazone and pioglitazone, enhance insulin sensitivity by improving adipokine profiles (Maeda et al., 2001, 2002) and reducing fasting blood glucose levels (Boyle et al., 2002; Chappuis et al., 2007). TZDs also promote insulin sensitivity by directing fatty acids to subcutaneous fat, rather than visceral fat. Subcutaneous fat expandability, even in the context of obesity and type 2 diabetes, correlates with insulin sensitivity in rodents and humans (Ross et al., 1996; Miyazaki et al., 2002; Kim J.Y. et al., 2007; Tran et al., 2008; Porter et al., 2009). Numerous *in vitro* and *in vivo* studies demonstrate TZDs enhance mitochondrial biogenesis, content, function, and morphology. Rosiglitazone also induces cellular antioxidant enzymes responsible for the removal of ROS generated by increased mitochondrial activity in adipose tissue of diabetic rodents (Rong et al., 2007) and humans (Bogacka et al., 2005; Rong et al., 2007; Ahmed et al., 2010). It is now well established that anti-diabetic PPARγ agonists also activate a BAT gene program in white adipocytes, converting them to "beige" cells that express UCP1 (Tiraby et al., 2003; Wilson-Fritch et al., 2003; Bogacka et al., 2005; Ohno et al., 2005; Petrovic et al., 2010). Taken together, TZDs impact WAT

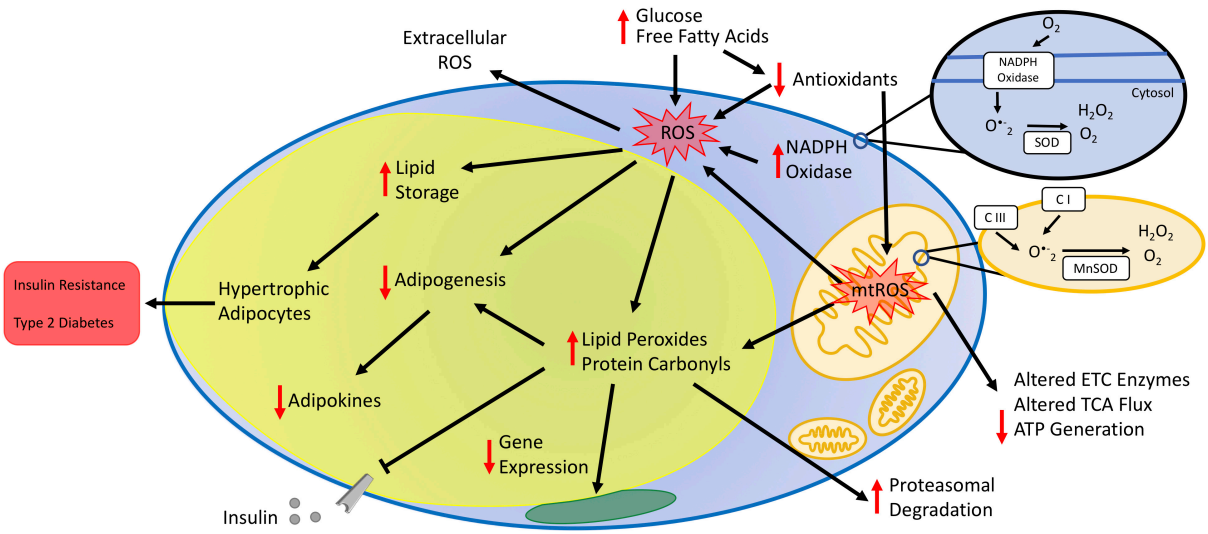

FIGURE 2 | Impact of oxidative stress on adipocyte function. Increased plasma glucose and free fatty acids contribute to increased oxidative stress by increasing the production of reactive oxygen species (ROS) and decreasing antioxidant concentrations. Increased oxidative stress occurs via enzymes in the cytoplasm, such as NADPH oxidase, and the mitochondria. The oxidative environment increases lipid storage resulting in hypertrophic adipocytes. Additionally, increased mitochondrial ROS (mtROS) alters the activity state of metabolic enzymes either directly or by changing the oxidative state of protein side-chains or by other post-translational modifications, including lipid peroxidation and protein carbonylation. Cumulatively, increased adipocyte oxidative stress decreases adipogenesis and secretion of adipokines, leading to unbalanced energy homeostasis, insulin resistance, and type 2 diabetes.

mitochondrial function in multiple ways that ultimately improve systemic fat metabolism and insulin sensitivity. Other therapeutic strategies include mitochondria-targeted scavengers (Smith et al., 2012) and chemical uncouplers that dissipate energy as heat (Perry et al., 2013; Goldgof et al., 2014). However, these methods to enhance mitochondrial function display a narrow therapeutic range that limits safe use for obesity.

Although the development of insulin resistance does not require impaired mitochondrial function (Hancock et al., 2008; Holloszy, 2013), pathways promoting insulin resistance may impair mitochondrial function and further increase ROS production, resulting in a detrimental feedback loop. Aerobic exercise and caloric restriction disrupt this vicious loop, potentially by preventing accumulation of injured mitochondrial proteins with substantial improvement of insulin sensitivity. In insulin-resistant people, aerobic exercise stimulates both mitochondrial biogenesis and efficiency concurrent with an enhancement of insulin action (Mul et al., 2015). Ultimately, exercise engages pathways that reduce ROS coupled with insulin sensitivity and improved mitochondrial function in WAT.

CONCLUSION

Obesity is the result of excessive expansion of WAT depots due to a chronic imbalance between energy intake and expenditure. Many studies demonstrate that oxidative stress in fat cells links obesity and its comorbidities. The fact that WAT remains the sole organ for storing surfeit lipid renders the macromolecules in adipocytes particularly vulnerable to carbonylation and other modifications driven by oxidative stress. Prolonged oxidative stress negatively influences endocrine and homeostatic performance of WAT, including disruption of hormone secretion, elevation of serum lipids, inadequate cellular antioxidant defenses, and impaired mitochondrial function (**Figure 2**). Metabolic challenges, such as persistent nutrient intake and sedentary behaviors that promote impaired glucose and lipid handling, also elevate mitochondrial ROS production to cause adipocyte dysfunction. Consequently, adipocytes cannot engage appropriate transcriptional and energetic responses to enable insulin sensitivity.

The increasing prevalence of obesity suggests lifestyle intervention as the principal method to treat obesity is unlikely to succeed. Currently, all available anti-obesity medications act by limiting energy intake through appetite suppression or inhibition of intestinal lipid absorption. However, these medications are largely ineffective and often have adverse side effects. The central role of mitochondria in nutrient handling provides a logical entry point for improving metabolism in obesity. While approaches to understanding and intervening in oxidative damage evolve, exploration of mitochondria redox balance may enable development of dietary and small molecule therapies for obesity and its comorbidities.

AUTHOR CONTRIBUTIONS

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