OBESITY, SMOKING, AND FATTY LIVER DISEASE

EDITED BY: Amiya P. Sinha-Hikim and Sushil K. Mahata

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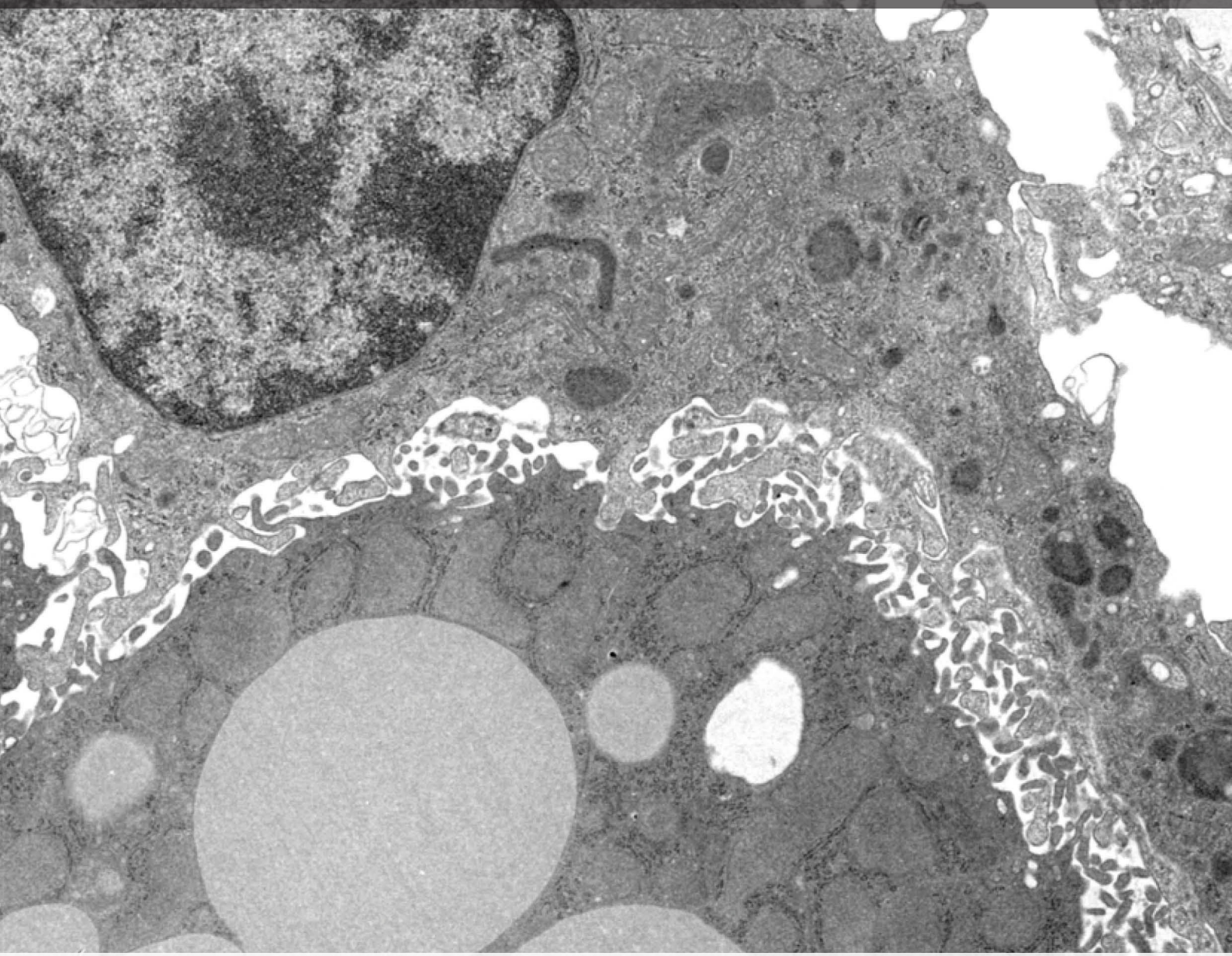

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OBESITY, SMOKING, AND FATTY LIVER DISEASE

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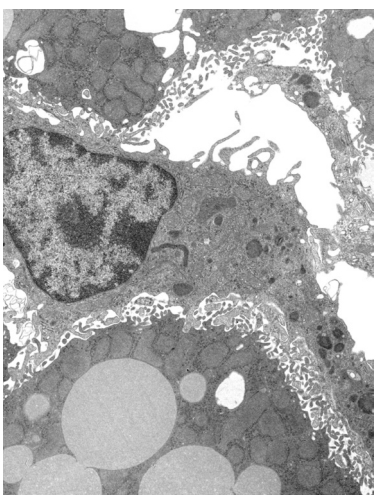

A Kupffer cell in liver sinusoid and lipid droplets in hepatocytes.

Image: Sushil K. Mahata.

The World Health Organization estimates that over 1.9 billion people worldwide are now obese or overweight [body mass index (BMI) > 27 Kg/m2]. Type 2 diabetes (T2D) is now recognized as the most devastating complications of obesity. Intimate relationship exists between obesity, innate (neutrophils, dendritic cells, macrophages, mast cells, and eosinophils) and adaptive (B and T lymphocytes) immune cells. Cells of the innate immune system produce inflammatory cytokines, and other factors leading to impaired insulin secretion and insulin resistance. Likewise, B lymphocytes (mostly B2 cells) are activated in obese adipose tissue and contribute to proinflammatory activation of adipose tissue macrophages and T cells resulting in insulin resistance. Thus, obesity-induced low-grade inflammation in adipose tissue, liver, skeletal muscle, and pancreas not only activates the innate and adaptive systems affecting metabolic homeostasis, it also results in fibrosis and necrosis. It is now becoming increasingly evident that fibrosis is a major contributor to metabolic dysregulation in obese and T2D patients and that advanced liver fibrosis leads to cirrhosis and death. The health risks associated with obesity are further exaggerated by smoking. This research topic consisting of 10 articles (9 reviews and one original) provide a comprehensive assessment of the impact of obesity on immunometabolism, cardiac functions, the connections of nicotine to non-alcoholic fatty liver disease (NAFLD), the expression of hepatic carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1), the role chromogranin A (CgA) and its peptides pancreastatin (PST) and catestatin (CST) in insulin sensitivity, the loss of skeletal muscle mass and function, and the alternate RNA splicing.

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EDITORIAL
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Editorial: Obesity, Smoking, and Fatty Liver Disease

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Keywords: obesity, diabetes, immunometabolism, cardiomyopathy, fatty liver disease, insulin sensitivity, RNA splicing, skeletal muscle regeneration

Editorial on the Research Topic

Obesity, Smoking, and Fatty Liver Disease

Obesity is on the rise worldwide and is doing so at an alarming rate. Obesity constitutes a major risk factor for diabetes and associated disorders like altered innate (neutrophils, dendritic cells, macrophages, mast cells, and eosinophils) and adaptive (B and T lymphocytes) immune cell responses to metabolism, diabetic cardiomyopathy (DCM), cardiovascular dysfunctions, non-alcoholic fatty liver disease (NAFLD), and certain forms of cancer. The health risks associated with obesity are further exaggerated by smoking. This research topic consisting of 10 articles (9 reviews and 1 original) provides a comprehensive assessment of the impact of obesity on immunometabolism, cardiac functions, the connections of nicotine to NAFLD, the expression of hepatic carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), the role chromogranin A (CgA) and its peptides pancreastatin (PST) and catestatin (CST) in insulin sensitivity, the skeletal muscle regeneration, and the alternate RNA splicing.

The first article by Mayoral Monibas et al. discusses the identification and contribution of hepatic non-parenchymal cells such as resident Kupffer cells (KCs), recruited monocyte-derived hepatic macrophages (RHMs), resident innate lymphocytes or natural killer cells, and fat storing hepatic stellate cells (HSCs) in the development of NAFLD, non-alcoholic steatohepatitis (NASH), and fibrosis through the use of cell surface markers. The authors underscore the polarization of hepatic macrophages from anti-inflammatory (M2) to proinflammatory (M1) types during obesity, macrophage regulation of NAFLD/NASH, and the expression of hepatic genes during obesity. In a schematic diagram, they have shown that in obese liver M1-KCs and Ly6C^{hi} macrophages stimulate HSCs that activate myofibroblast leading to fibrosis. The second article by Ray et al. highlights the interaction between the innate/adaptive immune system and the obesity-induced changes in metabolism. They discuss how TNF-α released by M1-macrophages during obesity and lipopolysaccharide released by gut bacteria signal *via* the TNF receptor and toll-like receptors, respectively, and induce inflammation and consequent upregulation of proinflammatory genes. The authors describe polarization of anti-inflammatory adipose tissue M2 macrophage to proinflammatory adipose tissue M1 during obesity. The authors also highlight that polarization of anti-inflammatory M2-KCs to M1-KCs and RHMs including Ly6C^{hi} during obesity results in decreased hepatic insulin sensitivity. The third article by Heinrich et al. highlights the role of hepatic CEACAM1 in obesity across multiple species and most notably demonstrates a significant reduction in hepatic CEACAM1 in obese subjects with fatty liver disease. The fourth article by Bandyopadhyay and Mahata underline contributions of two CgA-derived peptides, namely, PST and CST in regulation of obesity and insulin sensitivity. The authors discuss the mechanisms underlying

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inhibition of glucose-stimulated insulin secretion, hepatic gluconeogenesis, and insulin-stimulated lipid synthesis by PST. Furthermore, they underscore how PST induces inflammation and endoplasmic reticulum stress leading to the development of insulin resistance. CST, on the other hand, decreases hypertension by inhibiting catecholamine secretion and releasing histamine. They underline that CST alleviates adiposity by increasing lipolysis followed by increased β-oxidation of fatty acids. They also emphasize that CgA is proteolytically processed to counter-regulatory peptides such as PST and CST for fine tuning and maintenance of metabolic homeostasis. The fifth article by Sinha-Hikim et al. critically reviews the connections of nicotine and high-fat diet (HFD) to NAFLD. Nicotine when combined with an HFD leads to NAFLD through multiple mechanisms, including generation of severe oxidative stress and increased hepatocellular apoptosis as well inducing adipose tissue lipolysis resulting in excess delivery of free fatty acid and perturbation of hepatic lipid homeostasis through inactivation of AMP-activated protein kinase. Evidence also suggests a central role of the gut microbiota in obesity and its related disorders, including NAFLD. The pathogenesis of human NAFLD remains unclear, in particular in the context of its relationship to insulin resistance and visceral obesity. The sixth article by Sinha et al. underscores that skeletal muscle maintenance is a dynamic process and undergoes constant repair and regeneration. However, skeletal muscle regenerative capacity declines in obesity. They focus on obesity-associated changes in inflammation, metabolism, and impaired insulin signaling, which are pathologically dysregulated and ultimately result in a loss of muscle mass and function. The seventh article (original) by Heinrich et al. demonstrates that loss of hepatic CEACAM1 provides a unifying mechanism linking insulin resistance to obesity and NAFLD. The eighth article by Mishra et al. underlines the physiological steps leading to the development of DCM. The early steps include changes in substrate metabolism (abandoning glucose and relying mostly on fatty acids), oxidative and endoplasmic reticulum stress, formation of extracellular matrix proteins, and advanced glycation end products. The late steps embrace steatosis, apoptosis, fibrosis, and remodeling of cardiomyocytes resulting in DCM constituting left ventricular hypertrophy and reduced diastolic function. In a schematic diagram, they have shown how CCL7 released by activated B cells during obesity causes infiltration of monocyte-derived macrophages and subsequent stimulation of mast cells and infiltration of neutrophils. TGF-β secreted by activated monocyte-derived macrophages stimulates myofibroblasts to induce fibrosis. They also emphasize the differential expressions of various miRNAs in diabetic hearts and their roles in cardiac function and metabolism. The ninth article by Webster underlines the mechanisms underlying alternate RNA splicing and their implications in the development of liver, hepatic steatosis, and hepatocellular carcinoma. The author provides a detailed information on alternative splicing in liver and genetic manipulation of RNA-binding proteins *in vivo*. In addition, he has thoroughly described the RNA splicing SR proteins (with long repeats of serine and arginine) and their crucial roles in the development of hypertrophic and dilated cardiomyopathy, liver damages, and secretion of very low-density lipoproteins and triglycerides. The 10th article by Khullar et al. discusses how cumulative interactive effects of genetic and environmental factors result in the development of diabetes. In particular, the authors describe how modifications in histone acetyl transferases and histone deacetylases with consequent change in gene expression cause diabetes-induced microvascular complications.

AUTHOR CONTRIBUTIONS

AS-H and SM have made equal contributions.

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MINI REVIEW
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Connection of Nicotine to Diet-Induced Obesity and Non-Alcoholic Fatty Liver Disease: Cellular and Mechanistic Insights

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Non-alcoholic fatty liver disease (NAFLD) poses a serious health hazard affecting 20–40% of adults in the general population in the USA and over 70% of the obese and extremely obese people. In addition to obesity, nicotine is recognized as a risk factor for NAFLD, and it has been reported that nicotine can exaggerate obesity-induced hepatic steatosis. The development of NAFLD has serious clinical complications because of its potential progression from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma. Multiple mechanisms can be involved in nicotine plus high-fat diet-induced (HFD) hepatic steatosis. Emerging evidence now suggests that nicotine exacerbates hepatic steatosis triggered by HFD, through increased oxidative stress and hepatocellular apoptosis, decreased phosphorylation (inactivation) of adenosine-5-monophosphate-activated protein kinase and, in turn, up-regulation of sterol response-element binding protein 1-c, fatty acid synthase, and activation of acetyl-coenzyme A-carboxylase, leading to increased hepatic lipogenesis. There is also growing evidence that chronic endoplasmic reticulum stress through regulation of several pathways leading to oxidative stress, inflammation, perturbed hepatic lipid homeostasis, apoptosis, and autophagy can induce hepatic steatosis and its progression to NASH. Evidence also suggests a central role of the gut microbiota in obesity and its related disorders, including NAFLD. This review explores the contribution of nicotine and obesity to the development of NAFLD and its molecular underpinning.

Keywords: nicotine, high-fat diet, obesity, oxidative stress, non-alcoholic fatty liver disease

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INTRODUCTION

In 2009, approximately 20% (~60 million) of Americans smoked and about ~88 million non-smokers were exposed to secondhand smoke (1). Unless dramatic progress is made in diminishing the initiation and increasing cessation of combustible tobacco product use, a billion of preventable death will occur in twenty-first century worldwide (2). Thus cigarette smoking needs to be viewed as a chronic disease, and in addition to research on the difficult problem of smoking cessation, research also needs to be conducted on the detrimental effects of chronic cigarette use. The prevalence of smoking was 31.1% among persons below the federal poverty level (1), so smoking should be considered a health disparity. Cigarette smoking is the leading preventable cause of death and disability worldwide (3, 4). Smoking is a major risk factor for chronic obstructive pulmonary disease and lung cancer and devastating cardiovascular disease (CVD), such as myocardial infarction, sudden death, stroke,

and peripheral vascular disease (5–8), with a dose–response correlation between CVD morbidity and mortality and the number of cigarettes smoked (8). Furthermore, usages of nicotine only formulations, such as transdermal patches, nicotine gum, and electronic cigarettes, in particular, are increasing (9, 10). The lack of targeted and effective strategies to control tobacco consumption contribute to large burden of cardiovascular disorders in low- and middle-income people worldwide, where CVD has become the leading cause of morbidity and mortality (8). Moreover, smoking leads to substantial financial costs to society. Between 2009 and 2012, smoking cost the USA approximately $289–332.5 billion, with 46–53% of this amount spent on adult medical care and the rest due to loss of workplace productivity (4). The negative effects of smoking, thus, leads to reduced quality of life and loss of life and can lead to personal and national financial burden. The health risk associated with smoking can be exaggerated by obesity (11, 12).

Nicotinic acetylcholine receptors (nAChRs) are a family of ionotropic receptor proteins formed by five homologous or identical subunits and are involved in signal transduction between neurons and muscle cells (10, 13, 14). nAChRs are divided into muscle (α1, β1, γ/ε, and δ) and neuronal nAChRs (α 2–10 and β 2–4) (10, 14, 15). Neuronal nAChRs are further subdivided into those that form homomeric receptors when expressed in heterologous systems (α7-10) and those that form heteromeric receptors (α2-6 and β2-4) in different combinations (10, 14, 15). nAChRs are also expressed in various tissues, including adipocytes, pancreatic beta cells, hepatocytes, myocytes, and cardiomyocytes (16–19). The nAChRs, which are activated by nicotine or its metabolites cotinine, can activate various signaling pathways that can alter cellular metabolic homeostasis (10). This review discusses emerging evidence of contribution of nicotine when combined with obesity to the development of hepatic steatosis and insights into the molecular mechanisms by which nicotine contributes to non-alcoholic fatty liver disease (NAFLD).

NAFLD Is Highly Prevalent in Obese Individuals and Can Be Exaggerated by Smoking

Non-alcoholic fatty liver disease is the most common liver disorder and is associated with metabolic syndrome and diabetes mellitus. It includes the whole spectrum of fatty liver, ranging from simple steatosis to steatohepatitis [non-alcoholic steatohepatitis (NASH)], which can progress to liver cirrhosis and hepatocellular carcinoma (20–22). Data from the Framingham Heart Study showed that fatty liver is characterized by dysglycemia and dyslipidemia independent of visceral adipose tissue (23). There is increasing evidence that smoking can also contribute to NAFLD. Multiple logistic regression analysis from a retrospective follow-up study over a 10-year period, involving 2,029 Japanese subjects, demonstrated that cigarette smoking (adjusted odd ratio 1.91; 95% confidence interval 1.34–2.72) is an independent risk factor for NAFLD (24). A statistically significant association between smoking history and severity of liver fibrosis was demonstrated in a large multicenter cohort of 1,091 subjects with biopsy-proven NAFLD (25). Of further importance, the health risk associated with smoking, whether passive or active, is exaggerated by obesity, and smoking and obesity are the leading causes of morbidity and mortality worldwide (11, 12). The life expectancy of an obese smoker is 13 years less than that of a normal-weight non-smoker (11). Furthermore, smoking lowers the body weight and body mass index (BMI), which make many people reluctant to quit smoking (11).

In the United States, 72% of the adult male population is overweight or obese out of which 11% have a BMI of 35 kg/m^2 and 4% a BMI of at least 40 kg/m^2 (26). Obese men are at a higher risk to develop atherosclerosis, coronary heart disease, diabetes, hypertension, dyslipidemia, and NAFLD (27). NAFLD, in turn, can also be an independent risk factor of atherosclerosis and CVD (28, 29). Currently, 34% of the general population and over 75% of the obese and extremely obese individuals are estimated to have hepatic steatosis (30). Hispanics have the highest prevalence of hepatic steatosis followed by Caucasians and then African-Americans (31).

Mechanisms Linking Nicotine to NAFLD

The hallmark of NAFLD is accumulation of triglycerides (TG) in the hepatocytes (steatosis). Multiple mechanisms have proposed to explain the accumulation of TG in the liver, including (i) increased dietary fat intake, (ii) excess free fatty acid (FFA) delivery from lipolysis of white adipose tissue, (iii) increased *de novo* lipogenesis, (iv) reduced fatty acid β-oxidation, and (v) reduced fat export in the form of very low-density lipoprotein (VLDL) (21, 32). The precise molecular mechanisms of the pathogenesis of steatosis and its progression to NASH are not well understood. AMP-activated protein kinase (AMPK) is a central regulator of lipid homeostasis and mediates suppression of lipogenic gene expression, such as acetyl-coenzyme A-carboxylase (ACC) and fatty acid synthase (FAS) through inhibition of sterol regulatory element binding protein-1c (SREBP1-c) and carbohydrate response-element binding protein (ChREBP) (33–35). ACC is the rate determining enzyme for the synthesis of malonyl-CoA, both a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation (33). AMPK can phosphorylate and inactivate ACC leading to inhibition of *de novo* fatty acid and cholesterol synthesis (33). AMPK can also increase the activity of malonyl-CoA decarboxylase to further decrease malonyl-CoA levels (33). Lipogenesis is further regulated by glucose, which activates ChREBP, which, in turn, activates gene expression of most enzymes involved in lipogenesis (21).

Two-Hit or Multiple-Hit Hypothesis

Steatosis can prime the liver to develop more progressive liver pathologies in response to additional metabolic and/or environmental stressors. Mechanistically, this is commonly mediated by the prevalent "two-hit" hypothesis that implies accumulation of TG in hepatocytes (steatosis) in the first hit, followed by triggering progression to inflammation, oxidative stress, and apoptosis in the second hit (22, 35, 36). In more advanced cases, fibrosis is also exacerbated, leading to the progressive form of NAFLD, known as NASH. Environmental stressors [such as high-fat diet (HFD), cigarette smoke, drugs, and pollutants] or metabolic stressors (such as obesity, diabetes, hypertension, hypertriglyceridemia and hypercholesterolemia) are known to trigger progression to the second phase. Nonetheless, the molecular underpinning of

steatosis is not well understood. Oxidative stress coupled with hepatocyte apoptosis is believed to play a pivotal role in pathogenesis of NAFLD (22, 37, 38). In fact, emerging data suggest that hepatocyte apoptosis plays a key component in the progression of simple steatosis to NASH (22, 37). Notably, a proof-of-principle, randomized, double blind, placebo-controlled study of GS-9450 (selective inhibitor of caspases 1, 8, and 9) suggests that reducing hepatocellular apoptosis may be a valuable therapeutic strategy in patients with NASH (39).

Smoking Exacerbates Effects of Dietary Fat on Liver

Animal experiments using first-hand (delivered *via* a smoking device designed to puff the smoke into the inhalation chamber housing the animals), second-hand smoke (side-stream whole smoke solution delivered *via* a puffer box), or nicotine and models of genetic or diet-induced obesity (DIO) provide perhaps the strongest evidence linking nicotine to hepatic steatosis and NAFLD. Yuan and colleagues (40) demonstrated that HFD-fed apoB100 transgenic mice on C57Bl6J background exposed to second-hand smoke exhibit lipid accumulation in the liver and this effect was mediated by inactivation of AMPK and activation of its downstream target SREBP-1. In another study, Azzalini and colleagues (41) demonstrated that first-hand smoke exacerbates NAFLD in obese Zucker rats. The effect of first-hand smoke on the severity of hepatic steatosis was associated with increased oxidative stress, hepatocyte apoptosis, expression of key genes involved in hepatic fibrogenesis, and inactivation of Akt but stimulation of extracellular signal regulated kinase (ERK) signaling. We used the model of DIO in C57BL6J mice to study the mechanisms underlying the detrimental effects of nicotine and HFD in the development of fatty liver disease (42). Like humans, these mice, when fed a HFD deriving 60% of calories from fat, developed visceral adiposity, hyperglycemia, insulin and leptin resistance, as well as hepatic steatosis (43, 44). We elected to use a single drug (nicotine) as opposed to first-or second-hand smoke in order to eliminate the confounding effects of other components involved in cigarette smoking. Adult C57BL6 male mice were fed a normal chow diet or HFD and received twice daily injections of nicotine (0.75 mg/kg BW, IP) or saline for 10 weeks. Of note, the daily dosage of 1.5 mg/kg BW in mice results in a serum concentration of nicotine that is similar to the clinically relevant concentrations found in habitual cigarette smokers and nicotine-containing chewing gum users (19). We purposely used shorter (10-week) duration to examine the synergistic effects of these two insults in the initiation of NAFLD, as a longer exposure to HFD alone results in extensive steatosis (45) and systemic inflammation (46). We found that nicotine alone did not lead to hepatic steatosis, but it caused hepatic steatosis only when combined with HFD (**Figure 1**) (42). A significant ($p < 0.01$) increase in the Vv% of lipid droplets together with a reduction in the Vv% of endoplasmic reticulum (ER) (67.8%) and glycogen (49.2%) was also noted in hepatocytes from mice on HFD plus nicotine, compared to mice on HFD alone. The additive effects of nicotine on the severity of HFD-induced hepatic steatosis was associated with significantly greater oxidative stress, increased hepatic TG levels, higher incidence of hepatocellular apoptosis, inactivation (dephosphorylation) of AMPK, and activation of its downstream target ACC (42).

Indeed, these above studies, using various experimental models, demonstrated that nicotine further worsens HFD-induced hepatic steatosis. Summation of the results further indicate that increased oxidative stress and hepatocellular apoptosis, inactivation of Akt and AMPK, and activation of its downstream targets SREBP-1 and ACC, together with stimulation of ERK are involved in the pathogenesis of nicotine plus HFD-induced hepatic steatosis.

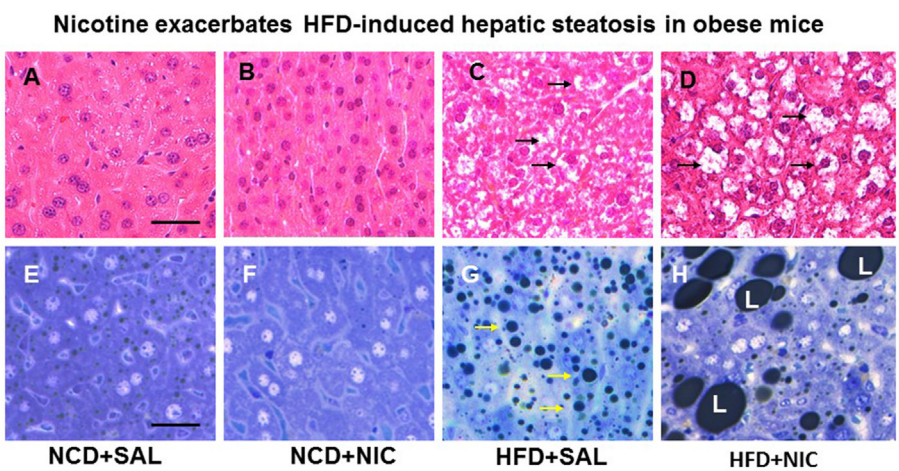

FIGURE 1 | Representative H&E-stained liver sections from mice fed with normal chow diet (NCD) without (A) or with (B) nicotine exhibit normal histological appearance. Compared with a mouse on a high-fat diet (HFD), where a modest increase in lipid accumulation (arrow) is detected **(C)**, combined treatment with nicotine and HFD causes a marked increase in lipid accumulation in the liver **(D)**. **(E–H)** Representative light microscopic images of glutaraldehyde-fixed, osmium tetroxide post-fixed, epoxy-embedded, and toluidine-blue-stained live sections from different treatment groups show nicotine plus a HFD **(H)** causes a striking increase in lipid accumulation of varying sizes in hepatocytes compared to those from mice on a HFD alone [**(G)**, arrow]. Mice fed with NCD with **(F)** or without nicotine **(E)** have normal liver morphology. Scale bar = 25 μm [reproduced with permission from Friedman et al. (42)].

Contribution of Adipose Tissue Lipolysis to Nicotine and HFD-Induced Hepatic Steatosis

Adipose tissue has the unique function of storing TG in lipid droplets and upon lipolysis, to provide FFA to other organs during time of energy shortage (47). In obesity and other conditions where cellular lipid homeostasis is perturbed, lipolysis can contribute to ectopic lipid accumulation (48). Mounting experimental evidence supports that nicotine considerably decreases HFD-induced adiposity in mice, as determined by dual-energy X-ray absorption densitometry, computed tomography, as well as by magnetic resonance imaging, with no change in lean body mass (19, 49). Nicotine when combined with a HFD, however, significantly increases the levels of serum, hepatic TG, and circulating FFA (19, 42, 50). These results indicate that nicotine in mice on a HFD promotes lipid distribution from adipose tissue to other organs. Decisive evidence that increased adipose tissue lipolysis contributes to nicotine plus HFD-induced hepatic steatosis derives from studies showing that acipimox, an inhibitor of adipose tissue lipolysis, treatment significantly prevented nicotine plus HFD-induced increase in hepatic TG levels and hepatic steatosis (**Figure 2**) (42). A recent study (19) has also demonstrated that acipimox treatment significantly prevented nicotine plus HFD-induced increase in serum FFA levels and serum and hepatic TG levels, as well as hepatic steatosis (**Figure 2**). This concept is supported by another evidence showing that inhibition of adipose tissue lipolysis by adipose-specific ablation of desnutrin prevented ectopic lipid accumulation in the liver even when fed with a HFD (51). Together, these results suggest that adipose tissue lipolysis plays a major role in the development of nicotine plus HFD-induced hepatic steatosis.

Mechanistically, nicotine activates AMPKα2 in adipocytes, which phosphorylates MAP kinase phosphatase-1 (MKP1) at serine 334, resulting its proteasome-dependent degradation (19). Nicotine-induced reduction in MKP1, in turn, activates both p38 mitogen-activated protein kinase (p38 MAPK) and c-jun-NH_2-terminal kinase (JNK), which phosphorylates insulin receptor substrate 1 (IRS1) at serine 307. Phosphorylation of IRS1 leads to its degradation and the subsequent inhibition of Akt, resulting in increased adipose tissue lipolysis and circulating FFA levels (19).

The Role of ER Stress

Chronic ER stress induces several pathways leading to oxidative stress, inflammation, perturbed hepatic lipid homeostasis, apoptosis, and autophagy that can lead to hepatic steatosis and its progression to NASH [reviewed in Ref. (52)]. ER stress is related with hepatic lipid metabolism by directly increasing lipogenesis and limiting VLDL formation. It has been demonstrated that

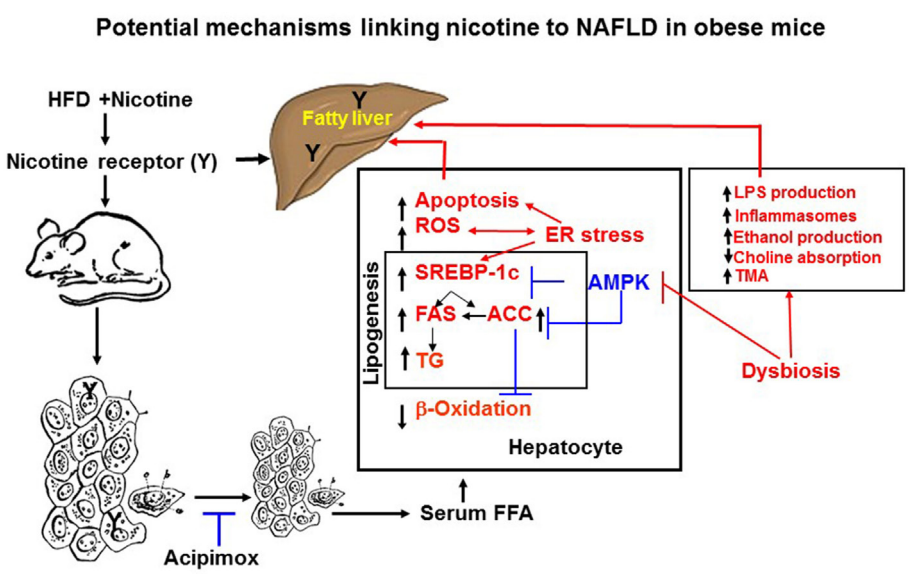

FIGURE 2 | **Potential mechanisms of nicotine plus HFD-induced hepatic steatosis in obese mice**. Nicotine plus a HFD promotes abdominal lipolysis, resulting in free fatty acid (FAA) release from adipose tissue into the circulation, thereby contributing to the buildup of lipids as triglyceride in the liver. In addition, nicotine plus a HFD may also promote *de novo* lipogenesis through inactivation of AMP-activated protein kinase (AMPK) and activation of its downstream target acetyl-coenzyme A-carboxylase (ACC), leading to the development of hepatic steatosis. Inactivation of AMPK can also stimulate lipogenesis through upregulation of key genes in the lipogenic pathway, such as fatty acid synthase (FAS) and ACC, by activating the transcription factor sterol regulatory element binding protein 1 c (SREBP-1c). Intrahepatic lipid accumulation can also trigger hepatocellular apoptosis through generation of oxidative stress coupled with activation of c-Jun NH_2-termina kinase (JNK)-mediated apoptotic signaling. AMPK inactivation could further sensitize liver cells to nicotine plus HFD-induced apoptosis. There is also growing evidence that chronic endoplasmic reticulum stress through regulation of several pathways leading to oxidative stress, inflammation, perturbed hepatic lipid homeostasis, apoptosis, and autophagy, can also induce hepatic steatosis and its progression to non-alcoholic steatohepatitis. Evidence also suggests a central role of the gut microbiota in obesity and its related disorders, including non-alcoholic fatty liver disease (NAFLD). It is possible nicotine plus a HFD through changes in short-chain fatty acids metabolism, increased intestinal permeability and lipopolysaccharides activation of Toll-like receptors and inflammasomes, endogenous ethanol production, decreased choline availability and increased trimethylamine (TAM) production could cause NAFLD. The multiple mechanisms of nicotine and obesity-induced hepatic steatosis can results from both its nicotinic acetylcholine receptor-mediated and non-receptor effects.

ER stress contributes to increased hepatic lipogenesis in *ob/ob* mice through SREBP1c activation while overexpression of ER chaperone BIP decreased ER stress and inhibited lipogenesis by inactivating SREBP1 (53). Furthermore, ER stress modulates several factors, including nuclear factor 2 erythroid-related factor 2 (Nrf2), JNK, nuclear factor κB (NF-κB), and c/EBP homologous protein (CHOP), all of which play a role in the inflammatory process, cellular defense against oxidative stress, and cell death. For example, Nrf2 serves as master regular of a cellular defense system against oxidative stress (54, 55). Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Keap1, which facilitates its ubiquitination and proteasomic degradation. Upon exposure to oxidative stress, the sequestration complex brakes down and the dissociated Nrf2 translocates into the nucleus, where it binds to cis-acting antioxidant response elements and promotes the transcription of a large number of cytoprotective genes (56, 57). However, under pathological conditions, such as NASH, NRf2 activity is impaired (52). Consistent with the role of NrF2 in NAFLD, it has been demonstrated that genetic ablation of Nrf2 markedly exacerbates NASH (58). Conversely, enhanced expression of Nrf2 in mice bearing a hepatocyte-specific knockdown of Keap1 attenuated the fatty liver induced by a methionine- and choline-deficient diet (59). JNK is activated in various animal models of obesity and also in patients with NASH and its deletion results in attenuation of fatty liver (22). Activation of JNK has also been documented in HFD-induced hepatic steatosis in apoplipoprotein E knockout mice (60) or nicotine plus HFD-induced hepatic steatosis in obese mice (42). NF-κB is a transcription factor and a primary regulator of inflammatory action. Activation of NF-κB dimers is due to inhibiton of NF-κB kinase (IKK)-mediated phosphorylation-induced proteasomal degradation of IκB, enabling the active NF-κB transcription factor subunits to translocate to the nucleus and induce target gene expression. Persistent activation of NF-κB signaling has been shown in animal models of NAFLD as well as in patients with NASH (35). Furthermore, CHOP plays a pivotal role in ER-induced cell death. Deletion of CHOP decreases hepatocyte apoptosis in alcohol-induced liver disease and reduces cholestsis-induced liver fibrosis (61, 62).

It is worth noting here that both nicotine (63, 64) and HFD (65, 66) are capable of generating hepatic ER stress. Thus, it is possible that nicotine plus HFD could generate severe hepatic ER stress leading to hepatic steatosis. Clearly, further studies are needed to define the role of ER stress in fatty liver disease triggered by nicotine and HFD.

Connections of Gut Microbiota to NAFLD

Evidence linking dysbiosis (also known as disruption of the normal gut microbiota) contributes to the pathogenesis of NAFLD has accumulated rapidly (67–69). Early studies have shown that patients with biopsy-proven NAFLD had significantly increased gut permeability compared to healthy volunteers (70). Both the increased gut permeability and prevalence of small intestinal bacterial overgrowth correlated with severity of steatosis in the patients with the NAFHD (70). The strongest evidence supporting the role of dysbiosis in NAFLD, however, stems from animals studies where the gut microbiome has been manipulated. It has been shown that microbiome from obese mice is linked to increased energy from the diet and this trail can be transmissible to lean adult germ-free mice by co-housing with obese mice (71). A growing number of studies examining how dysbiosis might drive NAFLD have identified a number of plausible mechanisms, including changes in short-chain fatty acids (SCFAs) metabolism, increased intestinal permeability and lipopolysaccharides (LPS) activation of toll-like receptors (TLRs) and inflammasomes, endogenous ethanol production, decreased choline availability, and trimethylamine production (69). For example, it has been shown that SCFAs can lower FAS activity and hepatic lipid synthesis in HFD-fed mice through activation of AMPK and inactivation of its downstream substrate ACC (72). Evidence exists that smoking can also induce profound changes in intestinal microbiota (73, 74). Taken together, it is possible that nicotine plus a HFD through changes in SCFAs metabolism, increased intestinal permeability and LPS activation of TLRs and inflammasomes, endogenous ethanol production, decreased choline availability and trimethylamine production could cause NAFLD.

CONCLUSION AND PERSPECTIVES

Nicotine when combined with a HFD leads to NAFLD through multiple mechanisms, summarized in **Figure 2**, including generation of severe oxidative stress and increased hepatocellular apoptosis as well inducing adipose tissue lipolysis resulting in excess delivery of FFA and perturbation of hepatic lipid homeostasis through inactivation of AMPK. There is also growing evidence that chronic ER stress through regulation of several pathways leading to oxidative stress, inflammation, perturbed hepatic lipid homeostasis, apoptosis, and autophagy, can also induce hepatic steatosis and its progression to NASH. Evidence also suggests a central role of the gut microbiota in obesity and its related disorders, including NAFLD. The multiple mechanisms of nicotine and obesity-induced hepatic steatosis is mediated by both its nAChR-mediated and non-receptor effects.

A better understanding of the mechanisms and various diverse signaling pathways responsible for nicotine plus HFD-induced NAFLD may also unveil novel pharmacological targets to treat fatty liver disease and adverse metabolic sequelae. The emerging knowledge about a direct connection of smoking or tobacco products to obesity and fatty liver disease should be considered during the evaluation of regulations on nicotine product manufacturing, distribution, and marketing.

AUTHOR CONTRIBUTIONS

AS-H and IS-H conceived and prepared the manuscript. TF critically appraised the manuscript and also wrote a part of the manuscript.

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REVIEW
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Loss of Hepatic CEACAM1: A Unifying Mechanism Linking Insulin Resistance to Obesity and Non-Alcoholic Fatty Liver Disease

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The pathogenesis of human non-alcoholic fatty liver disease (NAFLD) remains unclear, in particular in the context of its relationship to insulin resistance and visceral obesity. Work on the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in mice has resolved some of the related questions. CEACAM1 promotes insulin clearance by enhancing the rate of uptake of the insulin-receptor complex. It also mediates a negative acute effect of insulin on fatty acid synthase activity. This positions CEACAM1 to coordinate the regulation of insulin and lipid metabolism. Fed a regular chow diet, global null mutation of *Ceacam1* manifest hyperinsulinemia, insulin resistance, obesity, and steatohepatitis. They also develop spontaneous chicken-wire fibrosis, characteristic of non-alcoholic steatohepatitis. Reduction of hepatic CEACAM1 expression plays a significant role in the pathogenesis of diet-induced metabolic abnormalities, as bolstered by the protective effect of hepatic CEACAM1 gain-of-function against the metabolic response to dietary fat. Together, this emphasizes that loss of hepatic CEACAM1 links NAFLD to insulin resistance and obesity.

Keywords: insulin clearance, insulin resistance, lipogenesis, fatty liver oxidation, lipolysis, NAFLD, visceral obesity

PHYSIOLOGIC REGULATION OF CARCINOEMBRYONIC ANTIGEN-RELATED CELL ADHESION MOLECULE 1 (CEACAM1)

The CEACAM1 is a transmembrane glycoprotein that undergoes phosphorylation by the insulin receptor tyrosine kinase (1). Among insulin target tissues, CEACAM1 is predominantly expressed in the liver (2). This is consistent with its role in promoting insulin clearance, which occurs mostly in liver and to a lower extent in kidney. Consistent with the important role of the liver in regulating insulin and lipid metabolism, Ceacam1 transcription is coordinately regulated by insulin and fatty acids during fasting–refeeding conditions, with fatty acids at fasting repressing it *via* a mechanism depending on the peroxisome proliferator-activated receptor alpha (PPARα) (3, 4) and insulin inducing it in the first few hours of refeeding (3, 5).

CEACAM1 PROMOTES INSULIN CLEARANCE AND MEDIATES AN ACUTE NEGATIVE EFFECT OF INSULIN ON HEPATIC *DE NOVO* LIPOGENESIS

Insulin is released from pancreatic β-cells in a pulsatile manner (6). The acute rise of insulin in the portal vein causes phosphorylation and activation of the insulin receptor tyrosine kinase in the hepatocyte (7, 8). This, in turn, leads to phosphorylation of substrates, including CEACAM1 (1). Upon its phosphorylation, CEACAM1 promotes receptor-mediated insulin uptake into clathrin-coated pits/vesicles of the hepatocyte to be eventually degraded and cleared from the blood (9, 10). This process mediates the rapid extraction of ~50% of secreted insulin through its first pass into the liver.

Internalization of phosphorylated CEACAM1 as part of the insulin-receptor complex leads to its binding to fatty acid synthase (FASN) (11), a key enzyme that catalyzes the conversion of malonyl-CoA to palmitic acid during *de novo* lipogenesis. CEACAM1 association downregulates FASN enzymatic activity and restricts hepatic *de novo* lipogenesis, likely to protect the liver against the potential lipogenic effect of approximately twofold to threefold higher level of insulin in the portal than the systemic circulation (12). Thus, CEACAM1 phosphorylation by the insulin receptor in response to acute rise of insulin constitutes a key mechanism that underlies the maintenance of physiologic insulin levels, at the same time as mediating a suppressive acute effect of insulin on lipogenesis in liver. Combined, this restricts hepatic lipid production under normal physiologic conditions; assigning a major role for CEACAM1 in integrating the regulation of insulin and lipid metabolism in the hepatocyte. Under conditions of hyperinsulinemia, the pulsatility of insulin secretion is compromised (6), limiting insulin signaling in the hepatocyte, including CEACAM1 phosphorylation, and subsequently, the acute negative effect of insulin on FASN activity is removed to contribute to hyperinsulinemia-driven lipogenesis (11). This paradigm emphasizes the contrast between the previously unappreciated suppressive effect of acute insulin pulses on fatty acid synthesis and the well-recognized positive effect of chronically elevated levels of insulin on lipogenic genes' expression by the coordinated action of sterol regulatory element-binding protein (SREBP1c) (13) and the upstream stimulatory factor 1 (14). Suppression of hepatic FASN activity by pulsatile insulin release proposes to include elevation in *de novo* lipogenesis as a manifest of hepatic insulin resistance in addition to increased hepatic glucose production (*via* glycogenolysis and gluconeogenesis) (8, 15).

MUTATING CEACAM1 IN LIVER CAUSES INSULIN RESISTANCE AND NON-ALCOHOLIC STEATOHEPATITIS (NASH)

Mice with liver-specific inactivation (L-SACC1) or with global null mutation of *Ceacam1* (*Cc1*$^{-/-}$) exhibit impairment in insulin clearance leading to chronic hyperinsulinemia and systemic insulin resistance (owing to downregulation of insulin receptor expression) (16–18). They also exhibit elevated lipid production in liver and redistribution to the white adipose tissue to be stored; thus, contributing to visceral obesity and increased release of free fatty acid (FFA) and adipokines (19).

Mutant Ceacam1 mice also develop inflammation in liver, in part due to the loss of the anti-inflammatory effect of CEACAM1 (20), apoptosis, and oxidative stress. Additionally, they manifest chicken-wire bridging fibrosis, a characteristic feature of NASH, even when fed a standard chow diet, making them rare mouse models of spontaneous fibrosis on the C57BL/6J genetic background. The underlying mechanisms of fibrosis in Ceacam1 mutants are the subject of intense investigations in our laboratories.

DIETARY FAT REDUCES HEPATIC CEACAM1 EXPRESSION IN C57BL/6J MICE

In uncomplicated obesity with low-grade insulin resistance, FFA are mobilized from white adipose tissue mainly to the liver to be removed by β-oxidation (21). This is supported by experimental evidence in rodents showing occurrence within few days of the initiation of high-fat intake as a result of dysregulated hypothalamic control in the adipose tissue (22). While this early lipolysis occurs in the absence of insulin resistance in the adipose tissue, the released FFA can rapidly initiate hepatic insulin resistance (23), in part by activating PKCδ-mediated pathways (24). As the nutritional burden persists, hepatic lipotoxicity develops in response to progressively compromised β-oxidation relative to re-esterification. Concomitantly, hepatic insulin resistance progresses into systemic insulin resistance to be manifested in peripheral tissues, including the white adipose tissue with ensuing advancement of a pro-inflammatory state (25).

Recent reports from our laboratories show that high-fat diet progressively reduces hepatic CEACAM1 level in C57BL/6J mice until it reaches >50% after 3 weeks, at which point, insulin clearance is impaired and hyperinsulinemia develops with attendant hepatic insulin resistance and steatohepatitis (26). Consistent with the key role for CEACAM1 in diet-induced insulin resistance and hepatosteatosis, adenoviral-mediated redelivery of wild-type, but not phosphorylation-defective CEACAM1 to the liver, completely reverses these metabolic abnormalities even while maintaining mice on a high-fat diet (27), demonstrating a causative role for the decrease in hepatic CEACAM1 level in sustaining diet-induced systemic insulin resistance and hepatic steatosis. That impairment of insulin clearance plays a significant role in hepatic insulin resistance in response to high-fat diet has recently been demonstrated in Asian men (28). Using a two-step hyperinsulinemic-euglycemic clamp, Bakker et al. (28) showed that in contrast to age- and sex-matched Caucasians, young and healthy South Asian men develop impairment of insulin clearance as well as hepatic insulin resistance in the absence of other metabolic alterations in skeletal muscle and white adipose tissue following 5 days of a high-fat Western diet intake. Several other studies in humans (28) as well as dogs (29) have supported the

findings that defective hepatic insulin clearance is implicated in diet-induced insulin resistance.

The decrease in hepatic CEACAM1 by high-fat diet is attributed to lipolysis-derived FFA, in agreement with reducing hepatic CEACAM1 levels by intralipid–heparin infusion (24) and the negative effect of FFA on insulin clearance (30, 31). The underlying mechanism of CEACAM1 repression by FFA is *via* PPARα activation (4). In the presence of normoinsulinemia, this provides a positive feedback mechanism on fatty acid β-oxidation as it limits the negative effect of CEACAM1 on FASN activity (11) and subsequently, reduces malonyl-CoA-mediated inhibition of fatty acids translocation to the mitochondria (3). When CEACAM1 level is reduced by >50%, hepatic insulin clearance fails and chronic hyperinsulinemia develops, causing hepatic insulin resistance, at least in part by downregulating insulin receptors in the hepatocyte (32, 33) and triggering *de novo* lipogenesis by activating SREBP1c-mediated transcription of lipogenic genes (13), including acetyl-CoA carboxylase (ACC), a limiting enzyme in lipid biosynthesis. Elevation in ACC level (and activity) induces malonyl-CoA level, which in turn, inhibits fatty acid transport to the mitochondria and β-oxidation. Potentially contributing to the downregulation of β-oxidation under hyperinsulinemic conditions is the maintenance of insulin-stimulated phosphorylation and inactivation of Foxa2-mediated suppression of the transcription of genes involved in fatty acid β-oxidation (34, 35). Collectively, this limits fatty acid β-oxidation while promoting *de novo* lipogenesis, leading to hepatosteatosis. With the loss of the potential counter-regulatory anti-inflammatory function of CEACAM1, this causes a more robust change in the inflammatory milieu of the liver and steatohepatitis develops. Together, the data identify reduction in CEACAM1 expression as a novel molecular underpinning of the integrated regulation of lipid oxidation and hepatic insulin resistance (gluconeogenesis) by FFA mobilization from white adipose tissue (36–38).

REDUCED HEPATIC CEACAM1 LEVELS CAUSES OBESITY BY CONTRIBUTING TO ENERGY IMBALANCE

High-fat diet represses hepatic CEACAM1 levels to impair insulin clearance and cause hyperinsulinemia that in turn, drives increased hepatic lipid production and output to the white adipose depot for storage (39). This is consistent with the well-accepted association of hyperinsulinemia and liver steatosis with high plasma Apolipoprotein B levels and visceral obesity in humans and rodents (40–45). Together with visceral obesity, sustained hyperinsulinemia reduces glucose transporter 4-mediated glucose transport to cause insulin resistance in adipose tissue (46), as supported by hyperinsulinemic-euglycemic clamp analysis in Ceacam1 mutants (16–18, 47) and in the diet-induced model (26).

Consistent with the finding that reduction of hepatic CEACAM1 plays a critical role in diet-induced altered metabolic response, transgenic protection of hepatic CEACAM1 in L-CC1 mice prevents hyperinsulinemia, insulin resistance, and hepatosteatosis in response to high-fat diet (26). It also limits the size of adipocytes and total fat mass by countering the negative effect of high-fat diet on energy expenditure and spontaneous physical activity (26). Similarly, adenoviral-redelivery of wild-type CEACAM1 in the liver protects energy balance against high-fat intake, thereby reversing the gain in body weight and visceral adiposity (27). Given that CEACAM1 is not detected in the adipocyte at the protein level (2), it is likely that the gain-of-function of hepatic CEACAM1 drives this positive effect on energy expenditure and adipose tissue biology (limited adipocyte size, fibrosis, and inflammation) (27, 39). The beneficial effect of hepatic CEACAM1 gain-of-function on insulin response in white adipose tissue could be mediated, at least in part, by the rise in plasma FGF21 (48, 49) that induces the locomotor activity (50) and energy expenditure (51, 52).

Both L-SACC1 and *Cc1*$^{-/-}$ mutant mice display visceral obesity and a higher body mass than their wild-type counterparts (16–18). Visceral obesity, which is partly caused by elevated hepatic lipid production and redistribution to white adipose tissue (19), leads to hyperleptinemia, which could in turn, alter response to leptin and cause energy imbalance. Consistently, global *Cc1*$^{-/-}$ null mice develop elevated production and secretion of leptin from their expanded while adipose depot in addition to increased total fat mass and obesity resulting from hyperphagia and reduced spontaneous physical activity (53). In addition to leptin resistance,

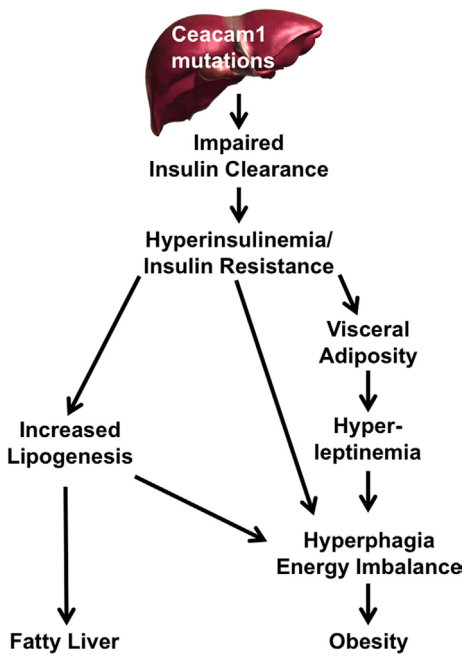

FIGURE 1 | A pivotal role for carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) reduction in the pathogenesis of fatty liver disease and obesity. Reduction or mutation of Ceacam1 in the liver results in decreased insulin clearance from the portal circulation. Reduced clearance leads to hyperinsulinemia followed by insulin resistance (owing to downregulation of the insulin receptor) and increased hepatic lipogenesis. Elevation in hepatic lipogenesis leads to lipid redistribution to the while adipose depot to increase visceral adiposity. This leads to hyperleptinemia, which along with hyperinsulinemia, increases food intake and energy imbalance, further exacerbating obesity. Hyperinsulinemia drives hepatic lipogenesis and fat accumulation in liver.

hyperinsulinemia also contributes to the obesity phenotype in these mice, at least in part, by inducing hypothalamic FASN level and activity (53), which in turn, causes hyperphagia (54) and lower physical activity (55, 56). Together, this demonstrates that altered CEACAM1-dependent insulin clearance pathways drive hyperinsulinemia-mediated link of hepatic steatosis to visceral obesity and increased total fat mass.

CONCLUDING REMARKS

The mechanisms underlying the pathogenesis of non-alcoholic fatty liver disease (NAFLD) in humans remain unclear (57) and whether insulin resistance plays a role in NAFLD has been debated, owing to the lack of appropriate animal models that replicate all features of the human disease and its progression to NASH (58, 59). As summarized in this review, our laboratory has demonstrated in the last couple of decades that loss in hepatic CEACAM1 expression and its defective phosphorylation impair insulin clearance and subsequently, play a pivotal role in insulin resistance, fatty liver disease, and obesity (**Figure 1**) (9, 10, 16–19, 25, 27, 39, 53, 60, 61). Demonstration of a role for impaired insulin clearance in insulin resistance in human disease is emerging (62–65). In this regard, compromised hepatic insulin extraction has been shown to constitute a risk factor for obesity (66, 67), type 2 diabetes (68), metabolic syndrome (65, 69), and fatty liver disease (70). The study by Lee (71) showing a marked decline in hepatic CEACAM1 levels in patients with high-grade fatty liver and obesity coupled with our mechanistic studies demonstrating that redelivering CEACAM1 to the liver reverses diet-induced insulin resistance, fatty liver, and visceral obesity (27) emphasizes a critical role for CEACAM1 in metabolic control. Of note, while our studies show that reduction of hepatic CEACAM1 causes insulin resistance, hepatosteatosis, and visceral obesity, they also show that diet-induced visceral obesity represses hepatic CEACAM1 to cause fat accumulation in liver and insulin resistance (3, 26, 27). Further emphasizing the metabolic role of hepatic CEACAM1, liver-specific overexpression of CEACAM1 curbs the metabolic abnormalities caused by high-fat diet and prevents insulin resistance and hepatosteatosis (26). Similarly, adenoviral-mediated redelivery of CEACAM1 to the liver reverses diet-induced metabolic derangement (27). Collectively, this positions the loss of hepatic CEACAM1 expression (and its resulting hyperinsulinemia and insulin resistance) on the crossroad of the pathogenesis of NAFLD and obesity.

AUTHOR CONTRIBUTIONS

GH wrote a first draft of the manuscript. HG, SG, HM, KR, QA-S, TB, DZ contributed to the writing. RG and LY reviewed the manuscript. SN was responsible for revising the manuscript. SN had full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript.

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REVIEW
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Alternative RNA Splicing in the Pathogenesis of Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is becoming increasingly prevalent due to the worldwide obesity epidemic and currently affects one-third of adults or about one billion people worldwide. NAFLD is predicted to affect over 50% of the world's population by the end of the next decade. It is the most common form of liver disease and is associated with increased risk for progression to a more severe form non-alcoholic steatohepatitis, as well as insulin resistance, type 2 diabetes mellitus, cirrhosis, and eventually hepatocellular carcinoma. This review article will focus on the role of alternative splicing in normal liver physiology and dysregulation in liver disease.

Keywords: non-alcoholic fatty liver disease, RNA splicing, hepatocellular carcinoma, splicing factors, microarrays

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INTRODUCTION

Publication of the human genome sequence in 1995, and subsequently other mammalian genomes in the following two decades, has revealed a surprisingly small number of genes that must account for tremendous species diversity. Indeed, recent estimates have suggested that the number of human protein-coding genes may be as low as 19,000 (1). This is surprising given that the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes encode 17,000 and 21,733 genes, respectively (2, 3), and even the lowly amoeboflagellate *Naegleria gruberi*, a free-living unicellular eukaryotic organism, has 15,727 genes (4). These observations posed a diversity paradox for genetics and challenged the one gene-one protein hypothesis. Unlike prokaryotic and lower eukaryotic genes, most mammalian genes are composed of multiple coding exons with intervening non-coding introns of variable length. Very often these exons encode discreet protein modules or substructures. Transcription of these split genes produces a primary transcript that requires further processing to remove the intronic sequences, a process called RNA splicing. Much of our understanding of the mechanism of RNA splicing comes from elegant biochemical and genetic studies in yeast and has been extensively reviewed (5). The presence of exons and introns provides a solution to the diversity paradox by allowing assembly of different proteins by modular construction of RNA transcript isoforms through a process termed alternative splicing (6, 7). The diversity of RNA transcripts is further amplified by the use of alternative transcription start sites and polyadenylation sequences. Transcriptome sequencing has shown that upward of 90% of mammalian genes have multiple transcript isoforms, and an estimated 160,000 alternatively spliced transcripts are protein encoding (8, 9). Although 85% of these genes have a predominant RNA transcript isoform (10), the minor isoforms can have different functions and may play an important role in disease.

Alternative splicing and the generation of protein diversity have broad implications for clinical disease. It is estimated that 50–60% of 31,250 disease-causing mutations in the Human Gene Mutation Database affect splicing (11, 12). Approximately 16% of these mutations are located directly in splice sites (13), and 66% are SNPs, microdeletions, or insertions within exons. While some of these latter

mutations have a pathogenic effect by altering protein sequence, a large proportion do not, but rather interfere with splicing by interrupting exonic splicing enhancers or silencers. Cancer has been termed a disease of the genome due to the accumulation of DNA damage and genetic alterations that cumulatively cause transformation and malignancy. Indeed many mutations alter protein function by creating constitutively active oncogenic proteins or disrupting tumor suppressor proteins. However, it is now increasingly recognized that many cancer-associated RNA transcripts do not result from point mutations in the RNA itself, but rather by changes in expression or function of splicing factors that regulate the ordered splicing of primary gene transcripts giving rise to aberrant expression of oncofetal isoforms with greater proliferative capacity.

MECHANISM OF ALTERNATIVE RNA SPLICING

Much of our understanding of mechanism of RNA splicing comes from genetic studies in yeast and biochemical reconstitution experiments (5, 14). These studies have shown that the ends of an intron are aligned for excision by a complex network of RNA and protein interactions involving both splice sites in a large complex called the spliceosome. Initially the 5′ splice site is recognized by the U1 small nuclear ribonucleoprotein particle (snRNP) by base pairing of the U1 small nuclear RNA to the 5′ splice site. The U2 snRNP is then recruited to the 3′ splice site and branch point sequence by the accessory factor U2AF. The complex containing the pre-RNA, and the U1 and U2 snRNPs is called the pre-spliceosomal complex and defines the intron. This complex then recruits the U4/U5/U6 tri-snRNP, and the spliceosome undergoes a number of rearrangements including replacement of the U4:U6 duplex with a U2:U6 duplex, loss of the U4 snRNP, and displacement of the U1 snRNP on the 5′ splice site by the U6 snRNP to create the catalytically competent splicing complex. The actual splicing reaction then proceeds by two transesterification reactions first by the branch point adenine at the 5′ splice site then by the exonic terminal hydroxyl group at the 3′ splice site resulting in ligation of the two exons and liberation of an intron-lariat structure.

What defines whether an exon is recognized in a primary RNA transcript? While U1 and U2 snRNPs can interact across short introns to define the intron in typical *in vitro* splicing reactions, this interaction is much less efficient when the size of the intron increases above 250 nucleotides (15). As most introns are kilobases in length, yet the average size of an exon is ~200 nt, definition of the splice sites *in vivo* is generally thought to occur across exons rather than introns, a process termed exon definition (16). Thus, exons are defined by binding of U1 and U2 snRNP across the exon in the primary transcript followed by the long-range splice site pairing across the intron to assemble functional spliceosomes. Support for this exon-definition model comes from the finding that mutation of the downstream 5′ splice site on an exon can alter splicing of the upstream intron, so the sequential splicing of introns is coordinated and does not occur independent of each other.

How does this process allow for the use of different exons or splice sites during alternative splicing? In general, alternative exons contain weak splice sites that are not recognized efficiently (14). For genes that are co-transcriptionally spliced, this can be explained by a kinetic competition for assembly of the U1–U2 complex across alternative exons, or for non-co-transcriptionally spliced genes, this could be explained by the differences in the stability of the resulting complexes. Whether these weak exons are recognized is determined to a large extent by the presence of *cis*-acting binding sites for RNA-binding proteins within the exon or adjacent introns in the primary RNA transcript (17–19). Two of the most well-studied families of RNA-binding splicing regulators are the SR proteins (16 members) and the hnRNPs (20 members) (20–24), but there are also a number of less-studied families of RNA-binding proteins that regulate splicing, including the CELF/BRUNOL family, the Zinc-finger proteins, and the RBM family (25–27). All proteins contain RNA-binding domains allowing sequence specific-binding to RNA. Proteins of the SR family have an RNA recognition motif (RRM) at the amino-terminus, and a C-terminal domain that is enriched in arginine/serine dipeptides (RS domain) and heavily phosphorylated. hnRNP proteins show greater structural diversity than SR proteins, with RRM, RGG (arginine/glycine rich box), or KH (K homology box) RNA-binding domains. Additionally, the hnRNPs have auxiliary functional domains, which mediate protein–protein interactions and/or localization, and are divergent in protein sequence and structure (28). Both SR proteins and hnRNPs can promote or inhibit exon recognition depending on sequence context, thus modulating the usage of alternative exons or splice sites (14, 29, 30).

ALTERNATIVE SPLICING IN LIVER DEVELOPMENT

While liver-specific transcriptional regulation is well established and has been studied for decades, liver-specific alternative splicing is less well understood. Alternative splicing has traditionally been studied on a gene-by-gene basis, which required prior knowledge of the gene transcripts, but the development of high-throughput array and RNA sequencing (RNA-seq) technologies has allowed an unbiased assessment of alternative splicing events (**Table 1**) (31). In a recent study, Nellore et al. aligned 21,504 human RNA-seq samples from the Sequence Read Archive to the human genome and compared exon–exon junctions to the known gene annotation databases (32). Approximately 19% of splice junctions (56,861) that were found in at least 1,000 samples were not previously annotated, indicating that a great deal of transcript diversity is still to be discovered. Brain, liver, and testis show the greatest diversity in transcripts with ~35–40% of genes showing alternative exon or splice site usage (33).

Changes in alternative RNA splicing have been detected during the development of many tissues including the brain, heart, and skeletal muscle, and more recently in liver (31). Fetal liver does not perform a metabolic function as nutrients are provided from the mother *via* the placenta. Instead the fetal liver supports hematopoiesis in the embryo (34). Hepatocytes in the embryo are proliferative but they growth arrest and differentiate after birth as the liver takes on a metabolic function (35–37). Hematopoiesis also switches from the liver to the bone marrow during late

TABLE 1 | Studies reporting alterations of RNA splicing factor expression or alternative splicing in liver.

Study	Objective	Method	Reference
Ameur et al.	Nascent transcripts and co-transcriptional splicing in brain and liver	RNA sequencing (RNA-seq) on human and chimpanzee RNA from brain and liver	(31)
Nellore et al.	Alternative splicing across Sequence Read Archive	Re-aligned 21,504 RNA-seq samples from SRA	(32)
Yeo et al.	Alternative splicing across human tissues	Re-aligned cDNA and EST alignments	(33)
Bhate et al.	Alternative splicing during mouse liver development	RNA-seq on FVB/NJ mice at embryonic day 18, and postnatal days 14, 28, and 90	(39)
Peng et al.	Transcriptome and alternative splicing during liver development	RNA-seq on male C57BL/6 mice ($n = 3$) at e17, postnatal days 0, 1, 3, 5,10, 15, 20, 25, 30, 45, and 60	(40, 41)
Lake at al	Transcriptome of non-alcoholic fatty liver disease (NAFLD)	Microarrays on 10 steatotic, 9 non-alcoholic steatohepatitis (NASH) with fatty liver, 7 NASH w/o fatty liver, and 19 normal subjects	(76)
Moylan et al.	Transcriptome in NAFLD	Microarrays on 40 mild NAFLD and 32 severe NAFLD subjects	(77)
Pihlajamaki et al.	Comparison of liver transcriptomes in obese and lean humans and mice	Microarrays on 5 lean non-diabetics and 8 obese subjects undergoing bariatric surgery	(79)
Zhu et al.	Liver transcriptome and alcohol-metabolizing genes in NAFLD	Microarrays on 40 mild NAFLD, 32 severe NAFLD, 15 alcoholic hepatitis, and 7 normal subjects	(83)
Ye and Liu	NAFLD transcriptional networks	Microarrays on 10 steatotic, 16 NASH, and 19 normal subjects	(86)
Ahrens et al.	Liver transcriptome and methylome after bariatric surgery	Microarrays on 15 NASH, 12 NAFLD, 18 obese and 18 control subjects, and 23 post-bariatric surgery	(87)
Teufel et al.	Comparison of liver transcriptomes in mouse models of NAFLD with human NAFLD or NASH	Microarrays on C57BL/6 mice, and 25 obese, 27 NAFLD, 25 NASH, and 39 normal human subjects	(88)
Lin et al.	Transcriptome in hepatocellular carcinoma (HCC)	RNA-seq on 56 paired tumor and non-tumor tissue; HBV+, HCV+, and non-viral	(104)
Burchard et al.	Liver transcriptome in HCC	Microarrays on 96 HBV-related HCC patients (paired tumor + adjacent non-tumor)	(105)
Shiraishi et al.	Transcriptome alterations and somatic mutations in liver cancer	RNA-seq on 22 paired HBV-related HCC (tumor and non-tumor tissue)	(107)
Huang et al.	Transcriptome of HBV-related HCC	RNA-seq on 10 paired HBV-related HCC (tumor and non-tumor tissue)	(108)
Tremblay et al.	RNA splicing in HCC	Reanalysis of 377 HCC samples from TCGA; HBV+, HCV+, HBV/HCV+, and non-viral	(109)

gestation. The transcription factors regulating this transition in hepatocytes and cholangiocytes have been well documented (37, 38), but many genes also show a switch in fetal-to-adult RNA isoform expression reflecting changes in alternative splicing. Bhate et al. profiled the mouse liver transcriptomes at embryonic day 18 and postnatal days 14 and 28 and at 3 months by RNA-seq (39). In addition to 4,882 changes in gene expression between e18 and adult, the authors found 529 genes that underwent a change in RNA splicing and 214 genes that underwent a change in polyadenylation. The majority of these changes in alternative splicing were conserved between mouse liver and in human fetal (22 weeks) and adult (51 years) liver tissue.

A more extensive study by Peng et al. profiled mouse liver transcriptomes at embryonic day 18, and postnatal days 0, 1, 3, 5, 10, 15, 20, 25, 30, 45, and 60 of mouse liver development (40, 41). They found 7,289 genes that were differentially expressed at some point during development, and 829 of these had multiple annotated splicing variants with 90 being differentially expressed. In addition, they found evidence for 2,383 novel splice isoforms, of which 1,455 were detected at multiple times suggesting that there is a great deal of liver transcript information yet to be annotated. As might be expected, both studies indicated that genes associated with amino acid, fatty acid, cholesterol, bile, glucose, steroid, urea, and drug metabolism were upregulated in adult liver, whereas those associated with hematopoiesis, DNA repair and metabolism, cell cycle, and chromosome reorganization were downregulated. The changes in alternative splicing were not the result of altered cell populations in the liver as the majority (88%) were still observed in purified hepatocytes (39). A number of splicing factors decreased in expression, including *Celf1, Celf2, Mbnl1*, *Ptbp1*, *Srsf1, 2, 3, 4, 6, 7, and 10, Hnrnpa1,* and *Hnrnph*, but *Esrp2* was increased in both studies.

A number of these genes have been studied *in vitro*, knocked out in the whole animal or deleted in hepatocytes (**Table 2**). Surprisingly, the *Mbnl* and *Celf* family proteins were identified in fetal liver. These proteins are expressed highly in muscle and have been studied extensively for their involvement in myotonic dystrophy (MD) (42–44). The whole-body knockout of *Mbnl1* causes muscle and eye abnormalities reminiscent of MD (45). Subsequently, two studies reported that loss of *Mbnl2* had no muscle phenotype but a third reported myotonia (46–48). Interestingly, one *Mbnl2* knockout mouse had a brain phenotype

TABLE 2 | Genetic manipulation of RNA-binding proteins *in vivo*.

Gene	Class	Model	Phenotype	Reference
Celf1	CELF/BRUNOL family	Homozygous knockout Transgenic overexpression	No liver phenotype/growth retardation? No assessment of splicing Hepatocyte proliferation in young livers. Myotonia and dystrophic muscle histology. Altered splicing	(50) (51, 55)
Esrp2	RBM family	Homozygous knockout	Increased proliferation, diploid and tetraploid hepatocytes, smaller hepatocytes, no metabolic changes, or liver damage. Altered splicing	(39)
Hnrnpa1	HNRNP family	Homozygous knockout	Perinatal lethality. Muscle developmental defects. Impaired cardiac function. Altered splicing	(58)
Mbnl1	Zn-finger protein	Homozygous knockout	No liver phenotype, muscle and eye abnormalities characteristic of myotonic dystrophy. Altered splicing	(45)
Mbnl2	Zn-finger protein	Homozygous knockout	No liver phenotype, defects in spatial memory, abnormal REM sleep. Altered splicing	(46–48)
Ptbp1	HNRNP family	Homozygous knockout	Embryonic lethal. No assessment of splicing	(60, 61)
Slu7	Zn-finger protein	AAV-shRNA knockdown in liver	Reduced gluconeogenesis, insulin resistance, enhanced glucose uptake and glycolysis, hepatocyte proliferation, dyslipidemia. Altered splicing	(111)
Srsf1	SR protein family	Homozygous knockout Hepatocyte knockout Cardiomyocyte knockout	Embryonic lethal postimplantation. No assessment of splicing No liver phenotype. No assessment of splicing Excitation coupling defects. Hypertrophic cardiomyopathy. Death due to heart failure	(66) (57) (66)
Srsf2	SR protein family	Homozygous knockout Hepatocyte knockout Cardiomyocyte knockout	Embryonic lethal postimplantation. No assessment of splicing Apoptosis, liver damage, liver failure. Altered splicing Dilated cardiomyopathy. Stress-induced death. No assessment of splicing	(56) (57) (56)
Srsf3	SR protein family	Homozygous knockout Hepatocyte knockout	Embryonic lethal at blastocyst stage. No assessment of splicing Metabolic dysfunction, steatosis, fibrosis, apoptosis and proliferation, liver damage, altered ploidy, hepatocellular carcinoma. Altered splicing	(67) (68, 110)
Srsf10	SR protein family	Homozygous knockout Heterozygous knockout	Late embryonic lethal with cardiac hypertrophy and liver degeneration. Altered splicing Increased VLDL secretion and plasma triglycerides. Altered splicing	(80) (79)

with impaired hippocampal plasticity and synaptic transmission consistent with high-level expression of *Mbnl2* in the brain (48). Loss of neither family member is associated with liver defects, however, but MBNL1 has been reported to regulate hematopoiesis in the fetal liver (49) potentially by regulating splicing of the *Ndel1* gene. In contrast, CUGBP1 (*Celf1*) is highly expressed in the liver but its effects appear unrelated to its role as a splicing factor, but rather are due to its role as a translational regulator as it forms a complex with eIF2 to support translation of proteins involved in liver function and regulates hepatic stellate cell activation (50–55).

Mice with complete deletion of Srsf2 die just after embryo implantation but mice with hepatocyte-specific deletion of *Srsf2* are viable and have normal size at birth (56, 57). The mice fail to thrive, however, and die by 2–3 weeks of age. The cause of death is liver failure. In contrast, hepatocyte-specific deletion of *Srsf1* did not have a phenotype and the mice were healthy. Livers in the newborn *Srsf2* KO mice appear normal in size and color but by day 11 the livers are pale and firmer. Histologically, the livers show hepatocyte ballooning with periportal fibrosis and inflammation. The liver failure is likely due to the lack of proliferation of hepatocytes in the neonatal liver, and increased apoptosis possibly due to endoplasmic reticulum and oxidative stress. Metabolically, the knockout livers show steatosis and lack glycogen. RNA-seq analysis indicated that the mice livers have altered cholesterol and bile homeostasis as SRSF2 stimulates expression of liver transcription factors *Srebp1c, Cebpa, Ppara, Nr1i3* (CAR1), *Nr1h4* (FXR), *Mlxipl* (CHREBBP), and *Foxa2*. Thus, SRSF2 has effects on liver RNA splicing that are not compensated by other SR proteins, unlike the role of SRSF1 that appears redundant.

Homozygous deletion of *Hnrnpa1* causes perinatal lethality within 30 min of birth because of muscle developmental defects. Death was due to cardiac dysfunction with higher blood pressure and heart rate, but defects were also observed in smooth and skeletal muscle (58). No liver phenotype was reported. The polypyrimidine tract binding protein PTBP1 (HNRNPI) binds to intronic sequences upstream of the 3′ splice site and represses splicing of pre-mRNAs (59). The effect of PTBP1 on liver function *in vivo* has not been studied as the homozygous deletion of *Ptbp1* is embryonic lethal at the implantation stage (60, 61). In HepG2 hepatoma cells, however, PTBP1 modulates splicing of multiple genes involved in cholesterol synthesis and uptake including *LDLR, MVK, HMGCS1,* and *PSCK9*. It also regulates splicing of the fatty acid desaturase genes 2 and 3 (*FADS2* and *3*) that are involved in fatty acid elongation and unsaturation (62, 63). Consequently, omega-3 and omega-6 poly-unsaturated fatty acids were reduced following *Ptbp1* knockdown, but saturated and mono-unsaturated fatty acids were not altered. Interestingly, PTBP1 is upregulated during hepatitis B virus infection and reduces expression of the proapoptotic form of FAS, which may contribute to the survival of infected hepatocytes (64).

Changes in alternative splicing during the mesenchymal to epithelial differentiation have been attributed to the splicing factors ESRP1 and ESRP2 (65). Expression of *Esrp2* was increased in the adult liver and ablation of *Esrp2* led to impaired adult splicing patterns implicating this splicing factor in the fetal-to-adult

transition in hepatocytes (39). The livers did not show changes in morphology, however, nor did they display signs of liver damage, or any alterations in lipid, cholesterol, or glucose metabolism.

Other splicing factors have also been implicated in hepatocyte differentiation. Mice with complete loss of *Srsf1* or *Srsf3* die during early embryogenesis, but mice carrying a hepatocyte-specific deletion of *Srsf1* or *Srsf3* are viable (66, 67). The hepatocyte-specific deletion of *Srsf1* did not show an overt liver phenotype but loss of *Srsf3* caused impaired hepatocyte maturation (57, 68). The impaired differentiation was consistent with mis-splicing of *Hnf1a* that is critical for liver development, leading to reductions in other liver-enriched transcription factors including HNF6α (*Onecut1*), HNF3α (*Foxa3*), and C/EBPα. Consequently, the livers continued to express fetal markers such as α-fetoprotein (*Afp*) and *H19.* The impaired differentiation was associated with disrupted hepatic architecture characterized by large irregular hepatocytes, with compressed sinusoidal spaces and bile canaliculi, and reduced binuclearity. Interestingly, expression of *Esrp2* is significantly reduced in the *Srsf3* knockout, which may partly explain the impaired differentiation phenotype. The entire phenotype cannot be explained by loss of *Esrp2,* however, as *Esrp2* ablated mice do not show changes in liver morphology or histology. Loss of *Srsf3* also causes alterations in glucose and lipid homeostasis characterized by reduced glycogen storage, fasting hypoglycemia, increased insulin sensitivity, and reduced cholesterol synthesis although the target genes are distinct from those altered in the *Srsf2* knockout. Like the *Srsf2* knockout, loss of *Srsf3* causes endoplasmic reticulum stress, hepatocyte apoptosis and proliferation, and liver damage but did not cause the liver failure seen in the *Srsf2* knockout.

ALTERNATIVE SPLICING AND FATTY LIVER

Overnutrition and obesity leads to non-alcoholic fatty liver disease (NAFLD) and its more severe form non-alcoholic steatohepatitis (NASH) (69, 70). These metabolic disturbances are becoming more common in the general population due to the current obesity epidemic (71–73). Both NAFLD and NASH are associated with the metabolic syndrome and insulin resistance, and are risk factors for type 2 diabetes, non-alcoholic liver cirrhosis, and for the development of hepatocellular carcinoma (HCC) (74, 75). So understanding the changes that occur in the fatty or NASH liver is important to elucidate mechanisms underlying the heightened risk for subsequent disease progression. Transcriptome profiling by microarray has been performed in humans with NAFLD (76, 77). While this allows gene expression changes to be monitored, most studies do not address changes in RNA alternative splicing (78). Toward the goal of understanding changes in splicing, Pihlajamaki et al. profiled gene expression in liver samples from insulin-resistant humans with obesity (79). The top-ranked pathway downregulated in obese liver samples related to RNA processing and splicing. A number of splicing factors were decreased including *SRSF10, SRSF7, SF3A1, SRSF2, SFPQ,* and *HNRNPs A1, K, D,* and *H*. The authors showed that knockdown of *SRSF10* increased lipogenesis *in vitro* in HepG2 cells and that heterozygous loss of *Srsf10* in mice increased plasma triglycerides due to increased secretion of VLDL and mis-splicing of the lipid storage protein LIPIN-1 (*Lpin1*). Homozygous deletion of *Srsf10*, however, causes embryonic lethality with liver degeneration (80). This was the first example of how a change in RNA splicing could cause a change in lipid metabolism in the obese liver. SRSF10 may also regulate the splicing of the scavenger receptor class B, member 1 gene (SCARB1) that encodes the SR-BI and SR-BII proteins that mediate reverse cholesterol transport (81). The loss of SRSF10 in obesity remains controversial, however, as it was not seen in another study (82).

Another large microarray study examined liver gene expression in 72 subjects with mild or advanced NAFLD, 10 normal liver, and 17 subjects with HBV-associated liver failure (77, 83, 84). Ninety-two splicing factor genes were altered in this dataset with 30 splicing factors being altered in either mild or advanced NAFLD. Many of these were also identified in the Pihlajamaki study. Another study has shown a decrease in SRSF4 in NASH (85). A systems biology weighted gene co-expression network analysis of 16 human NASH, 10 NAFLD, and 19 normal liver samples identified a highly significant module ($p < 2 \times 10^{-6}$) associated with RNA processing (86). These changes are not in all datasets, however, as a German study in 45 morbidly obese subjects with NAFLD or NASH did not show alterations in splicing factor expression (87, 88). Studies in mice have shown similar changes in the expression of splicing factors in diet-induced obesity and NASH models (79, 85, 88, 89). So NAFLD and NASH are associated with changes in RNA splicing factor expression in the liver, and this likely contributes to alterations in RNA splicing. Transcriptome profiling by RNA-seq could potentially provide a measure of RNA splicing although such an approach has not been published. It will be interesting to see whether these alterations in RNA splicing can contribute to the pathophysiology.

ALTERNATIVE SPLICING AND HCC

Worldwide, more than 700,000 people are diagnosed and 600,000 people die each year of liver cancer. HCC is the most common primary liver cancer (70–85%) (90) and usually arises after years of liver disease and inflammation (91) either due to chronic hepatitis B or C virus (HBV/HCV) infection (92), or alcoholic and non-alcoholic cirrhosis. The relative importance of these HCC subtypes depends on geography. HCC in HBV/HCV endemic regions in Asia and Africa is 80–90% virus associated, compared to only 20–50% of HCC in the US (93–95). Approximately 15–25% of HBV-infected individuals will develop chronic liver disease including cirrhosis, liver cancer, or failure, and 5–20% of HCV-infected individuals develop cirrhosis. A large majority (80%) of patients with HCC have cirrhosis, so cirrhosis is a major risk factor, but only 8% of patients with cirrhosis will develop HCC (96). In addition to chronic alcoholism, cirrhosis can have viral or metabolic causes (97–99), and alcohol use by at-risk individuals substantially increases the risk of cirrhosis and HCC. From a metabolic standpoint, obesity, NAFLD, and NASH are all risk factors for cirrhosis (75).

Alterations in RNA splicing in cancer have been known for over 30 years (11, 100, 101). Profiling the molecular alterations that occur in HCC has uncovered a number of targets with

altered RNA splicing including the *DNMT3b, AURKB, MDM2, TENSIN2, MAD1, KLF6, SVH, TP73, TP53,* and *FN1* genes (102). Many of these changes have been shown to have functional effects to promote proliferation, prevent apoptosis, and support transformation in cell culture experiments. RNA splicing is also important for HBV and HCV viral expression, and many viruses hijack the cellular splicing machinery to allow splicing of viral RNAs (103). More recent studies have utilized high-throughput technologies to survey the HCC transcriptome (104–107). A 2011 study sequenced the transcriptomes of 10 matched pairs of cancer and non-cancerous liver tissue from HBV-infected individuals (108). A total of 1,378 differentially expressed genes were identified in HCC, but more surprisingly 24,338 exons were differentially expressed, and the vast majority of differentially expressed genes also contained differentially expressed exons. A recent study utilized the RNA-seq data available through the TCGA database. Sequence data from 377 liver samples were reanalyzed to assess alterations in RNA splicing, uncovering ~45,000 alternative splicing events (109). These events were further filtered allowing the identification of 3,250 transcripts from 2,051 genes whose expression was altered in HBV-associated HCC, 1,380 transcripts from 907 genes that were altered in HCV-associated HCC, and 1,517 genes altered in non-viral HCC. Of these transcripts, 1,336 were shared by at least two groups. The authors also assessed splicing factor expression in these samples and found altered expression of 26 splicing factors, including ESRP2, SRSF2, CELF2, MBNL1, HNRNPA1, and HNRNPH, that were found altered in hepatocyte maturation study by Bhate et al. (39), that is consistent with oncofetal transformation. These studies are likely underestimates of the true dysregulated RNA splicing as most approaches rely on databases of known annotated RNA isoforms, so will exclude reads that do not correspond to known splicing events.

CONCLUSION AND FUTURE PERSPECTIVES

Although gross alterations in gene expression have been documented in nearly every disease state, recent data indicate that more subtle qualitative changes also occur, which may be just as important in disease pathogenesis. Recent high-throughput technologies are allowing a reassessment of these transcriptional changes with much higher resolution, providing a comprehensive documentation of individual transcript isoform identity and relative expression. These isoforms ultimately encode different proteins that could influence cellular function. Do these changes play a causal role in disease pathogenesis or are they simply a side effect of the disease? Traditionally, cancer was considered a disease of the genome and many of the changes in RNA splicing were thought to be a result of global alterations in gene expression in the cancer genome. Recent data, however, are indicating that subtle alterations in RNA splicing are observed in early disease, long before genomic alterations have occurred, and these alterations may play a role in predisposition to later disease. Data from mouse studies have suggested that altered splicing may cause cancer. Overexpression of the SR proteins SRSF1, SRSF3, and TRA2β (SRSF10) transforms fibroblasts and accelerates tumor growth in nude mice, and the proteins have been found to be elevated in certain cancers suggesting that they are proto-oncogenes. Interestingly, SRSF3 loss in hepatocytes also leads to liver cancer in mice (110), and SRSF3 is reduced in human HCC (111), so the properties of individual splicing factors may depend on cellular context. Aside from the SR proteins, other RNA-binding proteins that have been implicated as hnRNP proteins hnRNPA1, hnRNPA2, hnRNPH, and hnRNPI (PTB) are overexpressed in certain cancers (24, 100, 112–114), and knockdown of the proteins causes apoptosis *in vitro*. Overexpression of the zinc-finger protein MBNL2 in HCC correlates with smaller lower grade tumors and inhibits tumor growth and invasion in mice (115). Somatic mutations in splicing factor genes have also been found in cancers, the most frequently mutated being *SF3B1, U2AF1, SRSF2,* and *ZRSR2* (116). Thus, dysregulation of RNA splicing may precede and predispose to carcinogenesis, and changes in splicing may be an early event in cancer initiation and warrant further investigation. Further studies testing individual transcript changes will be required to complete our understanding of the subtleties of gene expression that underlie early disease pathogenesis.

AUTHOR CONTRIBUTIONS

NW conceived of and wrote the review.

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Distinct Hepatic Macrophage Populations in Lean and Obese Mice

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Obesity is a complex metabolic disorder associated with the development of non-communicable diseases such as cirrhosis, non-alcoholic fatty liver disease, and type 2 diabetes. In humans and rodents, obesity promotes hepatic steatosis and inflammation, which leads to increased production of pro-inflammatory cytokines and acute-phase proteins. Liver macrophages (resident as well as recruited) play a significant role in hepatic inflammation and insulin resistance (IR). Interestingly, depletion of hepatic macrophages protects against the development of high-fat-induced steatosis, inflammation, and IR. Kupffer cells (KCs), liver-resident macrophages, are the first-line defense against invading pathogens, clear toxic or immunogenic molecules, and help to maintain the liver in a tolerogenic immune environment. During high fat diet feeding and steatosis, there is an increased number of recruited hepatic macrophages (RHMs) in the liver and activation of KCs to a more inflammatory or M1 state. In this review, we will focus on the role of liver macrophages (KCs and RHMs) during obesity.

Keywords: obesity, insulin resistance, inflammation, hepatocytes, Kupffer cells, immunometabolism

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INTRODUCTION

The rising prevalence of obesity represents a major global health challenge, not least because it is considered a significant risk factor for a wide array of non-communicable diseases. Prominent among these are diseases of the liver, ranging from steatosis through to cirrhosis, collectively termed non-alcoholic fatty liver disease (NAFLD) (1). However, the etiology linking obesity with liver pathology is incompletely understood, hindering attempts to treat these conditions.

A landmark discovery offering therapeutic potential for the metabolic syndrome was the finding that the adipose tissue of obese mice and humans displays hallmarks of an inflammatory state, including increased concentrations of tumor necrosis factor alpha (TNF-α) and increased monocyte/macrophage infiltration (2–4). Indeed, TNF-α is sufficient to induce features of the metabolic syndrome, such as insulin resistance (IR), and many chemical and genetic depletion studies have demonstrated the importance of inflammation and inflammatory macrophages in this process [recently reviewed in Ref. (5)]. Macrophage accumulation also occurs in other key metabolic tissues including muscle (6–9), liver (10–12), and pancreas (13, 14), which contribute to the dysregulation of glucose homeostasis. In this review, we focus on the composition and behavior of hepatic macrophage populations in obese mice and highlight recent advances that could aid in the targeting of this axis to treat aspects of the metabolic syndrome.

THE LIVER AT THE INTERFACE BETWEEN METABOLISM AND IMMUNITY

The liver is a key metabolic organ, which regulates a variety of processes vital for maintaining metabolic homeostasis. These include control of glucose production and lipid metabolism, dysregulation of which are symptomatic of the metabolic syndrome. The liver also plays key roles as part of the immune system secreting acute-phase proteins, complement components, cytokines, chemokines, and being positioned, along with the gastrointestinal tract, at the major interface between ourselves and our external, even microbial environment (15, 16). This unique position where metabolism and immunity are intertwined is reflected in the liver architecture, whereby immune cells are intimately connected to hepatocytes and liver sinusoidal endothelial cells (LSECs) (17, 18), as well as the cross-regulation whereby metabolic stress can result in hepatic immune activation leading to metabolic dysregulation (19, 20).

The liver maximizes nutrient absorption as blood flows through a system of sinusoidal vessels and fenestrations through beds of hepatocytes (17). The majority of blood within the sinusoid derives from the intestines *via* the hepatic portal vein and is rich in both nutrients, and also potentially immunogenic microbial molecules, or in cases of opportunistic infection microbes themselves (17). Therefore, in addition to facilitating nutrient absorption, sinusoids must also enable the removal of immunogenic material and allow the immune system to combat of infection. Kupffer cells (KCs) are located in the hepatic sinusoids and play a key role in this process (18). They bind a range of microbes or microbial ligands *via* microbe-associated molecular patterns (MAMPs), and by phagocytosis prevent them penetrating into the general circulation (18). Lipopolysaccharide (LPS), for example, is readily detectable in portal blood, but only rarely detectable in systemic circulation (21). Compared with macrophages from other locations, KCs are predisposed to respond to activation signals in a less inflammatory fashion and are especially characterized by producing high concentrations of the anti-inflammatory cytokine, interleukin 10 (IL-10) (22). Furthermore, KCs, along with other antigen-presenting cells in the liver, express low levels of co-stimulatory molecules required to initiate an adaptive immune response and high levels of molecules that suppress T cell activation, such as programed death-ligand 1 (PDL-1) (17). Thus, during homeostasis KCs in collaboration with other hepatic immune cell populations clear microbial material while maintaining the inflammatory tone of the liver at a level sufficient for essential functions such as pathogen killing, tissue remodeling, and sinusoidal permeability, but below that which would result in overt inflammation and tissue damage (5, 18, 23). The factors maintaining KCs in this tolerogenic state are not completely clear but are critically important when we consider how these cells and the hepatic macrophage pool in general are altered during obesity.

The phenotype of tissue macrophages is thought to be dependent on their respective ontogeny, as well as their respective polarization state in the tissue environment (24). Polarization was most clearly described by *in vitro* studies, which used cytokines to induce different extremes of macrophage phenotype classified as M1 or classically activated macrophages, considered more pro-inflammatory, and M2 or alternatively activated macrophages that have an anti-inflammatory tone (25). M1 macrophage differentiation can be induced by interferon gamma (IFN-γ), alone or with microbial products such as LPS or inflammatory cytokine TNF-α. In contrast, interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), interleukin 33 (IL-33), transforming growth factor beta (TGF-β), and granulocyte colony-stimulating factor (G-CSF) activate macrophages to differentiate to M2. However, given the range of factors now known to influence macrophage polarization, including cellular metabolic state (26), it is likely that a spectrum of macrophage phenotypes occur *in vivo* even within the same tissue macrophage pool (25). In lean mice, KCs have an M2-like phenotype maintained by the type 2 cytokine, IL-4, and the nuclear hormone receptor peroxisome proliferator activator receptor delta (PPAR-δ) (27, 28). Thus, KCs are specialized by virtue of their derivation from the yolk sac early in development (24, 29), and by factors in the liver environment, which maintain them in a less inflammatory, M2-like state (27, 28).

PARENCHYMAL AND NON-PARENCHYMAL CELLS IN LIVER

Hepatocytes are the major parenchymal cells, while the non-parenchymal cells integrate five cell populations including resident macrophages or KCs (30), recruited hepatic macrophages (RHMs), resident innate lymphocytes or natural killer cells (NKs) (31, 32), fat storing cells termed Ito or stellate cells (HSCs) (33), and LSECs (34) (**Figure 1**).

These non-parenchymal cell populations can be identified by a variety of cell surface markers. In general, KCs and RHMs both express epidermal growth factor-like module-containing mucin-like hormone receptor-like 1 (F4/80) (35), NKs form two pools distinguished by mutually exclusive expression of CD49a or DX5 (36), HSCs express glial fibrillary acidic protein (GFAP) (37, 38), and LSECs express CD34 (39). In addition, these liver cell populations can also be distinguished by their physical location within the liver and specific ultrastructural characteristics. For example, hepatocytes contain many microvilli, which project into space of Disse (perisinusoidal space) between the endothelial cells and hepatocytes. KCs (~15% of all liver cells) represent the largest population of tissue macrophages (80–90% of resident macrophages in the whole body) (40). KCs are found attached to the luminal surface or inserted in the endothelial lining of hepatic sinusoids (41, 42), which make them the first macrophages to come into contact with gut-derived foreign and potentially noxious material. The size and function of KCs also depend on their specific location in the liver (43) with KCs in periportal regions being larger and more phagocytic with higher lysosomal enzyme activity than KCs in midzonal and perivenous locations (44). Unlike hepatocytes, KCs are amoeboid in shape. Fenestrae form open connections between the lumen of the sinusoid and the space of Disse (45). The transport and exchange of fluid, solutes, and particles between the sinusoidal lumen and the space of Disse containing the parenchymal cell surface are believed to occur through these open fenestrae (46). While KCs utilize phagocytosis

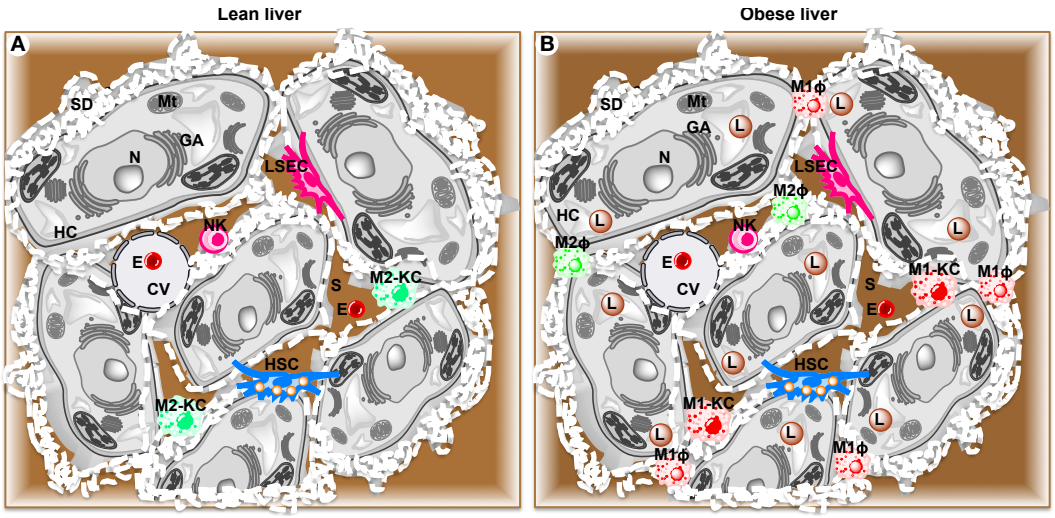

FIGURE 1 | **Schematic diagram showing parenchymal and non-parenchymal cells in liver**. **(A)** Lean liver showing parenchymal hepatocytes (HC) and non-parenchymal anti-inflammatory Kupffer cells (M2-KC), natural killer cells (NK), hepatic stellate cells (HSC), and liver sinusoidal endothelial cells (LSEC). **(B)** High fat diet-induced obese liver showing activated pro-inflammatory Kupffer cells (M1-KC), recruited hepatic macrophages (RHM), and lipid droplets (L). CV, central vein; E, erythrocyte; GA, Golgi apparatus; Mt, mitochondria; N, nucleus; S, sinusoid.

to incorporate large particles such as erythrocytes and bacteria, they take up small particles and molecules *via* pinocytic vesicles (47–50). NKs reside in sinusoids and eliminate virus-infected or transformed cells and regulate adaptive immune responses *via* contact-dependent signals and the secretion of cytokines (36, 51–53). HSCs are perisinusoidal cells, which contain characteristic lipid droplets. HSCs maintain vitamin A homeostasis as they store 80% of total vitamin A in the body. Inflammatory signals transform HSCs into myofibroblasts, resulting in collagen production and development of liver fibrosis (54, 55). LSECs possess a high-rate, high-capacity system to remove colloids and water-soluble waster macromolecules from the circulation (34, 56). At the ultrastructural level, LSECs constitute the only mammalian endothelial cells that combine non-diaphragmed fenestrae with a discontinuous basement membrane, which allows blood plasma to enter the space of Disse.

LIVER MACROPHAGE POPULATIONS DURING OBESITY

During the course of obesity, the adipose tissue's ability to store excess energy is compromised, leading to ectopic lipid accumulation in non-adipose tissues such as muscle and liver (57). Intracellular lipid accumulation in ectopic tissues is associated with a phenomenon known as lipotoxicity, which induces cell death, cytokine secretion, and activation of inflammatory processes, especially in the liver (58, 59). Furthermore, dietary stress and obesity can lead to excessive activation of the hepatic immune system due to increased penetration of microbial material (60–62). The response of the liver to damage and inflammation is a complex process involving parenchymal (hepatocytes) and non-parenchymal cells (KCs, NKs, HSCs, and LSECs), as well as monocyte-derived hepatic macrophages, RHMs (12, 63). The failure to regulate this inflammation during the progression of the obesity causes pathological chronic hepatic inflammation characterized by the advance of fatty liver to steatohepatitis, fibrosis, cirrhosis, and eventually liver failure (18, 64). Depletion of phagocytic cells in the liver (including both KCs and RHMs) through the administration of either liposome-encapsulated clodronate or gadolinium chloride protects against high-fat- or high-sucrose-induced steatosis, inflammation, and IR, demonstrating critical role of hepatic macrophages in the development of metabolic dysfunction (65).

MACROPHAGE REGULATION DURING NAFLD/NASH

Hepatic lipid accumulation and peroxidation leads to chronic hepatocyte endoplasmic reticulum (ER) stress, the production of reactive oxygen species (ROS), and toll-like receptor (TLR) activation, which converts KCs into an M1 phenotype defined by production of pro-inflammatory cytokines, oncostatin, and prostaglandins (**Figure 2**) (20, 66, 67). Circulating cytokines, adipokines, and free fatty acids (FFAs) released from inflamed adipose tissue in the obese state or immunogenic material derived from an altered intestinal microbiota can also contribute to KC polarization. M1-KCs secrete chemokine (C-C motif) ligand 2 (CCL2), pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), macrophage inflammatory protein (MIP)-1a, MIP1b, RANTES, oncostatin, and prostaglandins (PGE_2), which contribute to the alteration of the liver homeostasis and worsen the hepatic inflammatory response (29). PGE_2 regulates cytokine production (IL-1, IL-6, TNF-α, and TGF-β) (68, 69), acts synergistically with IL-6 to induce IR (70), and induces production of oncostatin M (OSM) in KCs (71). Increased OSM contributes to hepatic IR and the development of non-alcoholic steato hepatitis (NASH)

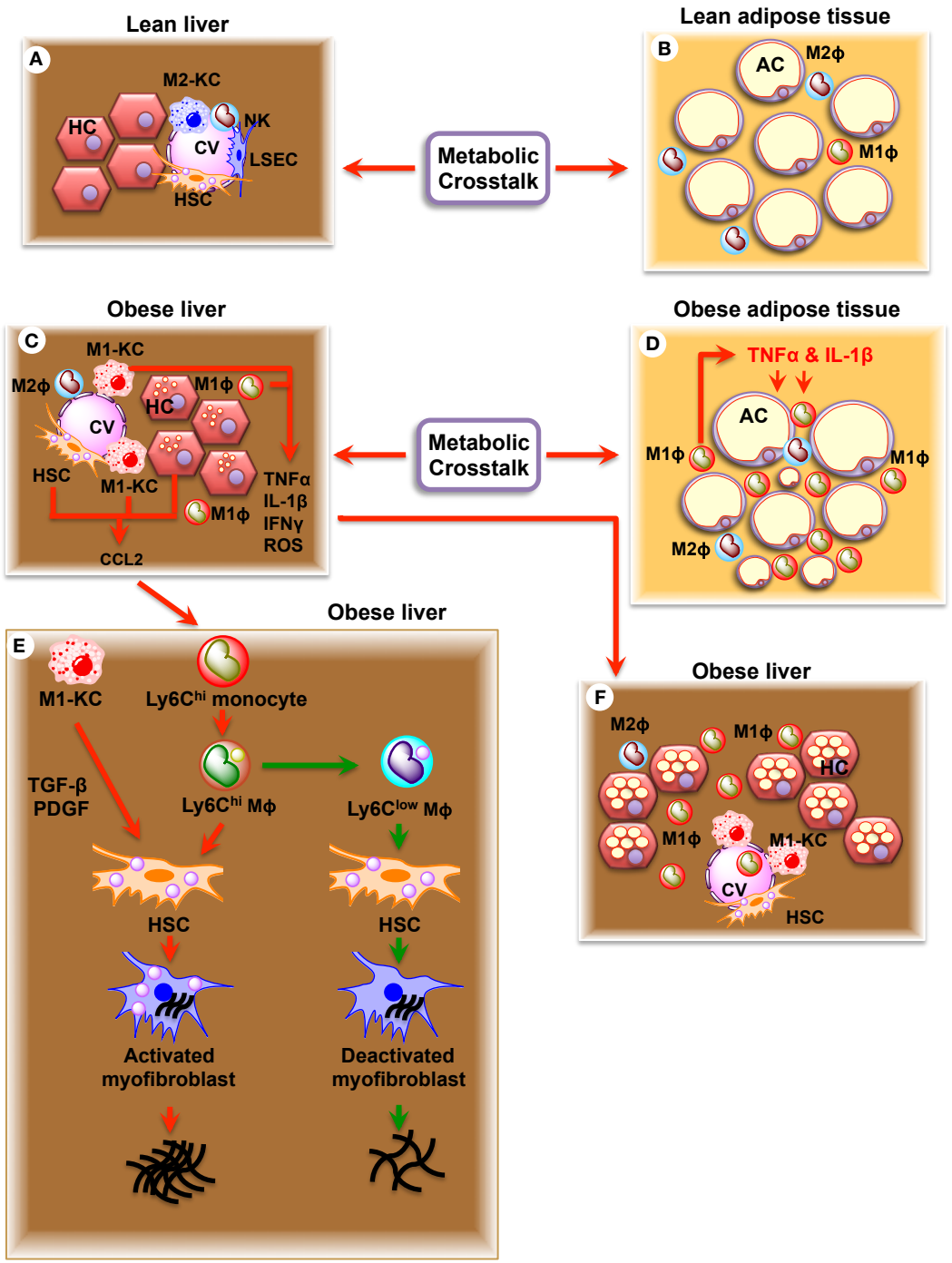

FIGURE 2 | **Schematic diagram showing the effects of resident (KC) and recruited hepatic macrophages (Ly6C^{high}) in regulation of non-alcoholic fatty liver disease (NAFLD) and fibrosis**. **(A)** Healthy liver showing parenchymal hepatocytes (HC) and non-parenchymal Kupffer cells (M2-KC), natural killer cells (NK), hepatic stellate cells (HSC), and liver sinusoidal endothelial cells (LSEC). **(B)** Healthy adipose tissue showing adipocytes (AC), adipocyte macrophage 1 (ATM1), and ATM2 macrophages. **(C)** Obese liver showing accumulation of lipid droplets in hepatocytes (HC), activated Kupffer cells (M1-KC), and activated hepatic stellate cells (HSC). Note increased production of TNF-α, IL-1β, IFNγ, ROS, and CCL2. **(D)** Obese adipose tissue showing larger adipocytes (AC), infiltrated ATM1 macrophages, and increased production of TNF-α and IL-1β. **(E)** Obese liver showing NAFLD and NASH. **(F)** Obese liver showing fibrosis. Increased production of CCL2 recruits Ly6C^{high} monocytes, which convert to Ly6C^{high} macrophages inside the liver. Ly6C^{high} macrophages produce TGFβ, connective tissue growth factor (CTGF), and PDGF, which act on HSC and transform HSC to activated myofibroblast. Activated myofibroblast in turn results in fibrosis. Ly6C^{high} macrophage is transformed into Ly6C^{low} macrophage upon eating dead hepatocytes and erythrocytes. Ly6C^{low} macrophage deactivates activated myofibroblasts and decrease fibrosis.

(71). High levels of TNF-α released by M1-KCs stimulate hepatic expression of CCL2 (also known as MCP1), a powerful monocyte chemoattractant, which recruits CCR2^{+}Ly6C^{high} monocytes from the vasculature into the liver (72), where they differentiate into Ly6C^{high} macrophages. The Ly6C^{high} macrophages amplify the severity of obesity-induced inflammation and hepatic IR through the secretion of TNF-α and interleukin 6 (IL-6) (12). C-C chemokine receptor type 2 (CCR2)-deficient mice are protected against weight gain and display reduced development of obesity, illustrating the importance of this chemokine system (73). Once established, this vicious circle of immune cell attraction, infiltration and activation, hepatocyte injury, and further inflammation promotes and defines the pathophysiology of NASH (74).

MACROPHAGE REGULATION OF HEPATIC FIBROSIS

Fibrosis is increasingly appreciated as a major contributor to metabolic dysregulation in obese humans and type 2 diabetic patients (75). Both KCs and recruited Ly6C^{high} macrophages contribute to the development of hepatic fibrosis. KCs activate HSCs through increased production of pro-fibrotic cytokine TGF-β and platelet-derived growth factor (PDGF) (76) leading to fibrosis. Ly6C^{high} macrophages also interact with HSCs to promote fibrosis through increased production of TGF-β, connective tissue growth factor (CTGF), and PDGF (77). Therefore, inhibition of monocyte recruitment through depletion of the pro-inflammatory signal CCL2 results in attenuation of liver fibrosis (77–79). In addition, pharmacological inhibition of CCL2 by the RNA-aptamer mNOX-E36 attenuates liver fibrosis, thereby strengthening a pro-fibrotic function of Ly6C^{high} macrophages (80, 81).

MACROPHAGE SURFACE MARKERS

Due to the distinct functions of RHMs and KCs in suppressing or perpetuating the immune activation (29, 82), it is important to be able to clearly isolate pure populations of each cell type. However, distinguishing RHM from KC has proven difficult mainly due to technical difficulties in isolating and identifying macrophages from the obese liver. KCs (CXCR1^{-}) appear histologically as larger cells with multiple phagocytic granules and have been defined by surface marker expression as CD45^{+}/CD11c^{-}/F4/80^{high}/CD11b^{low} (12, 83). RHMs (CXCR1^{+}) are smaller than KC, contain fine granules in the cytoplasm, and have been defined by surface marker expression as F4/80^{dim}/CD45^{+}/CD11b^{+}/CCR2^{+} (10), CD11b^{+}/Ly6C^{high}/Ly6G^{-} (83), or CD45^{+}/CD11c^{-}/F4/80^{low}/CD11b^{high} markers (83) depending on the publication. However, these factors alone do not sufficiently identify pure KC or RHM populations as there is significant size and surface marker overlap with other cell populations, including dendritic cells (DCs), eosinophils, and undifferentiated monocytes (84). KCs, unlike RHMs, have the unique ability to survive to lethal irradiation (85), which has enabled studies into these distinct cell types. The result of these investigations suggests that the number of KCs remains unchanged during the course of obesity, whereas accumulation of RHMs increases several-fold (12). Transcriptome analysis of these RHM and KC populations isolated from lean and diet-induced obese (DIO) mice revealed statistically marked differences between the two cell types on both diets. Furthermore, the Gene Ontology analysis of these transcriptomes showed a restricted list of 16 KC marker genes and 11 RHM markers genes differentially expressed from lean to DIO mice that could provide the opportunity for direct isolation strategies using specific surface markers (12). Interestingly, factors secreted in the culture media from isolated high fat diet (HFD)-RHMs, but not from isolated HFD-KCs, can promote hepatic glucose output and attenuate insulin's normal inhibitory effects on this aspect of hepatic metabolism suggesting that RHMs are the dominant immune cell type inducing hepatic IR (12, 82).

HEPATIC GENE EXPRESSION CHANGES DURING OBESITY

To identify potential mechanisms underlying the development of obesity and diabetes, many studies have been conducted to characterize changes in hepatic gene expression (86–91). Complex phenotypes such as obesity and IR involve many different interacting biological pathways, but recent technological advances in high throughput sequencing have greatly improved our ability to quantitatively detect gene expression changes in an unbiased way. Investigation of the hepatic gene expression profiles in obese db/db (leptin receptor deficient) mice compared with control mice revealed significant changes in lipid metabolism, gluconeogenesis, mitochondrial dysfunction, and oxidative stress (88, 89). Similar studies using HFD feeding to generate obesity resulted in increased hepatic expression of genes involved in fatty acid catabolism and ketone body synthesis, such as acyl-CoA oxidase1 (*Acox1*) and HMG-CoA lyase (*Hmgcl*), while genes involved in lipogenesis and cholesterol synthesis, such as fatty acid synthase (*Fasn*) and acetyl-CoA synthetase 2 (*Acsl6*), were drastically decreased in the HFD group (86). Further studies also identified upregulation of hepatic gluconeogenic genes and downregulation of expression of lipogenic genes in diabetic Zucker rats (92), with activation of distinct transcriptional regulatory networks during diabetic progression (93).

Due to the practical limitations in obtaining human liver tissue, the most detailed hepatic expression studies have, so far, been conducted in rodent models (86–89, 92, 93). However, with the increasing use of gastric bypass surgery in obese patients, obtaining liver biopsies has become more feasible (91). Comparison of hepatic gene expression before and after weight loss in morbidly obese women identified differentially expressed genes involved in lipid and energy homeostasis, pro-inflammatory tissue repair, and bile acid transport (91). Liver samples from morbidly obese patients with all stages of NAFLD and controls were analyzed by array, and NAFLD specific expression differences were seen for nine genes involved in intermediate metabolism including pyruvate carboxylase (*Pc*), ATP citrate lyase (*Acly*), and phospholipase C-gamma-1 (*Plcg1*) as well as insulin/insulin-like signaling including insulin-like growth factor-1 (*Igf1*), insulin-like growth factor binding protein 2 (*Igfbp2*), and protein kinase C epsilon (*Prkce*) (94). In additional studies, comparison of transcriptional profiles from NASH patients versus non-obese controls also

revealed significant changes in genes involved in metabolism, insulin signaling, and inflammation (90). For example, high levels of the central enzyme controlling unesterified arachidonic acid levels of Acyl-CoA synthetase long chain family member 4 (*Acsl4*) and lower levels of insulin signaling genes including *Igfbp2* were observed in NASH versus non-obese controls (95).

Therefore, many hepatic gene expression studies in rodents and humans have been conducted at the level of the whole liver, but whether these changes occur within the hepatocyte or non-parenchymal cells is yet to be fully investigated. Increased understanding of the changes induced in the obese state in the hepatocytes, liver-resident macrophages, and each immune cell population may allow us to specifically target potentially harmful populations while promoting anti-inflammatory populations (96). These studies will also help clarify the molecular mechanisms behind the development of IR and identify potential targets for therapeutic intervention. Furthermore, future integration of transcriptomics data with metabolomics and proteomics data will further our understanding of the mechanisms behind obesity-associated liver disease and help identify biomarkers for the development of disease progression (89).

CONCLUSION AND FUTURE PERSPECTIVES

Although KCs are reemerging in obesity and metabolic syndrome as a critical player in the onset of hepatic IR, as well as NAFLD, their role in metabolism is still largely unknown. We are yet to define the direct role of KCs in metabolic diseases as well as their interactions with neighboring cells and distant organs that modulate liver function and whole body metabolism. After a hepatic insult, KCs secrete important factors involved in the recruitment and transformation of blood monocytes, which are involved in the subsequent development of the hepatic IR. During obesity, the inflammatory state in the liver is associated with a large increase in RHMs with a M1 phenotype, targeting specifically these immune cells or manipulating the activation of KC may be an effective therapeutic strategy in obesity-related chronic liver and NASH. The use of new technologies such as next-generation or single-cell sequencing at different stages of obesity and IR and approaches to isolate and identify the diverse macrophage population and profile their transcriptomes in the liver could provide the opportunity for a direct targeting strategy using specific surface markers. Further research in the field of immunometabolism, including a better understanding of how changes in the microbiota affect the development of inflammation and more knowledge about the factors that direct the polarization state of macrophages toward either the pro- or anti-inflammatory state, is necessary to design new therapeutic strategies for treating T2D and NAFLD.

AUTHOR CONTRIBUTIONS

SM and RM conceived the idea. RM, AJ, OO, PT, and SM contributed equally to researching the data and writing of the manuscript.

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MINI REVIEW
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Obesity: An Immunometabolic Perspective

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Obesity, characterized by chronic activation of inflammatory pathways, is a critical factor contributing to insulin resistance (IR) and type 2 diabetes (T2D). Free fatty acids (FFAs) are increased in obesity and are implicated as proximate causes of IR and induction of inflammatory signaling in adipose, liver, muscle, and pancreas. Cells of the innate immune system produce cytokines, and other factors that affect insulin signaling and result in the development of IR. In the lean state, adipose tissue is populated by adipose tissue macrophage of the anti-inflammatory M2 type (ATM2) and natural killer (NK) cells; this maintains the insulin-sensitive phenotype because ATM2 cells secrete IL10. In contrast, obesity induces lipolysis and release of pro-inflammatory FFAs and factors, such as chemokine (C–C motif) ligand 2 (CCL2) and tumor necrosis factor alpha (TNF-α), which recruit blood monocytes in adipose tissue, where they are converted to macrophages of the highly pro-inflammatory M1-type (ATM1). Activated ATM1 produce large amounts of pro-inflammatory mediators such as TNF-α, interleukin-1β, IL-6, leukotriene B4, nitric oxide (NO), and resistin that work in a paracrine fashion and cause IR in adipose tissue. In the liver, both pro-inflammatory Kupffer cells (M1-KCs) and recruited hepatic macrophages (Ly6C^{high}) contribute to decreased hepatic insulin sensitivity. The present mini-review will update the bidirectional interaction between the immune system and obesity-induced changes in metabolism in adipose tissue and liver and the metabolic consequences thereof.

Keywords: obesity, insulin resistance, macrophages, ER stress, reactive oxygen species, type 2 diabetes, non-alcoholic fatty liver diseases

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INTRODUCTION

Multicellular organisms rely on two highly conserved mechanisms for their survival: the ability to store energy to prevent starvation (metabolic pathways) and the ability to fight infection (immune pathways). When nutrients are in excess, adipose tissue stores lipids and the liver stores glycogen for use during starvation or to combat stressful situations. In addition, both adipose tissue and liver are populated with innate and adaptive immune cells. Thus, immune cells modulate whole-body metabolism [in metabolic syndromes such as type 2 diabetes (T2D) and obesity] *via* effects on adipocytes and hepatocytes, and reciprocally, host nutrition and commensal microbiota-derived metabolites modulate immunological homeostasis. This bidirectional interaction between the immune system and whole-body metabolism has created the field of immunometabolism, which has witnessed a renaissance in the past 15 years. The landmark discovery by Hotamisligil et al. in 1993 suggested

that tumor necrosis factor (TNF) levels are elevated in the adipose tissue of obese and diabetic rodents and that its neutralization improves insulin-stimulated glucose uptake, which formed the cornerstone for immunometabolism (1). The second groundbreaking discovery in the field of immunometabolism came from Ferrante and Chen's group, who reported simultaneously that adipose tissue of obese mice is infiltrated with macrophages that contribute to adipose tissue inflammation and IR (2, 3). Since these initial discoveries in immunometabolism, it has been shown that a large number of immune cells and pathways regulate metabolic homeostasis in obese animals (4–11).

Obesity, an epidemic of the twenty-first century, continues to rise throughout the world, even in the countries where poverty and malnutrition are major problems. The World Health Organization estimates that globally there are more than 1.9 billion overweight adults [body mass index (BMI) > 27 kg/m^2]. Of them, 600 million people are obese with BMI more than 30 kg/m^2 (WHO obesity and overweight fact sheet, updated in June 2016: http://www.who.int/mediacentre/factsheets/fs311/en/). Obesity provides bacterial and metabolic danger signals that activate a plethora of inflammatory cascades that drives M1 macrophage phenotype. In addition, immune and metabolic pathways are tightly balanced in that the immune response is highly energy demanding and shifts energy away from non-essential functions (12). In contrast, infection and sepsis often result in metabolic disruptions including IR (13). Obesity- and T2D-induced alterations in components of the immune system are most apparent in adipose tissue, the liver, and the pancreatic islets. Therefore, this review will focus on obesity-induced changes in immune system and metabolism in adipose tissue and liver and the consequent development of disease states such as IR, T2D, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH).

OBESITY: INNATE AND ADAPTIVE IMMUNE RESPONSES AND THEIR SIGNALING

The mammalian immune system consists of two types of immune responses: innate and adaptive. Innate immune cells include neutrophils, dendritic cells, macrophages, mast cells, and eosinophils, which respond to general danger signals associated with invading pathogens. Neutrophils are the first responders to invading pathogens and are generally among the first immune cells to arrive at the site of inflammation. Macrophages are long lived and highly dynamic. They readily switch from anti-inflammatory M2 type to pro-inflammatory M1-type in resident tissues. Besides bacterial danger signals mediated by lipopolysaccharide (LPS), the toll-like receptor 4 (TLR4) ligand, obesity-associated metabolic danger signals also play an important role in macrophage polarization. To provide local immune responses, macrophages get assistance from other immune cells, such as TLR-proficient mast cells (14). Eosinophils are anti-inflammatory in nature and maintain the M2 macrophage population. Adaptive immune cells include B-2 and T lymphocytes, which exert specific and decisive adaptive immune functions and provide immunological memory (15). B-2 and T lymphocytes are also involved in sterile inflammation and autoimmune disorders (16, 17). TNF-α released by M1 macrophage initiates inflammatory signaling through its receptor TNFR1 with consequent regulation of gene expression. In the cytoplasm, NF-κB is sequestered by the inhibitor of κB (IκB) to prevent nuclear translocation. The activation of the IκB kinase leads to phosphorylation of IκB and release of NF-κB, which then translocate to the nucleus and bind to the promoters of pro-inflammatory genes and initiates transcription (9, 18) (**Figure 1**). Alternatively, the inflammatory signaling can be initiated by the microbial-derived LPS, which acts through the TLRs. TLRs can sense lipids and saturated fatty acids and are able to induce activation of TLR2 and TLR4 through myeloid differentiation primary response protein 88-dependent pathways, whereas unsaturated fatty acids block TLR-mediated signaling pathways and gene expression (**Figure 1**). Receptors of advanced glycation end product bind to lipids and nucleic acids resulting in oxidative stress, activate NF-κB, and promote transcription of pro-inflammatory factors (19, 20) (**Figure 1**). The inflammasome, an oligomeric protein complex, comprises scaffold, adaptor, and caspase proteins that mediate the maturation and secretion of inflammatory cytokines interleukin-1β (IL-1β) and IL-18 (21). The NLR family pyrin domain containing 3 inflammasome recruits and activates pro-caspase 1 to produce caspase-1, which then cleaves pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18, respectively (22).

IMMUNE CELLS AND THEIR POLARIZATION IN ADIPOSE TISSUE

The adipose tissue comprises adipocytes, immune cells (macrophages and lymphocytes), pre-adipocytes, and endothelial cells. Under lean conditions, Th2 T cells, T_{reg} cells, eosinophils, and ATM2-like resident macrophages predominate in the adipose tissue (**Figure 2**). ATM2 macrophages express CD11b, F4/80, CD301, and CD206 and promote local insulin sensitivity through production of anti-inflammatory cytokines, such as IL-10 (18). T_{reg} cells not only secrete IL-10 but also stimulate ATM2 macrophage to secrete IL-10. Eosinophils, on the other hand, secrete IL-4 and IL-13. In the lean state, IL-4, IL-10, and IL-13 maintain the anti-inflammatory and insulin-sensitive phenotype. In contrast, obesity induces lipolysis and release of pro-inflammatory free fatty acids (FFAs) and factors such as C–C motif ligand 2 (CCL2) and TNF-α that recruit blood monocytes in adipose tissue, where they become polarized to the highly pro-inflammatory M1-like state (**Figure 2**). FFAs serve as ligands for the TLR4 complex (23), activate classical inflammatory response, and drive accumulation of ATM (24, 25). Activated ATM1 express CD11c in addition to CD11b and F4/80 and produce large amounts of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, leukotriene B4, NO, and resistin that work in a paracrine fashion and causes IR in adipose tissue (26). The anti-inflammatory eosinophil population declines in obese adipose tissue. In addition, obesity decreases T_{reg} content and an increase in $CD4^+$ Th1 and $CD8^+$ effector T cells, which also secrete pro-inflammatory cytokines. Obesity increases B cell numbers and activates T cells, which potentiate M1-like

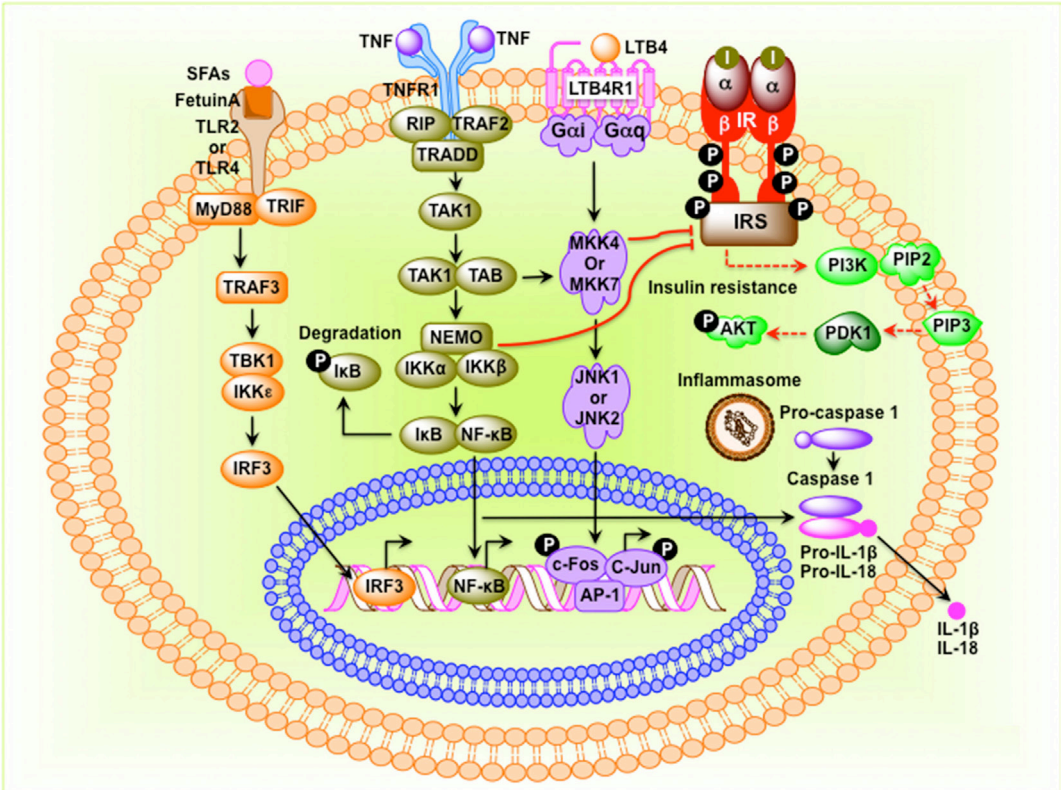

FIGURE 1 | **Molecular events that connect inflammation to insulin resistance in obesity**. Saturated fatty acids (SFAs) bind to Fetuin-A, an endogenous ligand of toll-like receptor 4 (TLR4) and TLR2, and initiate transcription of interferon regulatory factor 3 (IRF3) in a myeloid differentiation primary response protein 88 (MyD88)–TIR-domain-containing adapter-inducing interferon-β-dependent pathway. Activated IRF3 then translocates to the nucleus and binds to target DNA sequences. Tumor necrosis factor (TNF) protein binds to its receptor and initiates inhibitor of κB (IκB)–NF-κB signaling pathway leading to translocation of NF-κB to the nucleus where it binds to AP-1 DNA sequences. Stimulation leukotriene B4 receptor 1 (LTB41) activates the c-Jun N-terminal kinase pathway, leading to phosphorylation and binding of the c-Jun–c-Fos heterodimer to target genes. NF-κB, c-Jun–c-Fos, and IRF3 induce expression of inflammatory factors such as cytokines, chemokines, and components of the inflammasome. When inflammasome is assembled, pro-caspase-1 is converted to caspase-1, which then converts pro-interleukin-1β (IL-1β) and pro-IL-18 to IL-1β and IL-18, respectively. I, insulin; insulin receptor; IRS, insulin receptor substrate.

macrophage polarization, inflammation, and IR. Cytokines and chemokines are also released from the adipose tissue and promote inflammation and consequent IR in liver, muscle, and pancreas.

IMMUNE CELLS AND THEIR POLARIZATION IN LIVER

In the lean liver, hepatocytes are the major parenchymal cells, while the non-parenchymal cells integrate five cell populations including resident macrophages of M2-type or Kupffer cells (27), recruited hepatic macrophages, resident innate lymphocytes or natural killer cells (NKs) (28, 29), fat storing cells termed Ito or stellate cells (HSCs) (30), and liver sinusoidal endothelial cells (LSECs) (31). Under lean conditions, Kupffer cells (KCs) in collaboration with other hepatic immune cell populations clear microbial material while maintaining the inflammatory tone of the liver at a level sufficient for essential functions such as pathogen killing, tissue remodeling, and sinusoidal permeability, but below that they would result in overt inflammation and tissue damage (32–34). NKs eliminate virus-infected or transformed cells and regulate adaptive immune responses *via* contact-dependent signals and the secretion of cytokines (35–38).

Hepatic lipid accumulation and peroxidation lead to chronic hepatocyte endoplasmic reticulum stress, the production of reactive oxygen species, and TLR activation, which converts KCs into an M1 phenotype defined by production of pro-inflammatory cytokines, oncostatin, and prostaglandins (PGE_2) (39–41). Circulating cytokines, adipokines, and FFAs released from inflamed adipose tissue in the obese state or immunogenic material derived from an altered intestinal microbiota can also contribute to KC polarization. M1-KCs secrete chemokine CCL2 (also known as MCP1), pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), macrophage inflammatory protein (MIP)-1a, MIP1b, RANTES, oncostatin, and PGE_2, which contribute to the alteration of the liver homeostasis and worsen the hepatic inflammatory response (42). PGE_2 regulates cytokine production (IL-1β, IL-6, TNF-α, and TGF-β) (43, 44), acts synergistically with IL-6 to induce IR (45), and induces production of oncostatin M (OSM) in KCs (46). Increased OSM contributes to hepatic IR and

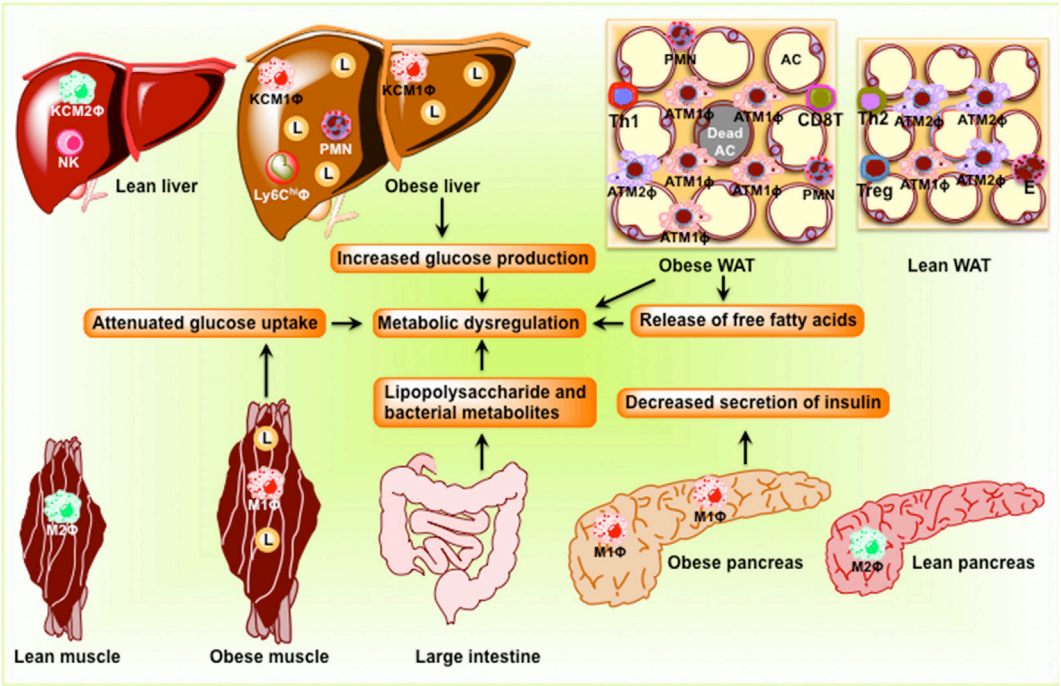

FIGURE 2 | Schematic diagram showing obesity-induced inflammation in peripheral organs including adipose tissue, the liver, skeletal muscle, and the pancreas to cause dysbiosis in the intestine. In adipose tissue, pro-inflammatory signaling induces lipolysis and release of free fatty acids eventuating in the development of insulin resistance. In the liver, obesity induces pro-inflammatory cytokine production and M1 macrophage recruitment, resulting in insulin resistance and steatosis. In skeletal muscle of obese rodents, accumulations of lipid and pro-inflammatory macrophage inhibit insulin signaling, which result in the development of insulin resistance. In the pancreas, obesity induces macrophage infiltration, interleukin-1β secretion, and decreases insulin secretion. Because of the change in the composition of the microbial population, dysbiosis occurs in the intestine. AC, adipocyte; KC, Kupffer cell; L, lipid droplets; M1Φ, classically activated macrophages/pro-inflammatory macrophages; M2Φ, alternatively activated macrophages/anti-inflammatory macrophages; NK, natural killer cell; PMN, polymorphonuclear neutrophil; WAT, white adipose tissue.

the development of NASH (46). High levels of TNF-α released by M1-KCs stimulates hepatic expression of CCL2, a powerful monocyte chemoattractant, which recruits CCR2^{+}Ly6C^{high} monocytes from the vasculature into the liver (47), where they differentiate into Ly6C^{high} macrophages. The Ly6C^{high} macrophages amplify the severity of obesity-induced inflammation and hepatic IR through secretion of TNF-α and IL-6 (48).

ADIPOSE TISSUE FIBROSIS AND METABOLIC DYSFUNCTION

Adipocytes and their progenitor cells (pre-adipocytes) are embedded in a network of extracellular matrix (ECM), which tightly regulates the function of adipose tissue (49). Fibrosis, the excessive accumulation of ECM components, is a highly conserved and coordinated protective response to tissue injury and is a common pathological consequence of inflammatory diseases (50). Fibrosis develops from an imbalance between excess synthesis of ECM components including collagens (I, III, and VI), elastins, and proteoglycans (51, 52), and an impairment in degradation of these proteins. Fibrosis limits the expandability of adipose tissue and contributes to ectopic fat accumulation and the development of IR (53). It has been recently shown that treatment with the antidiabetic drug metformin inhibits excessive ECM deposition in white adipose tissue (WAT) of leptin-deficient *ob/ob* mice and mice with diet-induced obesity (54). Fibrotic disorders cause 45% deaths in the United States (52). In adipose tissues, ECM undergoes constant remodeling to allow adipocytes to rapidly expand and shrink in parallel with weight gain and loss and function in adaptation to nutritional clues (55). Adipocytes undergo dramatic expansion during the development of obesity. Macrophages are believed to be the master "regulators" of fibrosis as they produce soluble mediators including TGF-β1 and platelet-derived growth factor (PDGF), which directly activate fibroblasts and control ECM dynamics by regulating the balance of various matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMP) (56). Myofibroblasts, macrophages, and endothelial cells also produce MMP and TIMP for ECM regulation (57). While MMPs are responsible for the degradation of virtually all ECM proteins (58), TIMP inhibits MMPs and is responsible for degrading excess ECM (59). Macrophages also regulate fibrogenesis by releasing chemokines and attract fibroblasts and other inflammatory cells. Thus, IL-13 produced by Th2 CD4^{+} T cells (52, 60, 61) and TGF-β1 activate fibroblasts to differentiate into α-smooth muscle actin (α-SMA) expressing myofibroblasts to produce ECM (62–64).

LIVER FIBROSIS AND METABOLIC DYSFUNCTION

Liver fibrosis results from the would-healing response of the liver to repeated injury such as hepatitis C virus (HCV) infection, alcohol abuse, and NASH (65, 66). Fibrosis is increasingly appreciated as a major contributor to metabolic dysregulation in obese humans and T2D patients (67). Advanced liver fibrosis leads to cirrhosis and death (68). Increased gut permeability and hepatic TLR4 signaling promotes fibrogenesis. Both KCs and recruited Ly6C^{high} macrophages contribute to the development of hepatic fibrosis (69). HSCs are the main collagen-producing cells in liver (70, 71). KCs activate HSCs through increased production of profibrotic cytokine TGF-β and mitogenic PDGF (72) leading to fibrosis. TGF-β leads to transdifferentiation of HSCs into myofibroblasts. PDGF stimulates myofibroblast proliferation. Inhibition of PDGF by anti-sense strategy attenuates liver fibrogenesis (73). HSC-derived myofibroblasts express α-SMA and collagen I. During fibrogenesis, LY6C^{high} monocytes are recruited to the inflamed liver *via* the CCL2/CCR2 (C–C chemokine receptor type 2) axis, forming a profibrotic Ly6C^{high} macrophage, which has been shown to be the predominant pro-fibrogenic population in the liver (74, 75). These cells express TNF-α and IL-1β, which perpetuate hepatocellular injury and enhance the survival of hepatic myofibroblasts. In addition, Ly6C^{high} macrophages express high levels of TGF-β-activating thrombospondin 1 (76). Macrophages also express the potent mitogen PDGF and the Th2 cell cytokines IL-4 and IL-13, which directly stimulate collagen synthesis in myofibroblasts. Chemokine expression such as CCL8 (also known as MCP2) and CCL7 (also known as MCP3) by these macrophages promotes the recruitment of monocytes, other inflammatory cells, and HSCs (77). Ly6C^{high} macrophages also interact with HSCs to promote fibrosis through increased production of TGF-β, connective tissue growth factor (CTGF), and PDGF (78). Inhibition of the main monocyte chemoattractant CCL2 in rats or genetic deletion of its receptor CCR2 in mice decreased macrophage infiltration in response to injury and markedly inhibited liver fibrosis, implicating monocyte recruitment as an essential component in liver fibrogenesis (78–82). In addition, pharmacological inhibition of CCL2 by the RNA-aptamer mNOX-E36 attenuates liver fibrosis, thereby strengthening a profibrotic function of Ly6C^{high} macrophages (83, 84). Hepatic myofibroblasts express TIMP1, which inhibits MMP activity and augments the accumulation of ECM in the scar tissue.

OBESITY, TISSUE INFLAMMATION, AND INSULIN RESISTANCE

Components of the immune system are affected in obesity and T2D and inflammation participates in the pathogenesis of T2D. Thus, obesity affects the immune system and promotes inflammation with consequent development of IR (85–87). Obesity-induced increased levels of glucose and FFAs create stress in pancreatic islets, adipose tissue, liver, and muscle, resulting in increased local production and release of cytokines and chemokines such as IL-1β, TNFα, CCL2, CCL3, and CXC-chemokine ligand 8 (CXCL8, also known as IL-8). These changes promote recruitment of immune cells in insulin-sensitive tissues and contribute to tissue inflammation and further production and release of cytokines and chemokines. The augmented release of cytokines and chemokines promotes inflammation in liver, muscle, and pancreatic islets. Obesity affects insulin signaling and causes IR by the following mechanisms: (i) inflammatory stimuli phosphorylate IκB resulting its dissociation from IκB/NF-κB complex followed by degradation in the cytoplasm. This allows translocation of free NF-κB to the nucleus, where it binds to cognate DNA response elements and transactivates the transcription of inflammatory genes. (ii) Phosphorylation and activation of c-Jun N-terminal kinase (JNK) leading to phosphorylation of the N-terminus of c-Jun. This initiates a switch of c-Jun dimers for c-Jun–c-Fos heterodimers with consequent stimulation of transcription of inflammatory target genes. (iii) Production of "second messengers," such as FFAs, that promote IR. (iv) Augmented transcription of genes involved in lipid processing, including the enzymes that synthesize ceramide, which inhibits the activation of AKT (88, 89).

Recent studies in both rodents and humans implicate gut microbiota as a contributor to metabolic disorders (90). The gut microbiota plays a part in the host's genomic profile and metabolic efficiency (91). Obesity in humans and rodents is associated with changes in the composition of the intestinal microbiota (92, 93). Dysbiotic microbiota in obesity enhances the digestion of complex carbohydrates and macronutrient absorption, leading to the development of obesity (94). In addition, gut microbiota has the capacity to harvest energy from nutrients and stores energy in the form of fat (95). The gut microbiota is also capable of inducing "metabolic endotoxemia" by increasing exposure to bacterial LPS coming from gut (96). LPS in the bloodstream contributes to IR by promoting tissue inflammation (97, 98).

OBESITY AND NAFLDs

Non-alcoholic fatty liver disease, the liver manifestation of the metabolic syndrome, has become the most common disorder in the United States and other developed countries, affecting over a third of the population (99). NAFLD begins with a simple steatosis that may evolve into NASH, a medley of inflammation, hepatocellular injury, and fibrosis, often resulting in cirrhosis and even hepatocellular cancer (100–102). KCs differ in their population density, morphological characteristics, and physiological functions depending on their position within the liver sinusoids (103, 104). Severity of human NAFLD is associated with higher population of KCs (105). However, NASH is associated with aggregates of enlarged KCs (106). Selective depletion of large KCs by administration of gadolinium chloride markedly attenuates liver injury induced by thioacetamide (107), carbon tetrachloride (108), alcohol (109), and ischemia/reperfusion (110), indicating the critical roles played by larger KCs in liver damage in these condition. In experimental NAFLD induced by methionine/choline deficient diet, liposome-encapsulated dichloromethylene bisphosphonate (clodronate) effective blunts all histological evidence of NASH (111). These findings indicate that the activation

of KCs positioned at the "frontline" is an essential element in the pathogenesis of NAFLD similar to other types of liver injury.

THERAPEUTIC PERSPECTIVES ON IMMUNOMODULATION

Although it is yet to be definitely established whether tissue inflammation causes IR in humans, several anti-inflammatory approaches have been tested in clinical studies of obese individuals with IR. Thus, salsalate, an analog of salicylate, has been shown to improve insulin clearance and insulin sensitivity (112–115). Anti-TNF antibodies were found to decrease blood glucose in obese individuals (116). Anti-IL-1β monoclonal antibody therapy improved glycemic condition and β-cell insulin secretion (117–119). The antidiabetic thiazolidinediones (e.g., rosiglitazone and pioglitazone) decreased adipose tissue macrophage content (120, 121) and increase circulating levels of adiponectin and FGF21, thereby mediating redistribution of adipose tissue lipid stores (122, 123). Orexin-1 receptor antagonist has been shown to exert anti-obesity effects in obese leptin-deficient *ob/ob* mice (124, 125). While obese mice fed a high-fat diet supplemented with ω-3 fatty acids caused a decrease in inflammation, improved insulin sensitivity, and normalized glucose tolerance (126), fish-oil supplementation yielded mixed results on metabolic end points in human studies (127, 128).

CONCLUSION AND FUTURE PERSPECTIVES

Although the last 15 years has witnessed a renaissance in the field of immunology and metabolism, immunometabolism is still a young field with many questions to be answered. (i) To what extent are obesity and inflammation triggered in parallel or in sequence? (ii) What is the ontogeny and fate of stromal cells that populate WAT and liver? (iii) Do macrophage localization and origin regulate immunometabolic phenotype? (iv) By what pathway(s) does inflammation provoke T2D? (v) Can genetic and environmental factors reinforce or dissociate the link between metabolic and immunological abnormalities? (vi) Do anti-inflammatory strategies target the underlying mechanisms of the disease, and if so, would starting these therapies early prevent progression or even the overt manifestation of the disease? Answers to the above questions and a more detailed understanding of immunometabolism will permit more focused immune therapies to target metabolic diseases.

AUTHOR CONTRIBUTIONS

IR and RD researched data and wrote the first draft of the article. SM researched data and extensively revised the draft, and made both the figures.

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MINI REVIEW
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Diabetic Cardiomyopathy: An Immunometabolic Perspective

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The heart possesses a remarkable inherent capability to adapt itself to a wide array of genetic and extrinsic factors to maintain contractile function. Failure to sustain its compensatory responses results in cardiac dysfunction, leading to cardiomyopathy. Diabetic cardiomyopathy (DCM) is characterized by left ventricular hypertrophy and reduced diastolic function, with or without concurrent systolic dysfunction in the absence of hypertension and coronary artery disease. Changes in substrate metabolism, oxidative stress, endoplasmic reticulum stress, formation of extracellular matrix proteins, and advanced glycation end products constitute the early stage in DCM. These early events are followed by steatosis (accumulation of lipid droplets) in cardiomyocytes, which is followed by apoptosis, changes in *immune responses* with a consequent increase in fibrosis, remodeling of cardiomyocytes, and the resultant decrease in cardiac function. The heart is an omnivore, metabolically flexible, and consumes the highest amount of ATP in the body. Altered myocardial *substrate and energy metabolism* initiate the development of DCM. Diabetic hearts shift away from the utilization of glucose, rely almost completely on fatty acids (FAs) as the energy source, and become metabolically inflexible. Oxidation of FAs is metabolically inefficient as it consumes more energy. In addition to metabolic inflexibility and energy inefficiency, the diabetic heart suffers from impaired calcium handling with consequent alteration of relaxation–contraction dynamics leading to diastolic and systolic dysfunction. Sarcoplasmic reticulum (SR) plays a key role in excitation–contraction coupling as Ca^{2+} is transported into the SR by the SERCA2a (sarcoplasmic/endoplasmic reticulum calcium-ATPase 2a) during cardiac relaxation. Diabetic cardiomyocytes display decreased SERCA2a activity and leaky Ca^{2+} release channel resulting in reduced SR calcium load. The diabetic heart also suffers from marked downregulation of novel cardioprotective *microRNAs* (miRNAs) discovered recently. Since immune responses and substrate energy metabolism are critically altered in diabetes, the present review will focus on immunometabolism and miRNAs.

Keywords: obesity, insulin resistance, inflammation, cardiomyopathy, innate and adaptive immunity, glucose metabolism, fat metabolism, miRNA

INTRODUCTION

Insulin deficiency and/or resistance and elevated plasma glucose level characterize diabetes, a chronic and progressive metabolic disorder. While type 1 diabetes mellitus (T1DM) accounts for 5–10% of all cases of diabetes (1), type 2 diabetes mellitus (T2DM) accounts for the remaining ~90% of all cases of diabetes (2). As of 2015, 415 million people across the globe have diabetes mellitus (DM) (www.diabetesatlas.org), which will cost 12% of all global health expenditures (accounting for $320 billion in the USA alone) (3). The International Diabetic Federation predicts that 552 million people will suffer from diabetes by 2030. T2DM is recognized as an independent risk factor for heart failure (HF). Patients with T2DM have a greater probability of death in established HF; suffer from worse prognosis after myocardial infarction (MI) (4–8); and accounts for 5.2% of all deaths globally (9, 10). T2DM is strongly associated with obesity and sedentary lifestyle coupled with increasingly westernized diet (2, 11). Diabetic patients are also highly susceptible to diastolic dysfunction, ventricular hypertrophy, and decreased myocardial strain (12).

Rubler and colleagues initially reported diabetic cardiomyopathy (DCM) from their observation of cardiac hypertrophy on post-mortem hearts from four diabetic patients who died of HF without cardiovascular disease, which was subsequently followed by various other studies (13–16). The Strong Heart Study, the Cardiovascular Health Study, and the Framingham Study revealed cardiac hypertrophy with compromised systolic and diastolic function in DCM patients (4, 17–19). Of note, diastolic dysfunction has been reported in diabetic hearts without hypertrophy (20–22). In fact, DCM starts with diastolic dysfunction in patients with T1DM or T2DM followed by systolic dysfunction (23–27). Rodent models of T1DM including streptozotocin (STZ)-treated (28) or alloxan-treated animals (29) and T2DM models such as Goto-Kakizaki rat (30), Zucker fatty rats, Zucker diabetic fatty rats, leptin-deficient *ob/ob* mice, and leptin receptor-deficient *db/db* mice consistently show the human DCM phenotypes (31–33). Of note, STZ- and alloxan-induced diabetes is characterized by myocardial atrophy including loss of contractile proteins as opposed to cardiac hypertrophy in T2DM models (34–36). In addition, in T1DM animals, the progress of systolic dysfunction is positively correlated with the progress of the magnitude and duration of hyperglycemia (hypoinsulinemic/hyperglycemia → systolic dysfunction) (31, 35–38). By contrast, mouse models of T2DM are characterized by hyperinsulinemia, hyperglycemia (later stages), and hyperlipidemia (hyperinsulinemic/hyperglycemic → hypertrophy and diastolic dysfunction) (31, 39–41).

Autophagy is reduced in the mouse hearts of OVE26 (a transgenic model of insulinopenic diabetes) and STZ-induced diabetic mouse hearts (42–44). Metformin has been shown to prevent DCM by stimulating AMP-activated protein kinase (AMPK) activity and enhancing autophagic capacity (43).

Recently, DM is identified as a microRNA (miRNA)-related disease (45), and several diabetic complications are associated with differential expressions of various miRNAs (46). Further, miRNAs play a vital role in the regulation of metabolism (47) and since DM is a metabolic disease it is logical to examine the role of miRNAs in DM. Thus the present review will focus on altered metabolism of glucose and fatty acids (FAs) as well as immune responses in diabetes.

DECREASED GLUCOSE UPTAKE AND METABOLISM

The heart consumes about 6 kg of ATP, or ~20 times its own weight, per day (48) that comes from the breakdown of fat, carbohydrate, protein, ketone bodies, or lactate. Of note, the amount of ATP in the heart is small (~10 mM, enough for only a few beats) compared with the demand (~10,000 times greater) (49). About 95% of total energy is generated from oxidative phosphorylation of FAs and glucose (50–52). A dramatic metabolic shift takes place in diabetic heart, as they rely almost completely on FAs for their energy source. As for example, 46 atoms of oxygen are required to generate 105 molecules of ATP from oxidation of 1 molecule of palmitate. By contrast, oxidation of 1 molecule of glucose utilizes 12 atoms of oxygen to generate 31 molecules of ATP. Therefore, oxidation of FAs consumes ~0.3 oxygen molecules more than glucose to generate each molecule of ATP. Thus, the diabetic heart suffers from metabolic inflexibility due to its reliance on FAs. The lack of insulin production in T1DM patients causes a dramatic decrease in cardiac glucose uptake (53, 54) where hyperglycemia increases glucose oxidation and mitochondrial generation of superoxide (55–57). Increased production of superoxide damages DNA and activates poly (ADP ribose) polymerase 1 (PARP-1) (58), which mediates inflammation and fibrosis in liver (59). PARP-1 inhibition improves cardiac function (60) and prevents hyperglycemia-induced pathological processes (61). While decreased glucose transporter type 4 (Glut4) expression in T1DM animals causes decreased glucose uptake in cardiac and skeletal muscle (62, 63), glucose uptake is impaired in T2DM hearts by decreased expression and translocation of Glut4/Glut1 (64, 65). Diabetic *db/db* mice show decreased glucose oxidation and increased reliance on FAs, indicating that insulin resistance is not responsible for metabolic switch (66–69). The high rate of FA oxidation in T2DM patients and rodents increases production of acetyl CoA and NADH, resulting in activation of pyruvate dehydrogenase kinase 4 (PDK4). PDK4 is also activated by peroxisome proliferator-activated receptor alpha (PPARα), which is overexpressed in diabetic rodents (70–73). Activated PDK4 inhibits pyruvate dehydrogenase complex, thereby preventing oxidation of pyruvate (74, 75) (**Figure 1**). In addition, increased accumulation of FAs and their derivatives fatty acyl CoA, diacylglycerol, and ceramide activate protein kinase C, c-Jun N-terminal kinases, mammalian target of rapamycin, and inhibitor of κB kinase β with consequent decrease in insulin signaling (76–79).

INCREASED FORMATION OF ADVANCED GLYCATION END PRODUCTS (AGEs)

In the diabetic heart, glucose forms covalent adducts with the plasma proteins through a non-enzymatic reaction between the free amino groups of proteins and carbonyl groups of reducing sugars, resulting in the formation of stable glycosylation products by Amadori rearrangement, which is called glycation (91–94).

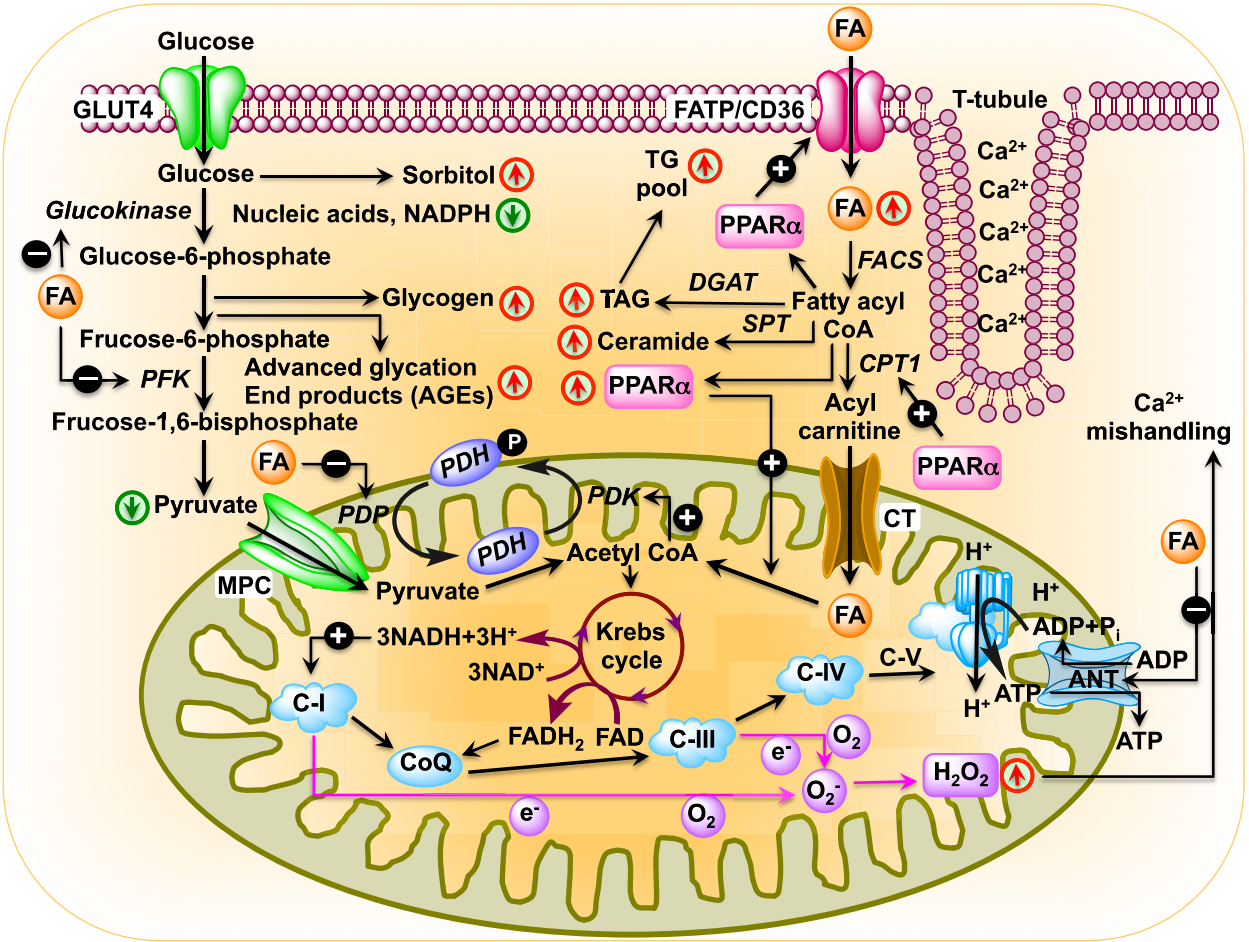

FIGURE 1 | Schematic diagram showing changes in cardiac metabolism in diabetic cardiomyopathy. In the diabetic heart, glucose oxidation is inhibited at multiple steps: (i) uptake of glucose is inhibited by reduced expression of glucose transporter Glut4 as well as by blunted translocation of Glut4 in response to insulin (64, 65); (ii) inhibition of hexokinase activity by fatty acids (FAs) resulting in reduced conversion of glucose to glucose-6-phosphate (80); (iii) inhibition of phosphofructokinase activity by FA, leading to reduced formation of fructose-1,6-bisphosphate by fructose-6-phosphate (69); (iv) inhibition of pyruvate dehydrogenase phosphatase activity by FA resulting in reduced pyruvate dehydrogenase (PDH) activity, which leads to reduced conversion of pyruvate to acetyl CoA. In the diabetic heart, PPARα expression is activated by increased FA uptake (81, 82). Activated PPARα upregulates PDH kinase 4 enzymes, which inhibits PDH resulting in reduced production of acetyl CoA (83–85). FA transporters CD36 and FA transport protein import FAs into the cell. After import, FAs can be stored as triacylglycerol (TAG) or converted to fatty acyl CoA by fatty acyl-CoA synthetase (FACS). Carnitine palmitoyltransferase 1 (CPT1) transfers the acyl group of fatty acyl CoA to carnitine, which then shuttles into the mitochondria by carnitine translocase (CT). PPARα activates transcription of CPT1 (86). In the matrix, CPT2 reconverts the acylcarnitine back into free carnitine and fatty acyl CoA (87), which is then converted to acetyl CoA that can be used in the tricarboxylic acid to produce adenosine triphosphate by β-oxidation. Diabetes upregulates mitochondrial generation of reactive oxygen species (57, 88, 89), which affects Ca^{2+} signaling (90).

Glycated proteins undergo a series of oxidation, dehydration, and cyclization reactions to form long-lived AGEs (95, 96). Both AGE and its receptor RAGE are overexpressed in diabetes (97) leading to the generation of reactive oxygen species (ROS) and subsequent activation of RAS–MAP kinase pathway (98). Activation of RAS–MAPK pathway in turn activates NF-κB pathway resulting in decreases in contractile proteins such as α-actin and myosin ATPase activity (35, 36) and shifts in myosin heavy chain isoforms from α to β with consequent development of decreased systolic tension (36–38, 97, 99). In diabetes, increased serum levels of AGEs show positive correlation with ventricular isovolumetric relaxation time, arterial stiffness, and carotid intimal thickness (100–102). Treatment of diabetic animals with aminoguanidine (an inhibitor of AGE formation) (103, 104) or with alagebrium (ALT-711; disrupts AGE cross-link) (105) restored LV function and reduced myocardial collagen, highlighting the importance of AGE in cardiac dysfunction. AGEs also impair collagen degradation by matrix metalloproteinases (MMPs), such as MMP2, resulting in increased fibrosis (106, 107). Fibrosis increases myocardial stiffness and impairs diastolic function (104). In T1DM heart, AGEs also induce cross-linking of SERCA2a pump, thereby attenuating sarcoplasmic reticulum (SR) Ca^{2+} reuptake (108, 109) with consequent attenuation of the maximum and minimum rate of pressure change in the ventricle and LV developed pressure (108). Of note, the type 2 ryanodine receptor-dependent Ca^{2+} release not only plays critical roles for

excitation–contraction coupling in cardiomyocytes but plays crucial roles in the regulation of insulin secretion and glucose homeostasis (110, 111). Genetic ablation of the RAGE gene improves hemodynamic dysfunction, thereby providing AGE/RAGE pathway as a potential therapeutic target to alleviate cardiac dysfunction in diabetes.

INCREASED FA UPTAKE AND METABOLISM

The heart has a limited capacity for *de novo* synthesis of FAs. Therefore, it relies heavily on the circulating FAs (112). FAs translocate from blood to cardiomyocytes using three FA transporters: cluster of differentiation 36 (CD36), FA transport protein 1, and the plasma membrane form of FA-binding protein (113–116). Increased PPARα expression in diabetic hearts (70–73, 117) augments transcription of FA transporters. About 75% of the translocated FAs are transferred to mitochondria for the generation of ATP and the rest are converted to triacylglycerol (TAG) for future use (118). Translocated FAs are activated by esterification to fatty acyl CoA by the action of cytosolic fatty acyl-CoA synthetase (FACS). Carnitine palmitoyltransferase 1 (CPT1) exchanges the CoA moiety of fatty acyl CoA for carnitine resulting in the formation of acylcarnitine. Acylcarnitine is transported across the inner mitochondrial membrane into the matrix by carnitine–acylcarnitine translocase. PPARα augments transcription of CPT1 (119, 120). In the matrix, CPT2 reconverts the acylcarnitine back into free carnitine and fatty acyl CoA. PPARα increases transcription of CPT2 (120). Fatty acyl CoA is then converted to acetyl CoA for β-oxidation and generation of ATP. PPARα increases conversion of fatty acyl CoA in the mitochondrial matrix to acetyl CoA. Thus, PPARα plays critical roles in metabolic reprograming in diabetic hearts.

Since the diabetic heart relies on FAs for ATP generation, it consumes ~30% more oxygen compared with non-diabetic heart to generate similar levels of ATP (87, 121) and generate the same or the reduced amounts of contractile force (41). This disproportionate use of FAs also alters cellular ATP shuttling as long-chain acyl CoA derivatives inhibit the adenine nucleotide translocator for the transport of ATP from mitochondria to the cytosol (122–124), eventuating in inefficient delivery of ATP to myofibrils that affects cardiac contractility.

INFLAMMATION, INNATE, AND ADAPTIVE IMMUNE RESPONSES

Metabolic disturbances induce subcellular low-grade inflammation in the heart (125). Inflammation is a key pathogenic feature of lipid excess and diabetes. The innate immune system comprising of neutrophils, dendritic cells, macrophages, mast cells, and eosinophils also induces chronic metabolic inflammation (126, 127). Myocardial inflammation is implicated in the development of DCM (128–131). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a primary regulator of inflammatory responses, is activated in the heart upon exposure to FAs or glucose (132, 133). NF-κB induces not only the expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), pro-IL1β, and pro-IL18, but it also induces the expression of NLR family pyrin domain-containing 3 (NLRP3) inflammasome (134). Activated RAGE also triggers an inflammatory response by heterodimerizing with TLR-4 leading to the production of pro-IL1β, Pro-IL18, and NLRP3 (135). Activated NLRP3 inflammasome activates caspase-1 and mediates the processing and release of pro-inflammatory cytokines IL1β and IL18 resulting in inflammatory cell infiltration and amplification of the inflammatory response (125, 136–138). Likewise, depletion of NLRP3 attenuates inflammation and cardiomyopathy in T2DM rats (137). Of note, activated inflammasomes play critical roles in the pathogenesis of HF (139). Resident immune cells in the resting heart include the following: macrophages, residing near endothelial cells or within the interstitial space (140–143); mast cells that are responsible for early triggers of immune responses (144); a small number of adaptive immune cells: B cells and regulatory T (T_{Reg}) cell subsets (142, 145, 146); and dendritic cells that test sample antigens (142, 147) (**Figure 2A**). The differential expression of major histocompatibility complex (MHC) class II and CC chemokine receptor 2 (CCR2) distinguishes three different subsets of cardiac macrophages: MHC class II^{high} ($CCR2^{-}$), MHC class II^{low} ($CCR2^{-}$), and $CCR2^{+}$ macrophages. The first two are the preponderant macrophages in the heart, derived from embryogenic progenitors and renewed through *in situ* proliferation, rather than through monocyte input. By contrast, $CCR2^{+}$ macrophages derive from and replenished by circulating blood monocytes, which comprise of $Ly6C^{high}$ and $Ly6C^{low}$ (148–150). Studies in *Ccr2* knockout mice (lacking circulating monocytes) reveal increased cardiac pathology (151, 152). The loss of $Ly6C^{high}$ monocytes prevents hypertension-induced cardiac fibrosis and improves cardiac function after MI (141, 153, 154). Monnerat et al. suggest that diabetes enhances IL1β production from cardiac MHC II^{high} pro-inflammatory macrophages through activation of TLR2–NLRP3 inflammasome axis (155). The increased level of IL1β leads to a reduction in potassium current and an increase in calcium sparks in cardiomyocytes, which cause cardiac arrhythmias (156). By contrast, M2-like macrophages ($CD206^{+}F4/80^{+}CD11b^{+}$) exert profound functions on tissue repair in heart depending on IL4 secretion (156). Recent studies implicate TNFβ producing B cells as a major contributor to myocardial fibrosis (153, 157). Antigen and cytokine stimulation are known to differentiate naive T cells into distinct T cell subpopulations that include T helper cells and $CD4^{+}CD25^{+}FOXP3^{+}$ T_{Reg} cells (158). T_{Reg} cells comprise a subset of $CD4^{+}$ lymphocytes that suppress activation, proliferation, and effector responses of both innate and adaptive immune cells (159–161). As opposed to B cells, depletion of T_{Reg} cells aggravates myocardial fibrosis and adoptive transfer of exogenous T_{Reg} cells into these mice attenuates the extent of myocardial fibrosis (158). The following pro-inflammatory changes with oxidative stress and decreased cardiac function were detected in STZ-induced rat model of T1DM (162): (i) significant increases in myocardial intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, (ii) increased expression of beta2-leukotrienes-integrins^{+} ($CD18^{+}$, $CD11a^{+}$, $CD11b^{+}$), (iii) increased expression of TNFα, and

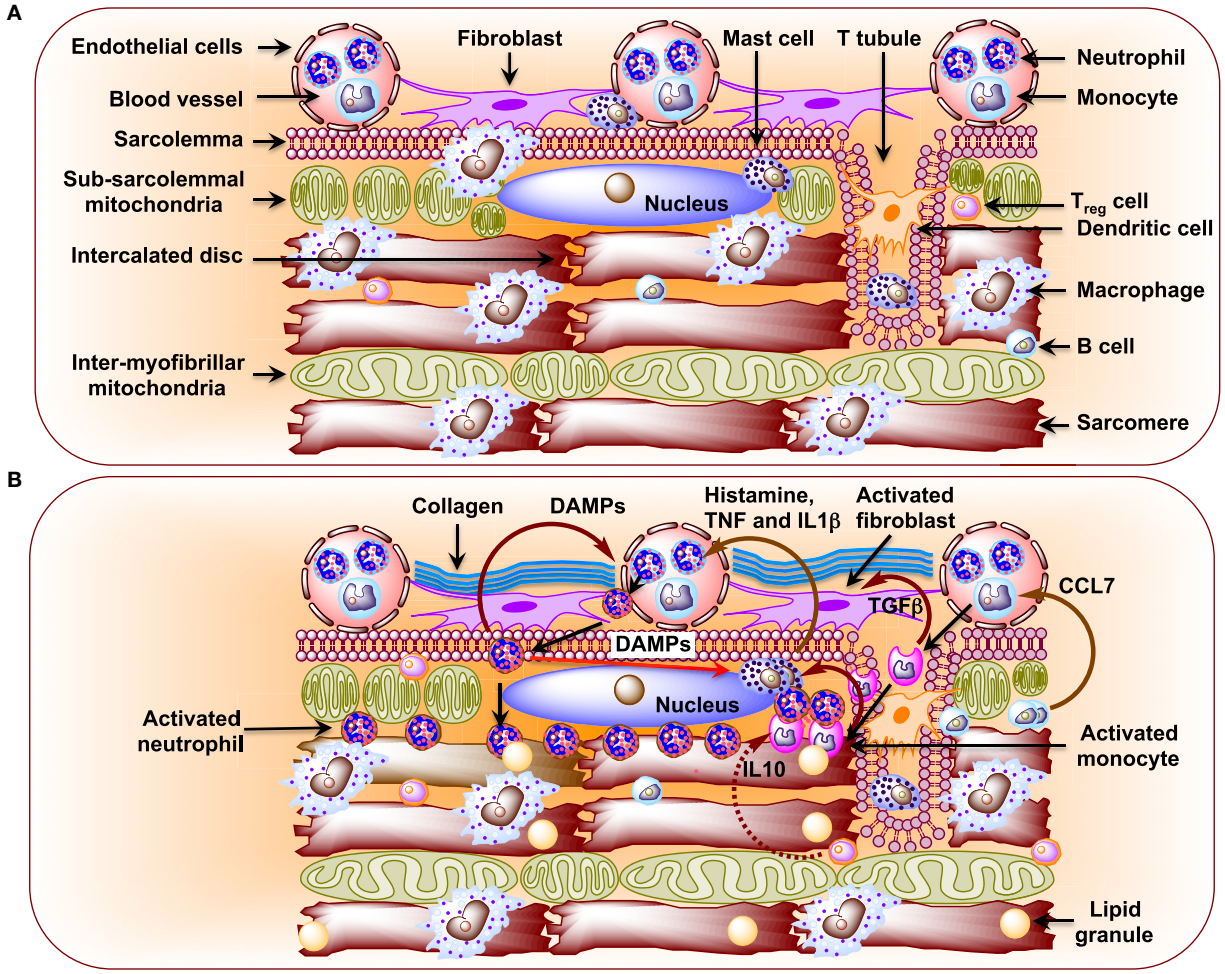

FIGURE 2 | **(A)** Schematic diagram showing immune cells in the healthy heart. Macrophages are the preponderant immune cells in the resting heart and reside primarily surrounding endothelial cells and also in the interstitium among cardiomyocytes (141–143). The less preponderant immune cells include the following: mast cells, dendritic cells, B cells, and regulatory T (T_{Reg}) cells (142, 144, 147). Neutrophils and monocytes, in general, are not detected in the resting heart. **(B)** Schematic diagram showing infiltration of neutrophils and monocytes from the circulation and their effects on resident immune cells in the diabetic cardiomyopathy (DCM) heart. In DCM, activated B cells release CCL7 that activates blood monocytes (146). Upon infiltration activated monocytes stimulate mast cells to release histamine, TNF, and interleukin 1β (IL1β), which activate neutrophils in circulation (144, 174). Activated neutrophils infiltrate heart and activate mast cells through damage-associated molecular patterns as well as blood neutrophils. Activated monocytes secrete TGFβ, which activates fibroblasts to induce formation of collagen.

(iv) IL1β (**Figure 2B**). Treatment of STZ-induced DCM rats with irbesartan (AT-1 receptor antagonist) has been reported to improve cardiac functions by attenuating cardiac inflammation (IL1β, TNFα, and TGFβ) and restoration of MMP activity with consequent decrease in fibrosis (107). Similar results were reported after neutralization of TNFα (163) or genetic deletion of neurokinin receptor B (164) in rodent models of T1DM. Subsequently, several studies confirmed the pro-inflammatory phenotypes in diabetic rodent heart (165–167). As opposed to metabolic responses, immune responses in T1DM and T2DM are comparable as both of them show consistent activation of pro-inflammatory transcription factor NF-κB. Cytokines (i) increase formation of peroxynitrite, which play critical roles in cardiac dysfunction (168), (ii) exert direct effects on the function of SR as well as on the regulation of SR calcium ATPase expression (168, 169), and (iii) increase fibrosis (170). Treatment of rats and humans with statins (171), renin angiotensin aldosterone system (RAAS) inhibitors (107), metformin (172), and thiazolidinediones (173) reduces inflammation in the heart and improve cardiac function.

miRNA IN DIABETIC HEARTS

MicroRNAs are highly conserved endogenous small non-coding RNAs, ~22 nucleotides in length, that regulate gene expression by binding to partially complementary sequences of mRNA (175). The failing hearts consistently show chronic immune activation and aberrant miRNA expression (176). Thus, miR-155 plays an important role in the mammalian immune systems as well as during HF and is abundantly expressed in T-cells, B-cells, and monocytes (177–180). miRNAs are also differentially expressed during HF (181). STZ-induced diabetic heart expresses higher levels of

miR-195 and silencing of miR-195 reduces DCM (182). Likewise, miR-141 is increased in diabetic heart and affects mitochondrial function and ATP generation (183). Palmitate-stimulated neonatal rat cardiomyocytes (NRCs) and diet-induced obese (DIO) mouse heart also showed increased expression of miR-451, which decreases LKB1/AMPK signaling (184). Expression of miR-133a reduces Glut4 expression with consequent decrease in insulin-mediated glucose uptake in NRCs (185). While overexpression of miR-223 in NRCs significantly increased glucose uptake by increasing total Glut4 level and its translocation, inhibition of miR-223 in the heart resulted in a significant decrease in Glut4 expression (186). In contrast to the findings in NRCs, expression of miR-133a is decreased in the hearts of diabetic mice and is associated with increased fibrosis. Of note, overexpression of miR-133a in the heart attenuates cardiac fibrosis (187). Murine miR-322 has recently been shown to provide cardioprotection against consequences of hyperinsulinemia and hyperlipidemia (188). In Ins+/– Akita mice, a model for T1DM, the majority of miRNAs are downregulated in the heart (189), including miR-133a which regulates contractility of the diabetic heart (190). Even after treatment with insulin, which normalizes blood glucose levels, there are several miRNAs that remain differentially regulated in the diabetic heart, and they can potentially contribute to pathological remodeling of the diabetic heart (191). These miRNAs could be a potential target for developing a novel therapeutic strategy for the treatment of diabetic HF.

Diabetes mellitus is a metabolic disease, and miRNAs play a crucial role in the regulation of metabolism (47). Increased levels of plasma cholesterol and triglyceride are common in diabetes, and liver specific ablation of miR-122, the most abundant miRNA in the liver, reduces plasma cholesterol and triglyceride levels (192, 193). The intracellular cholesterol and FA homeostasis are controlled by miR-33a and miR-33b, which target genes involved in cholesterol export including adenosine triphosphate-binding cassette transporters (194–196). Endogenous inhibition or knockout of miR-33 leads to increased plasma high-density lipid levels (194–197). MiR-223 controls the expression of *Glut4* gene in cardiomyocytes, and miR-223 is upregulated while Glut4 is downregulated in human diabetic hearts (186). The switch of glycolysis to FA oxidation is regulated by PPARδ, which is regulated by the miR-199/miR-214 cluster. The miR-199/miR-214 cluster downregulates PPARδ and impairs FA oxidation (198). ROS stimulates apoptosis by mitochondrial cytochrome *c* release and ceramide generation (199). In rat cardiomyocytes, high glucose upregulates miR-34a and miR-1 that reduces the levels of B-cell lymphoma 2 (*Bcl-2*) and insulin-like growth factor 1 (*Igf-1*) genes, respectively, and induces apoptosis (200, 201). Recently, Kuwabara et al. has elegantly shown that miR-451 plays a key role in exacerbating lipotoxicity in cardiac myocytes and high-fat diet-induced cardiac hypertrophy in mice through suppression of the LKB1/AMPK pathway (184). MiR-133a, the most abundant miRNA in the heart, is downregulated in the diabetic mice heart with consequent induction of cardiac hypertrophy (202) and fibrosis (187). Lack of miR-133a also causes contractile dysfunction in the diabetic mice heart (190). These changes cause diastolic dysfunction, which if untreated leads to potential systolic dysfunction (28).

CONCLUSION AND FUTURE PERSPECTIVES

Cardiovascular disease has remained the leading cause of mortality and morbidity in individuals with diabetes. DCM is emerging as an increasing health concern with the epidemic rise in DM worldwide. Animal studies have clearly shown that glycemic control at an early stage prevents the development of DCM, and that certain anti-diabetic drugs exert anti-remodeling effects. While a large body of epidemiological evidence (50,000 T2DM patients) indicate a positive correlation between blood glucose level and/or HbA1c and the risk of HF (203–205), a meta-analysis of randomized controlled trials (37,229 patients) showed no effect of intensive glycemic control on the risk of HF in T2DM patients (206).

Therapeutic approach for DCM depends mainly on (i) glycemic control, (ii) glucose-lowering drug administration, (iii) improvement of autophagy, and (iv) an active life style. Earliest detection, helped by current research on miRNAs, will enhance therapeutic efficacy. The current burst of scientific evidence for the potential use of circulating miRNAs as biomarkers for cardiomyopathy is generating hopes that someday soon detection of specific miRNAs in biofluids of patients will help early treatment of both diabetes and cardiomyopathy.

AUTHOR CONTRIBUTIONS

SM conceived the idea, wrote the immunometabolism part of the manuscript, and made the schematic diagrams. PM wrote the microRNA part of the manuscript and contributed in correcting the final draft of the manuscript. SN contributed to drafting and correcting of the final version of the manuscript. WY, KP, and GB participated in discussion and reviewed/edited the manuscript.

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Chromogranin A Regulation of Obesity and Peripheral Insulin Sensitivity

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Chromogranin A (CgA) is a prohormone and granulogenic factor in endocrine and neuroendocrine tissues, as well as in neurons, and has a regulated secretory pathway. The intracellular functions of CgA include the initiation and regulation of dense-core granule biogenesis and sequestration of hormones in neuroendocrine cells. This protein is co-stored and co-released with secreted hormones. The extracellular functions of CgA include the generation of bioactive peptides, such as pancreastatin (PST), vasostatin, WE14, catestatin (CST), and serpinin. CgA knockout mice (*Chga*-KO) display: (i) hypertension with increased plasma catecholamines, (ii) obesity, (iii) improved hepatic insulin sensitivity, and (iv) muscle insulin resistance. These findings suggest that individual CgA-derived peptides may regulate different physiological functions. Indeed, additional studies have revealed that the pro-inflammatory PST influences insulin sensitivity and glucose tolerance, whereas CST alleviates adiposity and hypertension. This review will focus on the different metabolic roles of PST and CST peptides in insulin-sensitive and insulin-resistant models, and their potential use as therapeutic targets.

Keywords: obesity, insulin resistance, inflammation, chromogranin A knockout, pancreastatin, catestatin

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INTRODUCTION

The human chromogranin A (gene, *CHGA*; protein, CgA) gene encodes a 439-amino-acid mature protein of approximately 48–52 kDa with a coiled-coil structure (1–6). Initially detected in chromaffin granules of the adrenal medulla, this evolutionarily conserved protein is ubiquitously distributed in secretory vesicles of endocrine, neuroendocrine, and neuronal cells. CgA plays a pivotal role in the initiation and regulation of dense-core secretory granule biogenesis and hormone sequestration at the *trans*-Golgi network in neuroendocrine cells (4, 7–9). Increased levels of CgA have been identified in the blood of patients suffering from carcinoids or other neuroendocrine tumors (10–14), heart failure, renal failure, hypertension, rheumatoid arthritis, and inflammatory bowel disease (15–23), indicating an important role of CgA to influence human health and disease (24). Structurally, CgA has 8–10 dibasic sites and is proteolytically cleaved by prohormone convertases (25–27), cathepsin L (28), plasmin (29, 30), and kallikrein (31), generating biologically active peptides including the dysglycemic peptide pancreastatin (PST) ($CgA_{250–301}$) (32, 33); WE14 ($hCgA_{324–337}$) which acts as the antigen for highly diabetogenic $CD4^+$ T cell clones (34–38); the vasodilating, antiadrenergic, and antiangiogenic peptide vasostatin 1 ($CgA_{1–76}$) (39–43); the antiadrenergic, antihypertensive, antibacterial, proangiogenic, and antiobesigenic peptide catestatin (CST) ($CgA_{352–372}$) (44–56); and the proadrenergic peptide serpinin ($CgA_{402–439}$) (57, 58). Several of these CgA-derived peptides

have opposing counter-regulatory effects. For example, cardiac contractility in rodents is controlled by vasostatin ($hCgA_{1–76}$) and CST ($hCgA_{352–372}$), which are antiadrenergic (51, 59) as well as serpinin ($hCgA_{402–439}$), which is proadrenergic (58) (**Figure 1A**). Likewise, angiogenesis is controlled by vasostatin acting in an antiangiogenic manner (43, 56) and CST acting as in a proangiogenic manner (50, 56). These CgA-derived peptides, with diverse functions, emphasize the importance of the CgA proprotein in the regulation of physiological functions (**Figure 1A**). Accordingly, *Chga* whole-body knockout mice present a complex set of metabolic phenotypes and are obese, hyperadrenergic, and hypertensive (48, 60–63). *Chga*-KO mice have become an important model to study the roles of individual CgA-derived peptides through analysis of phenotypes after supplementation (48, 55, 60, 61, 64). Here, we will focus on how two of these peptides, PST and CST, act as important modulators of insulin sensitivity and glucose metabolism.

PST INHIBITS GLUCOSE-STIMULATED INSULIN SECRETION (GSIS)

PST, a C-terminally glycine-amidated 49-mer peptide, was identified in 1986 as a potent inhibitor of glucose-stimulated insulin secretion (GSIS) (32). Two molecular forms were detected in human plasma: a 52 amino acid form ($CgA_{250–301}$) and a larger

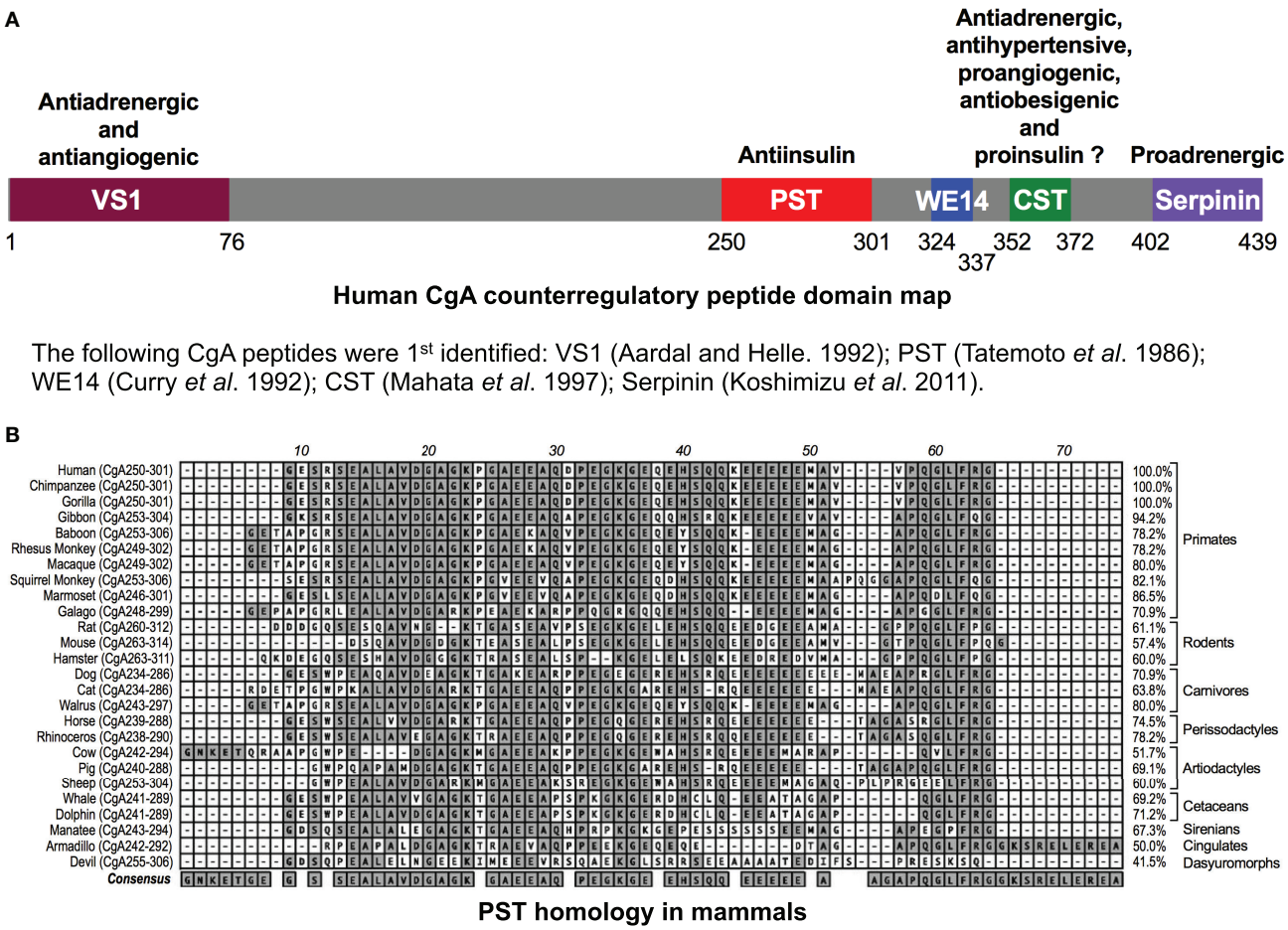

FIGURE 1 | **(A)** Schematic depiction of the domains of the chromogranin A (CgA) protein. Relative locations of vasostatin (VS1), pancreastatin (PST), WE14, catestatin (CST), and serpinin domains in CgA have been illustrated along with the description of their basic functional properties. **(B)** PST homology in mammals. Clustal-W program of MacVector (version 9.0) was used for PST domain alignments across 26 mammalian species. PST amino acid domains were shown on the left and percentage homology as compared to human sequence (100%) was shown on the right. The following gene accession numbers were used for this analysis: human (J03483), chimpanzee (XM_510135), western lowland gorilla (XM_004055595), northern white-cheeked gibbon (XM_003260903), olive baboon (NC_018155.1), rhesus monkey (XM_001092629), crab-eating macaque (AB_169793), Bolivian squirrel monkey (XM_003939842), white-tufted-ear marmoset (XM_002754214), small-eared galago (XM_003786997), Norway rat (XM_346781), house mouse (NM_007693), Chinese hamster (NW_003614307), dog (XM_003639191), cat (XM_003987967), Pacific walrus (XM_004394490), horse (NM_001081814), southern white rhinoceros (XM_004434217), cow (NM_181005), pig (XM_001925714), sheep (XM_004017959), killer whale (XM_004262352), bottle-nosed dolphin (XM_004315772), Florida manatee (XM_004376681), nine-banded armadillo (XM_004475519), and Tasmanian devil (XM_003756143). -, gaps in the alignment.

form with a molecular weight of 15–21 kDa (65). Although the PST sequence is well conserved in mammals, showing 41.5% homology between humans and the Tasmanian devil, no homology could be detected in submammalian vertebrates (**Figure 1B**) (66–68). PST inhibits GSIS *in vivo* in mice, rats, dogs, and pigs, as well as *in vitro* from isolated rat islets (69). In the perfused rat pancreas, PST inhibits unstimulated and stimulated insulin secretion (70–73). In PST-deficient *Chga*-KO mice, GSIS was ~1.7-fold higher at 7 and 15 min after administration of glucose, confirming the inhibitory role of PST in GSIS (60). In addition, PST inhibits glucagon secretion induced by low glucose (74) but had no effect on somatostatin secretion (75). In addition to inhibition of GSIS, PST inhibits insulin-stimulated glucose transport in primary rat and mouse adipocytes (60, 76, 77), differentiated 3T3-L1 adipocytes (68, 78), and primary hepatocytes (60). PST also increases nitric oxide (NO) levels in

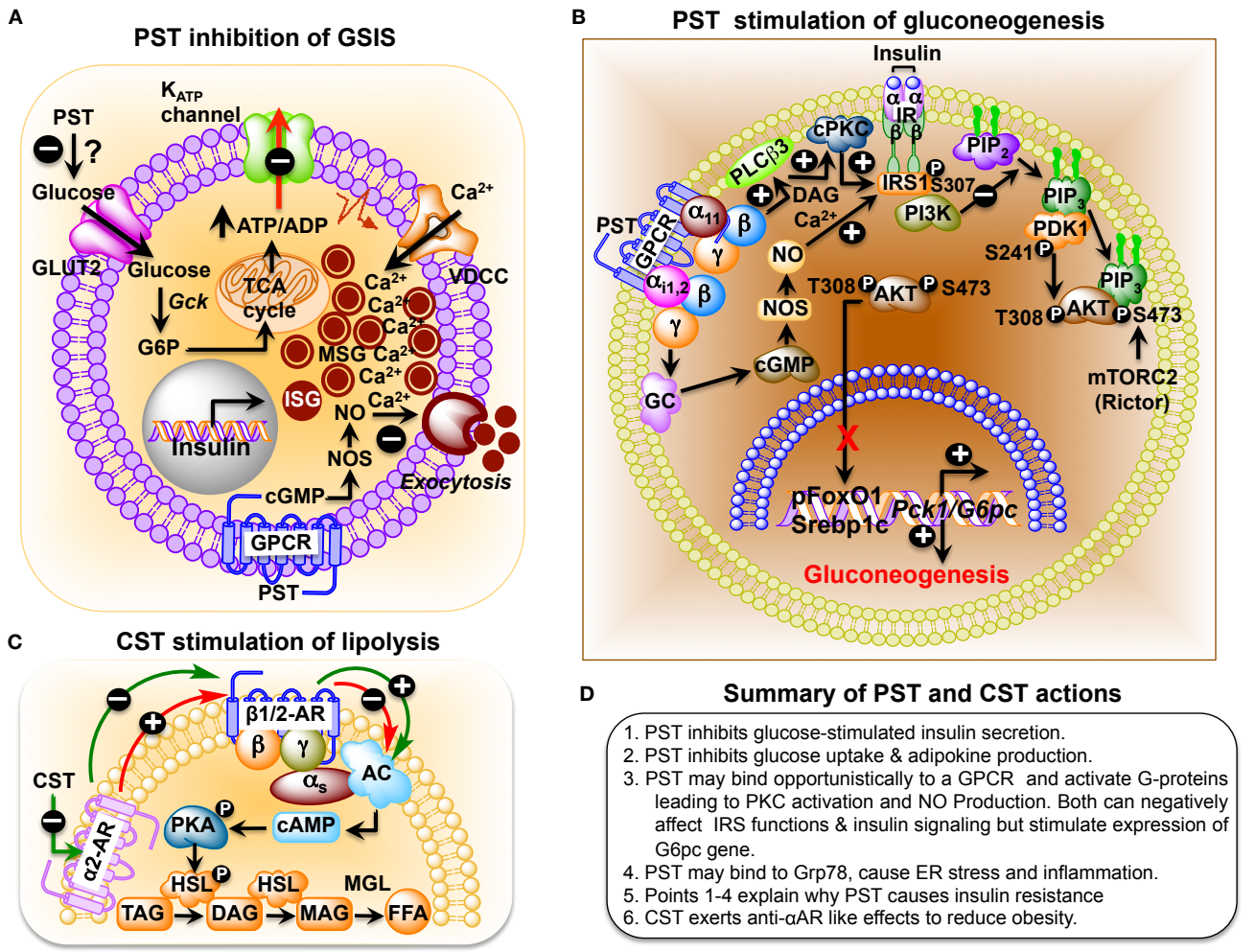

FIGURE 2 | **(A)** Schematic representation of the role of pancreastatin (PST) in the regulation of insulin secretion from pancreatic beta cells. PST-induced nitric oxide (NO) production, following a guanylate cyclase–cGMP–NOS pathway, inhibits glucose-stimulated insulin secretion (GSIS). **(B)** Schematic diagram showing PST inhibition of gluconeogenesis in hepatocytes. PST initiates a GTP-binding protein linked signaling cascade leading to activation of diacylglycerol (DAG) and calcium-dependent conventional PKC (cPKC), which attenuates IRS–PI3K–PDK1–AKT signaling pathway. In addition, stimulation of the cGMP–NOS pathway also assaults this signaling pathway by nitrosylation of IRS. Thus, PST-mediated suppression of this pathway allows forkhead box protein O1 (FoxO1) and sterol regulatory element-binding transcription factor 1c (SREBP1c) to stimulate expression of gluconeogenic genes, phosphoenolpyruvate carboxykinase 1 (*Pck1*) (also known as *Pepck*) and glucose-6-phosphatase (*G6pc*) (also known as *G6Pase*), and thus prevent insulin action. Under control conditions, insulin would have activated this signaling pathway, causing phosphorylation of FoxO1 (promoting its exclusion from the nucleus) and preventing processing of SREBP1 proprotein to SREBP1c with consequent inhibition of expression of gluconeogenic genes and gluconeogenesis. **(C)** Catestatin (CST) stimulation of lipolysis in adipocytes. Activation of α2-adrenergic receptor (α2-AR) inhibits β1/2-AR-induced lipolysis in a dominant way in obesity. CST enhances lipolysis by inhibiting α2-AR, which promotes β1/2-AR action and the consequent downstream signaling. Hormone-sensitive lipase (HSL) is an intracellular, neutral lipase that has broad substrate specificity, catalyzing the hydrolysis of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and cholesteryl esters. Its activity against DAG is about 10- and 5-fold higher than its activity against TAG and MAG, respectively, whereas its activity against cholesteryl esters is about twice its activity toward TAG. The hydrolytic activity of HSL against TAG and cholesteryl esters, but not against DAG, is stimulated by phosphorylation mediated primarily by PKA (84). AC, adenylyl cyclase; FFA, free fatty acids; MGL, monoacylglycerol lipase; PKA, protein kinase A. **(D)** Summary of PST and CST actions.

HTC rat hepatoma cells (79), L6 myotubes (68), and in livers of *Chga*-KO mice (60), showing that PST inhibits insulin action. Since NO inhibits GSIS (80) and PST increases NO production (60, 68, 79), we believe that PST likely inhibits GSIS through activation of the NO pathway (**Figure 2A**).

PST REGULATES HEPATIC GLUCOSE METABOLISM

PST treatment inhibits insulin-stimulated glycogen synthesis in primary hepatocytes (81) and activates glycogenolysis in the rat liver, implicating a direct anti-insulin effect on liver metabolism (82, 83). PST-deficient *Chga*-KO mice show greater suppression of hepatic glucose production (HGP) compared to wild-type (WT) mice during insulin clamp studies (60). Decreased glucose production in *Chga*-KO mice was also supported by decreased glucose production during pyruvate tolerance tests and decreased mRNA transcript levels of the gluconeogenic genes, such as the phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase (*G6pc*), compared to WT mice that were restored to WT levels after supplementation of PST to *Chga*-KO mice (60). PST activates gluconeogenesis by decreasing phosphorylation of insulin receptor substrate 2 at tyrosine residues through activation of conventional PKC and increases production of NO with subsequent attenuated phosphorylation of protein kinase B (AKT), forkhead box protein O1, and reduced matured sterol regulatory element-binding transcription factor 1c (SREBP1c) (**Figure 2B**) (60). These findings are consistent with the anti-insulin action of PST.

PST INFLUENCES LIPID METABOLISM

In addition to glucose metabolism, PST also modulates lipid metabolism. PST decreases insulin-stimulated synthesis of lipids in rat adipocytes (85), which is consistent with the PST-dependent increased expression of hepatic lipogenic genes in *Chga*-KO mice, including *Srebp1c*, peroxisome proliferator-activated receptor-gamma, and glycerol-3-phosphate acyltransferase (*Gpat*) (60). PST also stimulates release of glycerol and free fatty acids from rat adipocytes, which is completely inhibited by insulin (85). In humans, PST augments free fatty acid efflux into the circulation, resulting in an overall spillover of ~4.5-fold, which is consistent with the reported lipolytic action of PST (85), confirming the anti-insulin effects of PST.

PST PROMOTES INFLAMMATION AND INSULIN RESISTANCE

Since PST inhibits the action of insulin on glucose and lipid metabolism, one would expect improved insulin sensitivity in PST-deficient mice. Indeed, *Chga*-KO mice show improved hepatic insulin sensitivity as assessed by insulin tolerance tests (ITTs) showing increased hypoglycemia, and insulin clamp studies showing increased suppression of HGP. Improved hepatic insulin sensitivity was abolished when *Chga*-KO mice were treated with PST, implicating a positive correlation between PST and the development of insulin resistance (60). Similarly, type 2 diabetes mellitus (T2DM) patients show a substantial increase in plasma PST levels (~3.7-fold) (77). Gestational diabetic subjects and patients with non-insulin-dependent diabetes mellitus also show increased plasma PST levels (86, 87).

Feeding mice a high fat diet (HFD) creates obesity, leading to hyperinsulinemia and inflammation (88–92). ITT studies revealed that HFD-fed *Chga*-KO mice displayed improved insulin sensitivity compared to WT mice, demonstrating the importance of PST in the development of IR (64). This was reinforced by hyperinsulinemic–euglycemic clamp studies, where *Chga*-KO-HFD mice displayed increased glucose infusion rates, higher insulin-stimulated glucose disposal rates (IS-GDRs), and suppressed HGP. Recent studies implicate dissociation between obesity and insulin resistance as long as the inflammation is suppressed (64). The presence of supraphysiological levels of PST can reconnect obesity with insulin resistance by introducing inflammation. In the absence of PST, animals are insulin sensitive despite obesity. This is reminiscent of rosiglitazone-treated WT-HFD mice, which are insulin sensitive but obese (93–95).

The hallmarks of insulin resistance in HFD mice are obesity, hyperinsulinemia, and increased inflammation (88–92). Suppression of inflammation in HFD mice can improve insulin sensitivity (93–95). Therefore, the resistance to diet-induced insulin resistance in *Chga*-KO mice may reflect less inflammation in *Chga*-KO mice even after HFD feeding. PST treatment caused increased expression of the pro-inflammatory genes interleukin 1-beta, tumor necrosis factor alpha (*Tnfa*), interleukin 6 (*IL6*), chemokine C–C motif ligand 2 (*Ccl2*), and nitric oxide synthase 2a. Whereas expression of anti-inflammatory genes such as arginase 1 (*Arg1*), interleukin 10 (*IL10*), and C-type lectin domain family member 10a (*Clec10a*) in adipose tissues was higher in *Chga*-KO-HFD mice than WT controls, PST treatment significantly reduced the expression of *Arg1* and *IL10*. Consistent with gene expression data, the plasma levels of IL12p70, Ifng, and chemokine C–C motif ligand 3-like 1 (Ccl3l1), IL6, and chemokine C–X–C motif ligand 1 (Cxcl1) showed significantly decreased levels in *Chga*-KO-HFD versus WT-HFD plasma. PST treatment of *Chga*-KO-HFD mice raised plasma levels of IL12p70 and Ccl2, but had no effect on other proteins measured. PST also exerted direct effects on peritoneal macrophage cultures obtained from WT and *Chga*-KO mice. CgA-deficient peritoneal macrophages demonstrated attenuated response to LPS in the expression of pro-inflammatory cytokines as well as decreased chemotaxis in response to cytokines (64). PST treatment increased the expression of *Tnfa* and *Ccl2* in *Chga*-KO macrophages (64). Thus, it appears that PST acts as a pro-inflammatory peptide but its loss is likely only partially responsible for the improved inflammation seen in *Chga*-KO mice (**Figure 2D**).

Although clamp studies with *Chga*-KO mice fed normal chow diet (NCD) indicated decreased glucose disposal, meaning muscle insulin resistance (60), surprisingly, reduced muscle

insulin sensitivity in lean *Chga*-KO mice was reversed by HFD feeding as demonstrated by improved IS-GDR in muscle of HFD-fed *Chga*-KO mice. Can feeding a high amount of lipids to CgA-deficient mice regenerate cells and repair muscle dysfunction? What kind of lipid could that be? These unorthodox results on the regulation of muscle insulin sensitivity by a CgA-derived protein need further investigation. In this regard, one provocative speculation may deserve some investigation. HFD-induced ceramide and sphingolipids were implicated in the mobilization and differentiation of bone marrow-derived stem/progenitor cells, which are involved in the repair of tissues in ischemic heart disease (96). More specifically, sphingosine-1-phosphate (S1P) acts as a trophic factor for skeletal muscle cell regeneration (97). Sphingolipids are important structural components of cell membranes and are derived from ceramide. Ceramide production is increased in obesity and after HFD feeding (98, 99). Ceramide can be deacylated to sphingosine, which is then phosphorylated by sphingosine kinases to yield S1P. Since this improvement in muscle insulin sensitivity by HFD happened in *Chga*-KO mice, not in WT-DIO mice, absence of CgA protein or peptides triggered this unusual phenomenon. Therefore, it will be very important to investigate the roles of these dietary lipids in muscle repair and the functional relationship of these lipids with the CgA protein and CgA-derived peptides. Alternatively, it is also possible that the absence of CgA protein and its derivatives stimulated release of some myokines in response to dietary lipids, which would otherwise remain suppressed in WT-DIO mice. This response to HFD in *Chga*-KO mice could be muscle specific because muscle expresses CgA (100), and liver and adipose tissue do not (3, 46). Effects of CgA deficiency on liver and adipose tissue may be more systemic in nature, a part of which is carried out by CgA-deficient macrophages (64).

PST PROMOTES ENDOPLASMIC RETICULUM (ER) STRESS BY ATTENUATING EXPRESSION OF Grp78

The accumulation of unfolded and misfolded proteins in the ER lumen, termed ER stress, leads to activation of signaling pathways to counteract defects in protein folding (101–106). This unfolded protein response (UPR) increases repair activities, reduces global protein synthesis, and activates ER-associated protein degradation. However, if ER stress becomes chronic and UPR cannot cope with the repair demands, protein-folding homeostasis breaks down, leading to activation of apoptotic pathways (103, 107, 108). Thus, ER stress and the UPR play important roles in the pathogenesis of multiple human metabolic diseases including insulin resistance, diabetes, obesity, non-alcoholic fatty liver disease, and atherosclerosis (109, 110). The immunoglobulin binding protein (BiP) [also called glucose-regulated protein 78 (Grp78)], is an ER chaperone that is required for protein folding. BiP/Grp78 is a peptide-stimulated ATPase of the Hsp70 family that prevents protein aggregation by stabilizing intermediates in the protein-folding process.

Using ligand affinity chromatography with biotinylated human PST ($hCgA_{273–301}$-amide) as "bait" on a murine liver homogenate (as "prey"), we found that PST interacts in a pH-dependent fashion with Grp78 (78). Whereas NCD-fed *Chga*-KO livers show increased expression of Grp78, PST caused dose-dependent inhibition of Grp78 ATPase activity and inhibited increased expression of Grp78 during UPR activation (by tunicamycin) in hepatocytes (78). In hepatocytes, PST increased expression of *G6pc*. These results indicate that a major hepatic target of PST is the adaptive UPR chaperone Grp78 and that ATPase activity associated with Grp78 is involved in the suppression of glucose production by attenuating *G6pc* expression (78). Grp78s ATPase activity is required to suppress expression of *G6pc*; ER stress and suppression of glucose utilization appear to augment *Grp78* expression (111). Although it is not clear how circulating PST might contact the ER luminal protein Grp78 to modulate ER and insulin action, it has been reported that Grp78 translocates to the cell surface under some pathological conditions (112, 113).

MODULATION OF METABOLISM BY NATURALLY OCCURRING VARIANTS OF PST

Single-nucleotide polymorphism analysis of PST, both *in vivo* and *in vitro*, showed greater inhibition of insulin-stimulated glucose uptake by **Gly**297Ser variants followed by the **Glu**287Arg variants compared to WT-PST (77). The *in vitro* studies also revealed increased expression of gluconeogenic genes by PST variants as compared to WT-PST, with comparable potencies by **Glu**287Arg and **Gly**297Ser variants (68). The **Gly**297Ser subjects displayed markedly elevated plasma glucose and cholesterol compared to the **Gly**297**Gly** individuals. Interestingly, whereas the variants of PST in the C-terminal half of the molecule at 287 (Glu287Arg) and at 297 (Gly297Ser) enhance anti-insulin effects and elevate plasma glucose by inhibition of glucose uptake and stimulation of gluconeogenic effects, experimental deletion of the three N-terminal amino acids Pro–Glu–Gly on human WT-PST demonstrated the opposite effects by reducing plasma glucose level and hepatic gluconeogenesis in a rodent model of obesity (64). Therefore, finding variants in the N-terminal end of PST among the human population may lead to discovery of an allele which would confer protection against insulin resistance and can be used as an insulin-sensitizing peptide such as a N-terminal variant of PST (lacking three amino acids from the N-terminal end) called PSTv1 (64).

REGULATION OF INSULIN SENSITIVITY BY THE PST ANTAGONIST PSTv1

The elevated levels of plasma PST observed in T2DM patients (77) implied that preventing PST action might serve a therapeutic purpose of controlling insulin resistance and diabetes. To demonstrate a direct *in vivo* role of PST in the regulation of insulin sensitivity, WT-HFD mice were injected with the PST variant, PSTv1, which is a competitive antagonist of native PST. PSTv1 lacks the first three N-terminal residues of native PST and blocks

PST-mediated inhibition of glucose uptake and leptin secretion in 3T3-L1 preadipocytes. As predicted, chronic PSTv1 treatment lowered fasting plasma glucose levels in WT-HFD mice and improved glucose tolerance and insulin sensitivity (64). These results suggested that in WT-HFD mice, where the level of PST is high, PSTv1 administration competes with the native PST and phenocopies *Chga*-KO mice. This demonstrates the potential of PST as a therapeutic target for treatment of insulin resistance and diabetes.

CST DECREASES HYPERTENSION AND OBESITY

Hypertensive patients show elevated levels of plasma CgA but decreased plasma CST (114, 115). Low plasma CST predicts augmented pressor responses to environmental stimuli (114). In rats, CST reduces blood pressure responses to activation of sympathetic outflow by electrical stimulation (116). This vasodepressor effect of CST was mediated by massive release of histamine with subsequent vasodilation by histamine-induced production of NO. CST is a potent endogenous inhibitor of catecholamine secretion (44–47, 117–120) and catecholamine-mediated hypertension (48, 121). *Chga*-KO mice showed hyperadrenergic and hypertensive phenotypes that were normalized by intraperitoneal administration of CST (48). CSTs hypotensive effect was also documented in a polygenic model of high blood pressure mice (121). Other studies showed that CST also provides cardioprotection by inhibiting the opening of the mitochondrial permeability transition pore and stimulating the reperfusion injury salvage kinase pathway (122–127).

Catestatin-deficient *Chga*-KO mice are obese on an NCD (48). Chronic CST administration to *Chga*-KO mice reduced epididymal fat pad size to WT level (~25% reduction with respect to body weight of *Chga*-KO mice) (55). CST decreased plasma triglyceride levels in *Chga*-KO mice by increasing lipolysis (increased plasma glycerol and non-esterified fatty acids) through inhibition of α2-adrenergic receptor (α2-AR) (**Figure 2C**) (55). While inhibition of α2-AR by CST indirectly facilitates β-AR mediated lipolysis, CST can also have direct effect on ATGL (adipose triacylglycerol lipase) and HSL (hormone sensitive lipase) via activation of AMPK (128) as it has been demonstrated that activation of AMPK promote lipolysis in adipose tissue through ATGL and HSL. CST-treated *Chga*-KO mice show increased palmitate oxidation but decreased incorporation into lipids, which indicates that CST inhibits expansion of adipose tissue but promotes fatty acid uptake in the liver for oxidation. CST induced expression of several fatty acid oxidation genes including carnitine palmitoyltransferase 1a, peroxisome proliferator-activated receptor-a, acyl-CoA oxidase 1, and uncoupling protein 2, supporting increased fatty acid oxidation in the liver. In addition, CST increased expression of the fatty acid transporter gene *Cd36* and the lipogenic gene glycerol-3-phosphate acyltransferase 4 (*Gpat4*), indicating that CST stimulates fatty acid incorporation into triglycerides but not *de novo* lipogenesis. Overall, CST promoted lipid flux from the adipose tissue toward the liver for beta-oxidation (55). These obesity-reducing effects of CST are mediated by inhibition of α2-AR signaling and enhancement of leptin receptor signaling. In contrast to the negative metabolic effects of PST, CST has beneficial effects that could be utilized in therapeutic treatment of hypertension and obesity.

CONCLUSION AND FUTURE PERSPECTIVES

Chromogranin A is one of the few protein molecules, which can be processed into both negative and positive regulators such as PST and CST for fine-tuning and maintaining metabolic homeostasis. With respect to the pathway of lipid disposal, studies on the direct effect of CST, through activation of AMPK, on lipolytic activities of ATGL and HSL may generate exciting information. Although the metabolic effects of PST and CST have been well investigated, how they transmit signals into cells remains to be determined. Are there specific receptors for these peptides? Alternatively, can they opportunistically bind to some non-specific BiPs on the cell surface and get endocytosed? In some cells such as neutrophils, CST has been shown to be permeable (53). With respect to PST, its binding to Grp78 may occur opportunistically on the cell surface when Grp78, usually a luminal protein, translocates to the cell surface, which occurs under some pathological conditions (112, 113). Whether such interaction happens or not should be a matter of future investigation. If that happens, Grp78 would be able to carry PST to the luminal compartment and initiate a reaction with a small G-protein binding molecule leading to a cascade described in **Figure 2B**. In addition, although PST has been established as an anti-insulin peptide, the mechanisms underlying PST-dependent regulation of insulin secretion are poorly understood. Other CgA-derived pro-insulin peptides may also exist and need to be further investigated. These efforts, as well as generation of PST antagonists, may lead to development of powerful therapeutic treatments for insulin resistance and diabetes. Beyond PST and CST, additional studies should shed light on the role of other CgA-derived peptides in metabolism, with implications for treatment of metabolic disease.

AUTHOR CONTRIBUTIONS

SM conceived the idea. GB and SM contributed equally to researching the data and writing of the manuscript.

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Reduced Hepatic Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 Level in Obesity

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Impairment of insulin clearance is being increasingly recognized as a critical step in the development of insulin resistance and metabolic disease. The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) promotes insulin clearance. Null deletion or liver-specific inactivation of Ceacam1 in mice causes a defect in insulin clearance, insulin resistance, steatohepatitis, and visceral obesity. Immunohistological analysis revealed reduction of hepatic CEACAM1 in obese subjects with fatty liver disease. Thus, we aimed to determine whether this occurs at the hepatocyte level in response to systemic extrahepatic factors and whether this holds across species. Northern and Western blot analyses demonstrate that *CEACAM1* mRNA and protein levels are reduced in liver tissues of obese individuals compared to their lean age-matched counterparts. Furthermore, Western analysis reveals a comparable reduction of CEACAM1 protein in primary hepatocytes derived from the same obese subjects. Similar to humans, *Ceacam1* mRNA level, assessed by quantitative RT-PCR analysis, is significantly reduced in the livers of obese Zucker (*fa/fa*, ZDF) and Koletsky (*f/f*) rats relative to their age-matched lean counterparts. These studies demonstrate that the reduction of hepatic CEACAM1 in obesity occurs at the level of hepatocytes and identify the reduction of hepatic CEACAM1 as a common denominator of obesity across multiple species.

Keywords: insulin clearance, insulin resistance, obesity, carcinoembryonic antigen-related cell adhesion molecule 1, hyperinsulinemia, fatty liver disease

INTRODUCTION

The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is ubiquitously expressed (1). CEACAM1 protein is expressed highly in liver, but to an insignificant extent in white adipose tissue and skeletal muscle, among classical insulin target peripheral tissues (1). Upon its phosphorylation by the insulin receptor tyrosine kinase in the hepatocyte (2), CEACAM1 promotes the uptake of insulin *via* its receptor to be degraded and cleared (3, 4). Bolstering this function of CEACAM1, defective hepatic insulin clearance and subsequently, chronic hyperinsulinemia develops

in mice with global null mutation (*Cc1*$^{-/-}$) or with liver-specific overexpression of the dominant-negative phosphorylation-defective inactive isoform of Ceacam1 (L-SACC1) (5–7). At least in part by downregulating the insulin receptor number (8), chronic hyperinsulinemia causes insulin resistance in these mice (5–7). Consistent with its positive effect on *de novo* lipogenesis (9), hyperinsulinemia also causes hepatic lipid accumulation, as well as lipid redistribution to the white adipose depot for storage, resulting in elevated visceral obesity. Contributing to visceral obesity and increased total fat mass in *Cc1*$^{-/-}$ mice is leptin resistance, manifested by hyperphagia and reduced spontaneous physical activity (10).

In humans and rodents, high-fat diet causes insulin resistance and visceral obesity. Recent data from our laboratories show that high-fat intake causes a decrease in hepatic CEACAM1 level by >50% within 3 weeks (11), and that this appears to play a causative role in diet-induced insulin resistance insofar as adenoviral-mediated delivery of CEACAM1 in liver reverses the metabolic abnormalities associated with increased fat intake, including insulin resistance, hepatosteatosis, and visceral obesity (12). Similarly, transgenic overexpression of CEACAM1 in liver protects against diet-induced insulin resistance, visceral obesity, hepatosteatosis, and fibrosis in adipose tissue (11).

Together, this assigns a significant role for reduced hepatic CEACAM1 levels in hyperinsulinemia-driven metabolic abnormalities, including insulin resistance and hepatic steatosis in mice. It also provided the impetus to investigate whether reduction of hepatic CEACAM1 level occurs at the hepatocyte level and whether it is common in obesity across multiple species.

MATERIALS AND METHODS

Animal Care and Husbandry

Obese male Zucker fatty (*fa/fa*—8 weeks of age) and Zucker Diabetic Fatty rats (ZDF—12 weeks of age), and Koletsky spontaneous hypertensive rats (*f/f*—16 weeks of age) and their age-matched lean controls were purchased from Charles River Laboratories. Rats were fed *ad libitum* a regular chow diet and kept in a 12-h dark–light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee at the University of Toledo College of Medicine and Life Sciences (formerly known as the Medical College of Ohio). All experiments were conducted in accordance with the recommendations of the committee, confirming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Assessment of Plasma Biochemistry

Biochemical parameters were assessed in plasma drawn from overnight fasted rats. Plasma insulin and C-peptide levels were determined by radioimmunoassays (Linco Research) and their molar ratio at steady state was calculated as a marker of insulin clearance. Plasma triglyceride (TG) levels were assayed by Triglycerides reagent (Pointe Scientific) and plasma free fatty acids (FFA) by NEFA C kit (Wako). Hepatic TG content was assayed in tissues separated by chloroform–methanol, as previously described (12).

Human Primary Hepatocytes

Livers and freshly isolated primary hepatocytes derived from the same lean and obese subjects were purchased from Cellzdirect (www.cellzdirect.com). The subjects include seven anonymous coded obese (body mass index >30 kg/m^2) 45- to 50-year-old male subjects and four age-, sex-, and race-matched lean subjects. All subjects were non-smokers, non-alcoholics with no history of drug abuse, or other known health conditions or exposure to infectious diseases.

Specimens and cells were sent de-identified, labeled with a code with no other identifiable information. Hence, studies were exempted by the Institutional Review Board at the University of Toledo College of Medicine and Life Sciences (previously known as the Medical College of Ohio).

Western Blot Analysis of Human CEACAM1 Protein Levels

Lysates from primary hepatocytes and liver were analyzed by 4–12% SDS-PAGE followed by immunoblotting (Ib) with polyclonal antibody against CEACAM1 (13), and normalization against GAPDH (Santa Cruz).

Northern Blot Analysis of Rat *Ceacam1* mRNA Level

As previously described (11), Northern blot analysis was performed on total liver RNA extracted by TRIzol (Invitrogen), purified by MicroPoly (A) Pure Kit (Ambion), and sequentially probed with cDNAs for Ceacam1 followed by Gapdh for normalization, using the Random Primed DNA Labeling Kit (Roche).

Quantitative RT-PCR Analysis of Rat *CEACAM1* mRNA Level

qRT-PCR was performed in homogenized liver lysates as routinely performed (14). Briefly, total RNA was extracted by TRIzol (GIBCO BRL) and first strand cDNA was synthesized using Superscript II (Invitrogen) and oligo dT, and real-time RT-PCR was carried out using the Applied BioSystem. The long isoform of *CEACAM1* was amplified using the following primers: F: 5′-CAGCGCTGGCATACTTCCTT-3′, R: 5′-CACT TCCCCCGCCAGTCT-3′. As control, *β-Actin* was amplified using the primers: F: 5′-ATCAAGATCATTGCTCCTCCTGA-3′, R: 5′GAGCCACCAATCCACACAGAG-3′. At least one primer of each pair is located in the junction of two exons to avoid amplification of genomic DNA. Ct values (cycle threshold) were used to calculate the amount of amplified PCR product relative to *β-Actin*. The relative amount of mRNA was calculated as $2^{-\Delta CT}$.

Statistical Analysis

Data were analyzed with SPSS software using one-factor ANOVA analysis or Student's *t*-test. Values are expressed as mean ± SEM. $^{A}P < 0.05$ obese versus lean/genotype.

RESULTS

Reduced Hepatic CEACAM1 Levels in Tissues from Obese Humans

Northern analysis indicates that *CEACAM1* mRNA levels, normalized to *GAPDH*, are significantly lower (by ~>60%) in the liver of obese human subjects by comparison to those derived from their lean sex-, race-, and age-matched counterparts (**Figure 1A**). This translates into reduced hepatic CEACAM1 protein levels in lysates derived from livers (**Figure 1B**) of obese human subjects, as assessed by Western blot analysis using Ib with antibodies against human CEACAM1 and GAPDH (to normalize against total protein loading). Moreover, obese subjects exhibit hepatic fat accumulation, as assessed by the twofold to threefold higher hepatic TG level in obese subjects (50.2 ± 4.5 versus 20.3 ± 2.2 mg/g liver tissue, $P < 0.05$).

Reduced CEACAM1 Protein Content in Primary Hepatocytes from Obese Humans

Because metabolic factors such as insulin and fatty acids regulate Ceacam1 expression in hepatocytes, with insulin inducing its transcription (15) and fatty acids repressing it (16), we then aimed to examine whether the decline in hepatic CEACAM1 occurs at the hepatocyte level. To this end, we examined the protein level of CEACAM1 in primary hepatocytes derived from the same obese and lean subjects whose livers were used to assess hepatic CEACAM1 levels (see above). Western blot analysis using antibodies against human CEACAM1 for Ib indicates ~50% reduction (graph) in CEACAM1 protein level in primary hepatocytes derived from obese as compared to their sex- and age-matched lean counterparts (**Figure 2**).

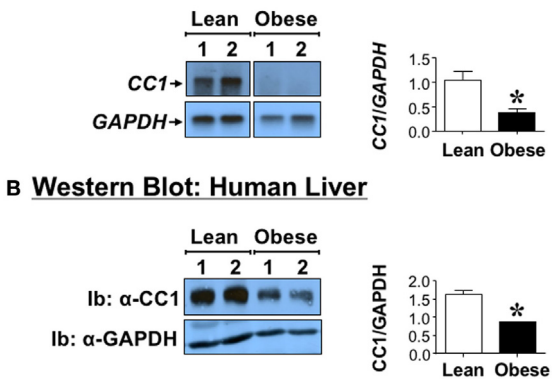

FIGURE 1 | Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) level in human livers. Livers derived from anonymous obese (body mass index >30 kg/m^2) 45- to 50-year-old male subjects and age-, sex-, and race-matched lean subjects. **(A)** *CEACAM1* mRNA (*CC1*) was analyzed by Northern blot analysis of total liver mRNA and sequentially probed with cDNAs for *CEACAM1* (*CC1*) followed by *GAPDH* for normalization. **(B)** Liver lysates from obese and lean subjects were analyzed by 4–12% SDS-PAGE followed by immunoblotting (Ib) with polyclonal antibody against CEACAM1 (CC1) and normalization against GAPDH. For simplicity, only two samples of each group are shown as representatives of three independent experiments. The graph on the right represents densitometry analysis of CEACAM1 bands relative to those of GAPDH in all tissues. Values shown as mean ± SEM with $^*P < 0.05$ being statistically significant.

Reduced Hepatic CEACAM1 Levels in Obese Rats

To investigate whether the reduction in hepatic CEACAM1 in obesity is common among species, we then examined mRNA levels of *Ceacam1* in the livers of obese male rats. These include obese Zucker hyperphagic rats without diabetes (*fa/fa*) or with diabetes (Zucker Diabetic Fatty rats—ZDF) (17), and obese spontaneous hypertensive Koletsky rats (*f/f*) (18). qRT-PCR analysis revealed a ≥50% decrease in hepatic *Ceacam1* mRNA levels in obese relative to lean rats (**Figure 3**). Consistent with a role for CEACAM1 in insulin clearance (6), obese rats display reduced insulin clearance (as measured by steady-state C-peptide/insulin molar ratio) and hyperinsulinemia (**Table 1**). As expected, they also exhibit elevated body weight, fasting plasma FFA, and plasma and hepatic TG levels (**Table 1**).

DISCUSSION

Using several genetically modified mouse models of loss- and gain-of-function of Ceacam1, we have demonstrated that

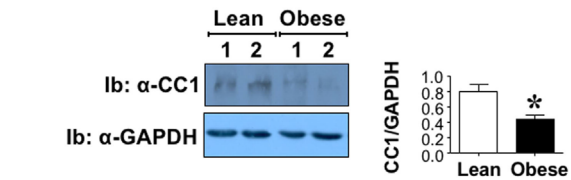

FIGURE 2 | Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) level in human primary hepatocytes. Primary cells were derived from the livers of the same anonymous donors and analyzed by Western analysis as in **Figure 1**, immunoblotting (Ib) with polyclonal antibody against CEACAM1 (CC1) and normalizing against GAPDH. For simplicity, only two samples of each group are shown as representatives of three independent experiments. The graph on the right represents densitometry analysis of CEACAM1 bands relative to those of GAPDH in all cells. Values shown as mean ± SEM with $^*P < 0.05$ being statistically significant.

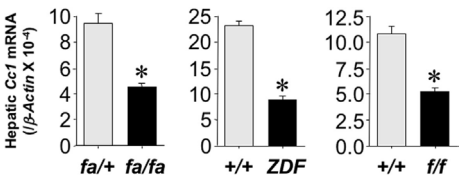

FIGURE 3 | Hepatic carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) level in obese rodents. *Ceacam1* mRNA was analyzed by qRT-PCR analysis of total liver RNA, normalized to *β-Actin*, from livers derived from obese *fa/fa*, ZDF, and Koletsky *f/f* rats and age-matched lean controls (n = 10/lean or obese/each strain). Values shown as mean ± SEM with $^*P < 0.05$ being statistically significant.

TABLE 1 | Biochemical parameters in obese rats.

	Zucker *fa/fa*		Zucker ZDF		Koletsky (*f/f*)	
	Lean	**Obese**	**Lean**	**Obese**	**Lean**	**Obese**
Body weight (g)	254 ± 10	330 ± 9[A]	275 ± 11	352 ± 10[A]	315 ± 9	458 ± 12[A]
Insulin (ρM × 10^2)	3.0 ± 0.8	18.2 ± 1.6[A]	0.9 ± 0.1	7.2 ± 1.0[A]	1.0 ± 0.3	25.5 ± 0.6[A]
C-peptide (ρM × 10^3)	1.1 ± 0.2	3.3 ± 0.2[A]	1.3 ± 0.1	3.0 ± 0.4[A]	1.5 ± 0.4	5.2 ± 0.2[A]
C-peptide/insulin ratio	3.8 ± 0.6	1.8 ± 0.1[A]	15.7 ± 1.3	5.0 ± 0.5[A]	14.6 ± 0.6	2.0 ± 0.1[A]
FFA (mEq/l)	0.5 ± 0.0	1.4 ± 0.1[A]	0.6 ± 0.1	1.1 ± 0.2[A]	0.7 ± 0.1	1.1 ± 0.2[A]
TG (mg/dl)	14 ± 3	246 ± 42[A]	65 ± 3	531 ± 58[A]	112 ± 8	385 ± 29[A]
Hepatic TG (mg/g tissue)	23 ± 2	63 ± 7[A]	27 ± 3	73 ± 5[A]	34 ± 4	78 ± 9[A]

Frozen liver tissues and plasma were extracted from 10 obese and 10 age-matched lean overnight fasted rats. These include fa/fa (8 weeks of age); ZDF (12 weeks of age), and Koletsky f/f (16 weeks of age). Values are expressed as mean ± SEM. [A]P < 0.05 obese versus lean/genotype. C-peptide/insulin molar ratio was used as determinant of insulin clearance. FFA, free fatty acids; TG, triglycerides.

CEACAM1 plays a critical role in promoting hepatic insulin clearance, and that its loss in the liver causes chronic hyperinsulinemia followed by systemic insulin resistance, altered lipid homeostasis, hepatosteatosis, and visceral obesity (5–7). That defective insulin clearance contributes significantly to these obesity-associated metabolic abnormalities has been demonstrated in several species, including humans (19–22). Thus, it has become imperative to investigate whether hepatic CEACAM1 level is commonly reduced among species. The current studies demonstrate that by comparison to lean controls, CEACAM1 level is reduced in the liver of age- and sex-matched obese human subjects and in three rat models of obesity resulting from null mutation of leptin receptor (17, 23).

Although the data on human subjects need to be strengthened by a much larger cohort of patients, they are consistent with a report finding a marked decline in hepatic CEACAM1 levels in 29% of 99 obese subjects with insulin resistance and non-alcoholic fatty liver disease, with a higher incidence of CEACAM1 loss in individuals with high-grade fatty liver and severe obesity, independently of type 2 diabetes (24). That this occurs independently of diabetes and fasting hyperglycemia is consistent with normal insulin secretion and fasting normoglycemia in Ceacam1 mutant mice (7). Moreover, sustained reduction of CEACAM1 protein content in primary hepatocytes derived from the same steatotic livers of obese donors demonstrates that the defect in CEACAM1 expression occurs at the hepatocyte level. We have recently shown that the rise in fatty acids release from adipocytes during high-fat feeding of mice progressively represses Ceacam1 expression in the hepatocyte by activating a mechanism depending on the activation of peroxisome proliferator-activated receptor α by fatty acids (25) and that this bestows a positive feedback mechanism on fatty acid β-oxidation (12). When the loss of hepatic CEACAM1 reaches more than 50% and impairment of insulin clearance develops, chronic hyperinsulinemia followed by hepatic steatosis ensues (12). Increased lipolysis-driven hepatic fatty acid β-oxidation in humans with uncomplicated obesity (26) and its role in regulating hepatic *de novo* lipogenesis (27, 28) propose an important role for the loss of hepatic CEACAM1 in the regulation of lipid homeostasis in hepatocytes derived from obese humans.

Obese Zucker and Koletsky hyperphagic obese rats display a decline in their hepatic CEACAM1 content likely causing impaired insulin clearance and hyperinsulinemia. They also manifest elevated visceral obesity with high fasting plasma FFA, and an increase in plasma and hepatic TG levels, consistent with the phenotype of *Ceacam1* mutant mice (5–7).

Similarly, rats selectively bred for low aerobic running capacity (LCR) exhibit metabolic syndrome, including hyperinsulinemia, insulin resistance, obesity, and hypertension. By comparison to age-matched high capacity runners (HCR) (29), they also exhibit hepatic steatosis (30). Hyperinsulinemia in LCR rats is associated with impaired hepatic insulin clearance in correlation with reduced *Ceacam1* mRNA (29) and protein levels (14). Caloric restriction reduces their hyperinsulinemia, and subsequently, hepatic fatty acid synthase level and steatosis, in parallel to inducing hepatic CEACAM1 levels and normalizing hepatic insulin extraction to the level of HCR (14). Whether low hepatic *Ceacam1* level in LCR by comparison to HCR is a cause or a consequence of increased release of plasma FFA from the white adipose tissue in these rats (12, 25) remains to be determined, but it is intriguing that CEACAM1 expression is modulated by the selection for low aerobic running capacity that leads to the metabolic anomalies of LCR rats.

In summary, the current studies demonstrate a strong association between reduced CEACAM1 expression in hepatocytes with obesity, hepatic steatosis, and dyslipidemia across species and multiple rat strains.

AUTHOR CONTRIBUTIONS

GH researched data, designed experiments, and wrote the manuscript. HM, HG, SG, KR, QA-S, AD, TB, and DZ researched data. RG designed experiments, and extracted tissues and plasma from rats. LY designed experiments in human tissues and reviewed the manuscript. SN was responsible for study design, conceptualization, data analysis, and results interpretation, and reviewing the manuscript; had full access to all the data of the study and takes responsibility for the integrity and accuracy of data analysis and the decision to submit and publish the manuscript.

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MINI REVIEW
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Systemic Regulators of Skeletal Muscle Regeneration in Obesity

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Skeletal muscle maintenance is a dynamic process and undergoes constant repair and regeneration. However, skeletal muscle regenerative capacity declines in obesity. In this review, we focus on obesity-associated changes in inflammation, metabolism, and impaired insulin signaling, which are pathologically dysregulated and ultimately result in a loss of muscle mass and function. In addition, we examine the relationships between skeletal muscle, liver, and visceral adipose tissue in an obese state.

Keywords: skeletal muscle, obesity, inflammation, metabolism, insulin

INTRODUCTION

Current estimates are that one-third of the United States population is obese, and this number is rapidly escalating (1). Many of these patients additionally suffer from preclinical or overt type 2 diabetes mellitus (T2DM) (1, 2). Multiple studies suggest that skeletal muscle wasting in these patients, especially those above the age of 60 years, can be severe (3–5). Diminished capacity for skeletal muscle regeneration likely contributes to the loss of lean muscle mass seen in diabetic patients (6). Obesity, a common precursor to T2DM, is also noted to have significant and independent negative effects on lean skeletal muscle mass (7). This is correlated with insulin resistance and reduced muscle performance (8). Overall, these patients suffer from a significant decline in muscle strength, as compared to age-matched controls, and a loss of functional independence (3–5). However, the effects of obesity on skeletal muscle regeneration remain largely unknown. Stimulation or preservation of skeletal muscle regeneration could possibly enable these patients to improve their strength and functional activity, as well as maintain skeletal muscle mass (8, 9).

Recent studies demonstrate that mice fed a high-fat diet (HFD) exhibit reduced hind limb muscle mass and form fewer and smaller fibers following skeletal muscle injury (10). Additionally, there exists a reduction in the total number of satellite cells, which are required for skeletal muscle regeneration (10, 11). Therefore, it is of significant clinical importance to understand how obesity impacts muscle regeneration and identify mechanisms that may be targeted for therapeutic benefit. Skeletal muscle mass in these patients is not only essential for ambulation but also necessary for glucose utilization and maintaining insulin sensitivity (12). Multiple factors affect muscle mass in patients with obesity including satellite cell function, inflammation, insulin signaling, and metabolic derangements. Furthermore, obesity-related increases in visceral adipose tissue (VAT) and fatty acid accumulation in the liver, as with non-alcoholic fatty liver disease (NAFLD), are intimately linked to the maintenance of muscle mass. When evaluating obesity and corollary loss of skeletal muscle mass, systemic mediators and their effect on muscle regeneration must be considered.

SATELLITE CELLS

Satellite cells in skeletal muscle are located beneath the basal lamina of mature muscle fibers and are thought to be the major source of regeneration following muscle injury (13). It is now known that the satellite cell population is heterogeneous and contains both myogenic and non-myogenic cell populations. Using fluorescence-activated cell sorting (FACS), unique,

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myogenic stem cells, or skeletal muscle precursors (SMPs), within the satellite cell pool can be identified and isolated for further study (14). Early life obesity, induced by HFD, results in both a reduction in SMP cell frequency and impaired differentiation (10). Specifically, myogenic differentiation (MyoD), a critical factor in promoting skeletal muscle differentiation, is significantly reduced in satellite cells isolated in a diet-induced obesity (DIO) murine model (15).

In addition, satellite cell activation in a murine DIO model is impaired, which can be partially attributed to a loss in hepatocyte growth factor (HGF) signaling in skeletal muscle (16). Skeletal muscle-specific decrease in active HGF following injury limits activation of satellite cells from their quiescent state. HGF activation of SMPs requires AMP-activated protein kinase (AMPK), a protein essential to maintain satellite cell number and induce myotube formation. The active, or phosphorylated form of AMPK, promotes skeletal muscle glucose uptake and increases insulin sensitivity. Recent studies suggest that the satellite cells isolated from injured muscles of DIO mice demonstrate decreased AMPK activity and impaired regeneration (17). Fibrogenic/adipogenic precursors (FAPs) are a separate and distinct population in the satellite cell compartment (18). These cells are unable to directly form myofibers but can promote the differentiation of SMPs or form adipose tissue based on the local environment (18–20). A common observation in conditions associated with impaired skeletal muscle insulin sensitivity is an accumulation of ectopic lipids within (*intracellular*) and between (*extracellular*) skeletal muscle fibers (21), which is linked to reduced insulin sensitivity and diminished muscle function (22). The contribution of FAPs to these intramuscular lipid deposits remains unknown, as does their precise contribution to skeletal muscle regeneration in a model of obesity.

INFLAMMATION

Obesity results in chronic, low-grade skeletal muscle inflammation (23). Recent studies further suggest DIO alone can reprogram both skeletal muscle and liver to increase the production of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), and IL-6 (24). Increased IL-6 has been shown to limit skeletal muscle differentiation *in vitro* (25). In murine models of cachexia, both IL-6 or nuclear factor-kappa B (NF-κB) overexpression in skeletal muscle causes severe muscular atrophy (26, 27). Separately, in persistent inflammatory conditions, IL-6 actions are associated with increased muscle wasting (28). Despite multiple studies suggesting that skeletal muscle-specific upregulation of proinflammatory cytokines induces muscle wasting; this area warrants further research in regard to obesity. To date, no studies have shown that IL-6 or NF-κB inhibition, either systemically or in skeletal muscle, improves regeneration in an obesity model. In addition, it remains unclear whether local inflammation from skeletal muscle, increased cytokine release from liver or visceral fat, or a combination are required for impaired muscle regeneration and loss of muscle mass.

TNF-α, another proinflammatory cytokine, also has catabolic effects on muscles in chronic inflammatory state. Elevated TNF-α circulation in obese models can cause muscle wasting, inflammatory myopathies, and insulin resistance by regulating activation and secretion of other proinflammatory cytokines (29, 30). TNF-α supplementation additionally limits C2C12 muscle myoblast cell differentiation *in vitro* by repressing MyoD synthesis. The effects of TNF-α on skeletal muscle regeneration in an obese model remains unknown.

In addition, obesity further promotes deposition of macrophages in VAT, which contributes to inflammation, increased lipolysis, and subsequently ectopic fat deposition in skeletal muscles (31). In the early stages of obesity, an increase in these macrophages precedes T cell accumulation. T cells, in turn, are polarized into proinflammatory Th1 cells that cause myocyte inflammation through interferon secretion. The inhibition of ectopic macrophage accumulation in fat may reverse insulin resistance and thereby improve muscle function (32, 33). These points further highlight that muscle wasting in obesity is a systemic issue, instead of secondary to local changes in skeletal muscle alone.

INSULIN RESISTANCE

An array of growth factor signaling cascades, regulated by insulin, are required for the proper maintenance of skeletal muscle mass. Obesity-associated insulin resistance alters these pathways and can variably inhibit muscle regeneration. Insulin signaling is a highly complex pathway within skeletal muscle, mediated by insulin growth factor-1 (IGF-1) (34). Specifically, downstream of IGF-1, both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways are known to regulate skeletal muscle regeneration (35).

Mitogen-activated protein kinases are enzymes that become catalytically activated in response to diverse stimuli such as mitogens, osmotic stress, and proinflammatory cytokines. MAPK activity mediates the crosstalk between canonical and non-canonical transforming growth factor (TGF-β) in a DIO model (36). In skeletal muscle, TGF-β1 inhibits differentiation of fetal myoblasts (37). Separately, increased levels of TGF-β can cause muscle injury to heal with fibrosis, rather than regenerated skeletal muscle (38). Increased p38 MAPK and TGF-β activity within ectopic adipocytes may induce satellite cell senescence (39). Paradoxically, results from C2C12 studies, a murine myoblast model for skeletal muscle development, demonstrates a positive role for activated MAPK in cell migration (40). MAPK signaling and activity remain controversial with respect to skeletal muscle regeneration in obesity, and this topic warrants further research. Interestingly, follistatin supplementation improves muscle growth in circumstances with elevated TGF-β signaling (41).

In models of muscular dystrophy, an increase in PI3K activity can be beneficial for regeneration, as it increases Akt activity and downstream, promyogenic factors, which stimulate muscle growth. Akt activation also helps in preventing muscle atrophy by inducing the expression of mammalian target of rapamycin and ribosomal protein S6 kinase beta-1 (S6K1) (42). Specifically, in DIO models, an increase in Akt activity by

phosphatase and tensin homolog (PTEN) inhibition restores skeletal muscle regeneration (11). The role of decreased insulin signaling with regard to skeletal muscle injury remains a topic of active research.

METABOLISM

Obesity and chronic overnutrition are closely associated with increased mitochondrial-derived oxidative stress (43, 44). Skeletal muscle from obese or diabetic patients shows decreased mitochondrial content and a corollary loss of fatty acid oxidation (45, 46) associated with excess caloric consumption and non-inherent mitochondrial dysfunction (47). In patients with T2DM, targeted overexpression of catalase within mitochondria can protect skeletal muscle from ischemic injury, but the role of oxidative stress and mitochondrial dysfunction in obesity-related loss of skeletal muscle regeneration remains unknown (48, 49). In the context of obesity, skeletal muscle undergoes a protective shift to retain its functional capacity by converting to glycolytic, type II muscle fibers, mediated by Brg1/Brm-associated factor (Baf60c) (50–52). The Baf60c pathway increases Akt activation, which, as discussed previously, improves diet-based glucose tolerance and increases insulin sensitivity. Independently, muscle-specific Akt activation also leads to hypertrophy of type II muscle fibers with subsequent resolution of hepatic steatosis, decreased fat mass, and improved metabolic parameters (53). However, Baf60c signaling is decreased in obese rodent models, possibly due to the inhibitory effects of TNF-α (51, 52).

In contrast, many studies suggest that hypertrophy of oxidative muscle fibers (type I) can also promote metabolic homeostasis. Muscle-specific overexpression of peroxisome proliferator-activated receptor-delta (PPAR-δ) (54, 55) promotes higher levels of type I fibers relative to type II fibers, improved performance in endurance, exercise, and resistance to DIO. Conversely, mice that are deficient in peroxisome proliferator-activated receptor-gamma (PPAR-γ) coactivator 1-alpha (PGC-1α) display abnormal oxidative fiber growth and develop an increase in body fat (56). These studies suggest that increased energy expenditure in skeletal muscle mediated by hypertrophy can protect against weight gain and metabolic dysfunction. In addition, myostatin-deficient mice are resistant to DIO (57), but this metabolic effect may be due to either changes in type I or type II fibers, or from the direct action of myostatin on adipose tissue (58). Overall, it remains unclear whether or not a type I or type II fiber majority contributes to the improvement in metabolic parameters in DIO, but an increase in muscle mass, in general, appears to counteract the metabolic derangements seen in obesity.

In the absence of muscle hypertrophy, reactive oxygen species (ROS) accumulate in skeletal muscle. In obese conditions, increased ROS production is associated with contractile dysfunction, chronic oxidative stress followed by protein loss, and muscle atrophy (59). ROS is also capable of modulating the insulin signaling pathway, although the exact mechanism remains unclear. Studies suggest that ROS decreases insulin response and contributes to impaired mitochondrial activity (60). Sirtuin (SIRT), a NAD(+)-dependent histone deacetylase (HDAC) localized in mitochondria, has been found to regulate several mitochondrial genes and is important in muscle differentiation, activation of myogenesis, and skeletal muscle metabolism. Specifically, SIRT1 promotes glycolysis and inhibits adipogenesis, thereby attenuating obesity-related insulin resistance (61–63). Conversely, in T2DM, inhibition of SIRT1 alters mitochondrial metabolism and increases the production of ROS (64).

Histone deacetylases, in general, are a group of enzymes that regulate gene expression by altering chromatin structure. In obesity models, HDAC inhibition restores PPAR-γ function improving skeletal muscle glucose and fatty acid metabolism. HDAC inhibition also generates non-traditional effects such as reducing adipose tissue expansion, resistance to obesity, and improvement in insulin sensitivity (65, 66). HDAC inhibitors have proven their potency in hampering fibrosis and favorably encouraging therapeutic muscle regeneration (67). Evaluation of HDAC inhibitors for the treatment of obesity-related muscle wasting is underway (68).

In skeletal muscle, glucose transporter 4 (GLUT4) levels are directly associated with increased oxidative capacity (69). Increases in GLUT4 translocation to the plasma membrane promotes improved rates of satellite cell proliferation and differentiation (70). AMPK increases GLUT4 gene expression in human skeletal muscles (71). AMPK is also a widely recognized regulator of energy metabolism. Decreased AMPK activity is associated with metabolic disorders such as obesity and T2DM (18, 72). AMPK also plays a key role in upregulating the transcription levels of paired box protein 7 (Pax7), myogenic factor 5, myogenin, and MyoD, all of which are necessary for muscle growth. Although metabolic rate is stimulated through AMPK activity, ATP/AMP ratios for the AMPK activation pathways are not affected by obesity (18, 73).

Skeletal muscle isolated from patients with T2DM shows reduced levels of diacylglycerol kinase-delta (DGKδ), a key enzyme in triglyceride biosynthesis required for appropriate AMPK function. DGKs control the expression levels of diacylglycerol (DAG) by catalyzing its conversion to phosphatidic acid utilizing ATP (74). Elevated plasma free fatty acid (FFA) levels from enlarged adipose tissue in obese models force intramyocellular DAG accumulation (75). In an obese population, increased DAG accumulation, secondary to reduced DGK or increased, circulating FFA, results in inhibition of both glucose uptake and glycogen synthesis. This further exacerbates insulin resistance.

LIVER AND FAT

As previously noted, obesity-associated liver dysfunction can have a profound impact on skeletal muscle maintenance and regeneration. NAFLD commonly occurs in obesity and is correlated with sarcopenia, even in the absence of insulin resistance (76, 77). Loss of muscle mass reduces a key cellular target for insulin action, contributing to glucose intolerance and, in turn, further muscle depletion. In addition, NAFLD is associated with

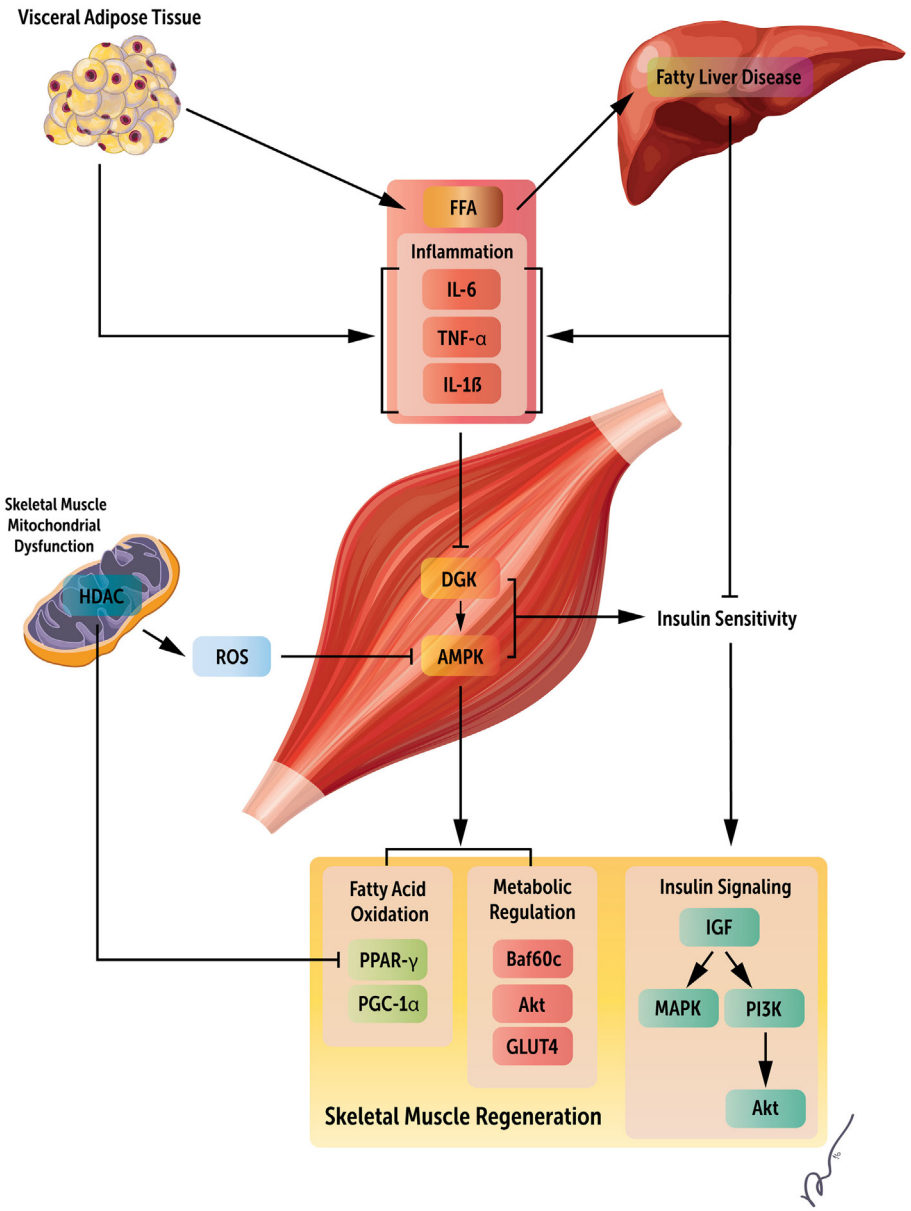

FIGURE 1 | Systemic regulators of obesity mediated loss of skeletal muscle regeneration. Obesity results in both increased visceral adipose tissue and fatty acid accumulation in the liver. These changes manifest as increased circulating fatty acids, inflammatory mediators, and insulin resistance, leading to metabolic derangements within skeletal muscle, and ultimately, decreased skeletal muscle regeneration by the deregulation of multiple signaling pathways. This figure summarizes key factors limiting muscle regeneration in an obese state.

the production of multiple proinflammatory factors, including NF-κB, IL-6, and TNF-α, all of which are known to be protein catabolic (78). VAT also releases circulating FFA, leading to further liver damage (79). Independently, VAT can also result in higher levels of proinflammatory cytokines, similar to the liver (80). Thereby, liver damage and VAT accumulation work synergistically to impair skeletal muscle regeneration in obesity by increasing FFA circulation, proinflammatory cytokines, and limiting promyogenic insulin actions on muscle. These pathways are depicted in **Figure 1**.

CONCLUSION AND PERSPECTIVES

Obesity is accompanied by significant health concerns, including severe loss of skeletal muscle mass. The maintenance of skeletal muscle is necessary for ambulation, proper insulin signaling, and glucose homeostasis. Obesity-related loss of muscle mass perpetuates a cycle of increasing metabolic abnormality, associated liver dysfunction, and further muscle loss. Effective methods to target obesity-associated muscle wasting must account for multiple systemic changes that occur,

including increased inflammatory mediators, circulating FFA, metabolic dysfunction, and insulin resistance. Further research is warranted to determine specific molecular mechanisms that limit skeletal muscle regeneration and induce atrophy in an obese state.

AUTHOR CONTRIBUTIONS

IS and DS drafted the manuscript and DV developed the figure. All the authors contributed to its intellectual content and critical revisions.

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REVIEW
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Emerging Evidence of Epigenetic Modifications in Vascular Complication of Diabetes

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Genes, dietary, and lifestyle factors have been shown to be important in the pathophysiology of diabetes and associated microvascular complications. Epigenetic modifications, such as DNA methylation, histone acetylation, and post-transcriptional RNA regulation, are being increasingly recognized as important mediators of the complex interplay between genes and the environment. Recent studies suggest that diabetes-induced dysregulation of epigenetic mechanisms resulting in altered gene expression in target cells can lead to diabetes-associated complications, such as diabetic cardiomyopathy, diabetic nephropathy, retinopathy, and so on, which are the major contributors to diabetes-associated morbidity and mortality. Thus, knowledge of dysregulated epigenetic pathways involved in diabetes can provide much needed new drug targets for these diseases. In this review, we constructed our search strategy to highlight the role of DNA methylation, modifications of histones and role of non-coding RNAs (microRNAs and long non-coding RNAs) in vascular complications of diabetes, including cardiomyopathy, nephropathy, and retinopathy.

Keywords: diabetes, cardiovascular complication, epigenetics, DNA methylation, histone modifications, non-coding RNAs

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INTRODUCTION

In spite of adequate glycemic control, incidence of vascular complications associated with diabetes, such as diabetic cardiomyopathy, retinopathy, nephropathy, and neuropathy, remains high contributing to increased morbidity and mortality in diabetic patients. Recent studies suggest that a complex interplay between genes and environment may significantly contribute to pathogenesis of microvascular complications associated with diabetes (1–3). Emerging evidence suggests that environmental factors modulate aberrant expression of several key genes through epigenetic mechanisms in type II diabetes mellitus (T2DM) (4). Epigenetic changes, such as DNA methylation, histone modifications, and interference of RNAs, comprise the major epigenetic regulators of gene expression. A large volume of data has emerged supporting aberrant DNA methylation, histone modifications, and expression of microRNAs and long non-coding RNAs (lncRNAs) contributing to deregulation of signaling pathways (oxidative stress, inflammation, and apoptosis, etc.) in T2DM. However, our knowledge on epigenetic regulation in diabetes-associated microvascular complications remains limited. Thus, elucidation of epigenetic changes could provide better understanding of pathophysiology and therapeutic management of these diseases. In this article, we briefly summarize recent findings on the role of DNA methylation, histone modifications, and post-transcriptional RNA regulation in microvascular complications of diabetes.

SEARCH METHODOLOGY

Literature searches of several electronic databases including Embase, Google Scholar, Ovid SP, Pubmed/Medline, and Web of Science were searched using the following search terms (free text, truncation, and MeSH or EMTREE terms): "DNA methylation" OR "histone acetylation," non-coding RNAs, microRNAs, long non-coding RNAs, post-transcriptional RNA regulation, epigenetic modifications, vascular, cardiovascular, renal, and retinal complications of diabetes for relevant publications in English language from 2006 to till date to evaluate the association between the role of DNA methylation, post-transcriptional RNA regulation, and histones modifications in diabetes-associated microvascular complications. Reference lists of included studies were hand-searched to identify other potentially eligible studies. Three authors (Madhu Khullar, Satish K. Raut, and Balneek Singh Cheema) reviewed the titles and abstracts to identify potentially eligible papers. These papers were examined in full detail. Final decision regarding inclusion was resolved by discussion. A manual review has been used for related publications and references of retrieved articles. We included randomized or non-randomized controlled clinical trials with or without blinding as well as cross-sectional and interventional studies that provided sufficient information.

EPIGENETIC MODIFICATIONS IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Evidence from both animal studies and clinical studies in diabetic patients has provided strong evidence linking histone modifications, post-transcriptional RNA regulation, and DNA methylation in microvascular complications of diabetes by regulating molecular pathways involved in pathophysiology of microvascular complications in diabetes (**Figure 1**).

These changes are inheritable and persist even after adequate glycemic control and contribute to metabolic memory and have been suggested to significantly contribute to diabetes-induced vascular complications (5).

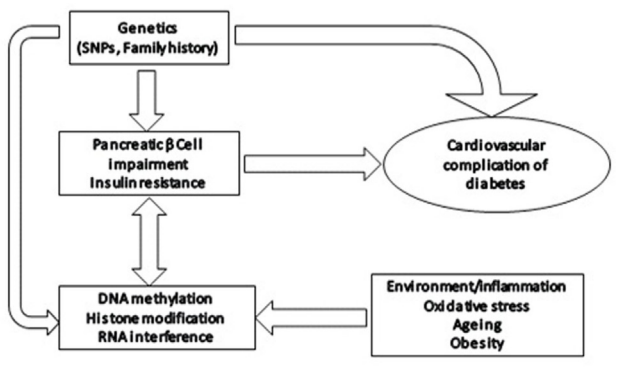

FIGURE 1 | Epigenetic modifications in diabetes: effect of various environmental/physiological factors on gene expression through epigenetic modifications, such as altered DNA methylation, histone modifications, and post-transcriptional RNA regulation.

HISTONE MODIFICATIONS IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Histone acetyl transferases (HATs) and histone deacetylases (HDACs) are the enzymes involved in histone acetylation/deacetylation and have been recently shown to be involved in regulating gene expression of several key molecules involved in microvascular complication of diabetes (6).

HDACs IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Histone deacetylases silence gene expression by deacetylating histone tails resulting in condensed euchromatin. Recent studies have implicated HDACs in diabetes and its associated microvascular complications; for example, HDAC1 and HDAC2 were shown to modulate expression of cardiac hypertrophy genes (6).

O-linked β-N-acetylglucosamine (O-GlcNAc) is an important signaling molecule which regulates cell function through O-GlcNAcylation of serine and threonine residues of proteins (7). O-GlcNAc plays a central role in regulating cardiovascular function. Increased O-GlcNAc levels observed in diabetic hearts and have been linked to diabetic cardiomyopathy (8). Cox and Marsh have reported decreased levels of Mammalian switch-independent 3 A (*mSin3A*), *HDAC1*, *HDAC2,* and increased expression of *HDAC2* mRNA and HDAC1/2 deacetylase activity in hearts from diabetic rats. These authors have suggested that there is a decreased physical association of O-GlcNAc with mSin3A/HDAC1/2 in the heart which results in their altered activity and expression in the diabetic heart and impacts its function. However, physical exercise increased cardiac O-GlcNAc of these proteins resulting in beneficial effects on cardiac function and proposed that anti-hypertrophic effects of exercise on diabetic hearts were mediated by O-GlcNAc mediated post translation modification of HDAC1, 2 and mSin3A (9).

HDAC3 has been shown to exert pro-hypertrophic effect in diabetic mice. Xu et al. have reported significantly increased cardiac HDAC3 activity in the OVE26 diabetic mice. They showed that HDAC3 was exerting its pro-hypertrophic activity by downregulating DUSP5 (a MAP Kinase phosphatase) expression, by deacetylation of histone H3 in the primer region of *DUSP5* gene (10). There are several studies showing beneficial and preventive effects of HDAC inhibition on diabetes-induced cardiovascular function. (This has been given in detail in a separate section.) However, further research is warranted to identify the specific HDAC isoforms that are dysregulated and their molecular targets that result in diabetic cardiomyopathy.

The role of HDACs in diabetic nephropathy has been reviewed recently by Li et al. (11). The available literature suggests that different HDAC isoforms targeting different molecular pathways are involved in pathophysiology of diabetic nephropathy. For example, HDAC1, HDAC2, and HDAC5 were shown to

modulate expression of genes induced by TGF-β1 (12), TGF- β (13), and HDAC4 inhibited autophagy by deacetylating STAT1 (14).

Increased histone acetylation has been also reported in diabetic retinopathy and has been partly attributed to high glucose-mediated decreased HDAC activity in retinal cells. HDAC activators and HDAC inhibitors were found to mitigate or potentiate diabetes-induced histone acetylation and expression of pro-inflammatory proteins in high glucose-treated cultured retinal Müller glia cells, confirming contribution of histone acetylation of retinal cells in pathophysiology of diabetic retinopathy (14). Decreased IL-10 levels are seen in diabetic retinopathy patients that have been suggested to be due to increased HDAC11 activity in conjunction with miR-19a in peripheral B cells of diabetic retinopathy patients (15).

Thus, available evidence supports a pathogenic role for aberrant HDAC activity in DC, DN, and DR by promoting histone acetylation and repression of genes of various signaling pathways, such as pro-inflammatory, pro-fibrotic, and antioxidant pathways.

HATs IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Histone acetylation mediated by HATs is another important epigenetic mechanism in gene regulation. HATs acetylate specific lysine residues of core histones at the N-terminal tail, causing DNA uncoiling, increased accessibility to transcription factors, and increased gene expression. Thus, altered HAT activity could regulate gene expression and affect cell function. Indeed, HATs have been implicated in several diseases, such as cancer, diabetes, cardiac hypertrophy, asthma, and so on.

Recent evidence suggests that HATs may participate in the pathophysiology of microvascular complications of diabetes by regulating the expression of inflammatory pathway genes. For example, high glucose treatment of monocytes was found to increase transcriptional activity of HATs CBP and p/CAF, resulting in increased histone lysine acetylation of promoter regions of inflammatory genes, cyclooxygenase-2 (*COX-2*) and *TNF*-α gene, and increased gene expression of these cytokines in cultured monocytes (16). An increased promoter histone lysine acetylation of inflammatory genes has been reported in monocytes from both T1DM and T2DM patients (17). Furthermore, HAT-mediated lipid oxidation has been also found to increase inflammation by increasing histone acetylation of inflammatory genes (18). Yun et al. observed that HATs-mediated increased pro-inflammatory cytokine expression could be attenuated by curcumin in high glucose-treated human monocytes (19). *Curcumin was shown to decrease high glucose-induced HAT activity, p300 gene expression, and acetylation of CBP/p300, a complex that functions as a coactivator of NF-κB.* The role of HATs in diabetic nephropathy has been recently reviewed by Li et al. (11) and provides evidence that high glucose-induced increased activity and levels of HATs, such as p300, CBP, and p/CAF, are mediating the activation of pro-inflammatory cytokines, ECM proteins, endothelial function, and fibrotic processes in diabetic nephropathy, *via* acetylation of both histone and non-histone proteins, such as Smads, p53, SP1, and NF-κB.

SIRTUINS IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Recently, another class of HDACs, Sirtuins has been shown to regulate key cellular and metabolic processes by deacetylating the lysine residues of proteins involved in these processes. Sirtuins are a highly conserved protein family of HDACs and have been found to have protective effects against several diseases, such as diabetes, cancer, cardiovascular, and neurodegenerative diseases (20). Sirtuins exert these beneficial effects by modulating the expression of the genes involved in energy metabolism, DNA repair, inflammation, fibrosis, and oxidative stress (21).

Sirtuins regulate enzymes of carbohydrate metabolism, lipid metabolism, adipogenesis, and insulin secretion in diabetic patients (15). SIRT1 regulates glucose metabolism in liver, pancreas, muscle, and adipose tissue, mainly by regulating PGC-1α (22). SIRT1 induces gluconeogenic genes through deacetylation of PGC-1α in fasting state. FOXO group of transcriptional factors promote gluconeogenesis *via* STAT3; SIRT1 inhibits gluconeogenesis by inhibiting gluconeogenic genes *via* deacetylation of FOXO transcription factors and STAT3 in liver (22). Increased SIRT1 is also shown to increase glucose-induced insulin secretion in pancreatic β-cells which is partly due to SIRT1-mediated inhibition of UCP-2 in pancreatic islet β-cells (22). SIRT3, a mitochondrial protein deacetylase was found to be effective in increasing insulin sensitivity and decreasing serum glucose (16). SIRT4, another sirtuin involved in glucose homeostasis acts by repressing enzyme glutamate dehydrogenase (GDH) inhibiting insulin secretion (23). Decreased levels of SIRT1, 3, and 4 have been observed in diabetic patients and were associated with hepatosteatosis. Apart from this, sirtuins have been also shown to regulate activity of NFkB and expression of its downstream inflammatory genes in diabetes (18, 19).

Recent studies show that cardiac Sirtuins expression is dysregulated in diabetic patients. Bagul et al. reported a decrease in cardiac SIRT-1 and increase in SIRT-3 activity in the T2DM rat and downregulation of all sirtuins except SIRT-2, which was increased in T1DM rat heart (24). In a recent review on sirtuins in cardiac complications of diabetes, sirtuins were suggested to attenuate the effects of insulin resistance and oxidative stress pathways in heart (25). SIRT-1 has been found to be the most important modulator of vascular function and is being targeted for therapeutic potential in various pre-clinical studies to improve cardiovascular functions. Bagul et al. have recently shown beneficial effect of reservatol on diabetic rat heart through modulating expression of SIRT-1 in T2DM and SIRT-1, 2, 3, and 5 in T2DM (24).

Recently, role of Sirtuins in vascular homeostasis has been reviewed nicely (26). Sirtuins were shown to regulate endothelial damage and vascular repair mechanisms. Sirtuins, by acting on specific endothelial targets, regulate several processes, including inflammation by modulating cytokine expression (IL-6, TNF-α, NF-KB, MMP-14), oxidative stress [manganese superoxide dismutase (MnSOD), FOXOs], and deacetylation of histone H3K14 and H4K16 (27).

High glucose milieu has been found to induce endothelial cell senescence and functional abnormalities by repressing

SIRT1 expression high glucose-treated endothelial cells. SIRT1 upregulation in these cells was found to be protective against glucose-induced endothelial dysfunction indicating its potential protective role in diabetic vascular complications (21). Advanced glycation end products (AGEs), important mediators of diabetes, induced vascular abnormalities. AGEs have been shown to decrease SIRT1 levels and promote apoptosis in human endothelial Eahy926 cells which could be reversed by increasing SIRT1, confirming that AGEs were inducing apoptosis by repressing SIRT1 in endothelial cells (28). The downregulation of SIRT1 by high glucose and in diabetic has been proposed to be mediated by glucose-induced oxidative stress in endothelial cells. Mortuza et al. showed that high glucose-induced downregulation of SIRT1 was accompanied by FOXO1-mediated decreased levels of antioxidant enzyme, suggesting that SIRT1/FOXO1 axis was regulating oxidative status in endothelial cells (27).

Decreased SIRT1 expression has been also implicated in increased cellular senescence in renal glomerulus and retinal blood vessels in diabetic male C57BL/6 mice which were mediated by p300 and FOXO1 mediated reduction in mitochondrial antioxidant enzyme MnSOD in these cells (27). Furthermore, SIRT1 overexpression has been also shown to be protective in diabetes-induced renal and retinal injury in diabetic mice, through attenuated p300, endothelin-1 (ET-1), and TGF-β1 expression (29). Downregulation of SIRT1 has been also shown to promote diabetic retinopathy by inducing increased MMP-9 expression in retinal endothelial cells (RECs) *via* acetylating transcriptional factor AP-1 (30). AGEs have been recently reported to decrease SIRT3 levels and SIRT3 knock down was associated with endothelial dysfunction in endothelial progenitor cells (EPCs). Moreover, SIRT3 augmentation ameliorated cellular dysfunction and enhanced antioxidant machinery (31). SIRT6 deficiency has been found to impair wound healing in diabetic db/db mice and induce pro-inflammatory cytokines and oxidative stress, and decrease angiogenesis, suggesting its potential role in diabetic vasculopathy (32).

The role of other sirtuins in diabetic vascular complications is not known and needs to be investigated. Overall, diabetes-induced downregulation of sirtuins (SIRT1, 3 and 6) appears to promote oxidative stress and endothelial dysfunction, and induce cellular fibrosis, suggesting these molecules to be of potential therapeutic use in diabetes and associated vascular complications.

HISTONE METHYLATION IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

Methylation of core histone tails at lysine or arginine residues are known to modulate gene expression by changing chromatin structure. For example, methylation at H3-K9 and H3-K27 mediates heterochromatin formation and results in silencing gene expression. Aberrant histone lysine methylation has been found to be involved in several pathological processes such as cancer, diabetes, cardiovascular diseases, etc. High glucose has been shown to induce increased histone H3 lysine 9 dimethylation in THP1 monocytes. Miao et al. showed that high glucose exposure caused increased H3K4me2 and H3K9me2 of specific chromatin regions and their associated genes. They reported increased H3K4me2 was associated with increased methylation of nine genes, including *ICAM3, FOS, GSTA-4, IL-8,* and *BCL-9*, showed decreased methylation following HG exposure. Similarly, H3K9me2 methylation resulted in increased methylation of 39 genes and decreased methylation of 11 genes. They further observed increased H3K9me2 at the coding and promoter regions of two candidate genes (*IL-1A* and *PTEN*) in blood monocytes of diabetic patients, indicating that diabetic milieu induced aberrant histone methylation is an important contributor to diabetes-associated complications (33).

Histone methyl transferases (HMTs) carry out methylation at specific lysine or arginine residues. HMTs Suv39 and G9a family methylate histone H3 at Lys9 and cause gene silencing whereas SET1/2 family HMTs methylate histone H3 at Lys4 and correlate with gene activation. Okabe et al. reported sustained vascular gene expression of H3K4 methyl transferase, Set7 as a responsive measure to hyperglycemia in vascular endothelial cells. They showed that metabolic memory of prior exposure to hyperglycemia was induced by Set7 and proposed that Set7 was a potential molecule for the phenomenon of hyperglycemic memory (34). This was further supported by an another study which showed that high glucose exposure altered ratio of cytoplasmic/nuclear ratio of Set7 protein without changing overall level of Set7 in vascular endothelial cells, indicating a role of Set7 and its role in hyperglycemia-induced gene activation of vascular endothelial cells (35).

The role of histone methylation in diabetic retinopathy has been also documented. For example, Zhong et al. showed that retinal superoxide dismutase gene (*SOD2*) was epigenetically regulated in diabetes through methylation/acetylation of H4K20me3, acetyl H3K9, and NF-kB p65 on the histones at the promoter/enhancer location of retinal *SOD2* in diabetes (36). These authors showed that these modifications continued after termination of hyperglycemia, supporting a diabetes-induced epigenetic regulation of retinal *SOD2* (36). Their study suggests that promoter region methylation of *SOD2* histones might play an important role in progression of diabetic retinopathy. Similarly, H3K9-specific demethylase JHDM2A (also known as JHMJD1A and KDM3A) has also been shown to be involved in regulating the expression of metabolic genes, strengthening the role of epigenetic regulation of metabolic genes in microvascular complications of diabetes (37). These authors observed that JHDM2A regulates the expression of PPARα and β-adrenergic signaling pathway genes and suggested that JHDM2A might regulate energy mediated β-adrenergic signaling pathway (37).

The fetal exposure to maternal milieu such as nutrition is known to result in intrauterine growth restriction (IUGR) and influence susceptibility to several diseases such as insulin resistance in adults. Hepatic insulin growth factor 1 (IGF-1) modulates insulin sensitivity, thus decreased IGF-1 levels are linked to insulin resistance. Decreased post natal plasma IGF-1 levels have been reported in IUGR infants and in new born rats with induced IGUR (38). Fu et al. have shown that IUGR affects *IGF-1* gene expression by modulating the region

and gender-specific histone modifications (methylation and acetylation) along the length of *IGF-1* gene. The authors showed that IUGR significantly increased H3K4me2 in males and H3 K4me3 in females new born rats with induced IUGR. Since, there is a dynamic association between histone methylation and associated DNA methylation which affects gene transcription, these histone modifications resulted in decreased IGF-1 expression in new born rats. These findings suggest that that aberrant methylation of core histone tails of hepatic IGF-1 regulate IGF-1 expression.

Yu et al. (39) have shown that combination of diabetes and renal failure accelerated cardiomyopathy by epigenetic alterations (increased acetylation, phosphorylation, K4 dimethylation, and reduced K9 dimethylation) of the cardiac histones H3. They observed increased H3 dimethylation at lysine 4 and 9 and decreased H3 dimethylation at lysine 9 in hearts of uninephrectomized db/db mice resulting in transcriptionally active chromatin and proposed that these changes were associated with increased expression of cardiac hypertrophy related genes. However, factors causing these changes are not known and need to be determined.

Histone methylation in etiology of diabetic nephropathy has been widely investigated and reviewed recently (40). Diabetic nephropathy is characterized by glomerular mesangial expansion, inflammation, renal fibrosis, and hypertrophy. In a recent study, Li et al. (41) showed that increased p21 expression seen in high glucose-treated mesangial cells was mediated by reduced histone H3-lysine9-dimethylation (H3K9me2), increased histone H3-lysine4 methylation (H3K4me1/3) and increased translocation of SET7/9 at the p21 promoter region. Similarly, Yuan et al. (12) also showed that oxidized lipid products such as 2(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] increased transcriptional activity of SET7, which in turn increased expression of pro-fibrotic genes in HETE treated mesangial cells. Losartan, an AT1R inhibitor, a common drug used in treatment of diabetic nephropathy has been shown to decrease H3K9/14Ac at RAGE, PAI-1, and MCP-1 promoters, in mesangial cells from db/db diabetic mice, suggesting that AT1R action may be also mediated by attenuation of epigenetic changes of the key genes involved in diabetic nephropathy.

Altered histone methylation of RECs has been reported in diabetic retinopathy. For example, decreased expression of MnSOD was found to be associated with altered H3K4me1/me2in diabetic retinas and endothelial cells (42). These changes were found to persist even after normalization of blood glucose levels, indicating that these changes acted as markers of metabolic memory (42).

Decreased H3K9me2 promoter methylation of MMP9, promoting increased expression has been also seen in diabetic retinas and suggested to be associated with increased ECM accumulation (42). Increased expression of PRMT4, a methyltransferase which specifically methylates H3R17 histones and promotes cell death has been observed in retinal pigment epithelial layer of diabetic rats even before development of diabetic retinopathy (43). Wang et al. (44) reported differential methylation on H3, H4, H2A, H2B, and H1 sites in diabetic retinas specifically they observed increased mono- and dimethylation of histone H4 lysine 20 (H4K20me1/me2), and were associated with DNA damage in retinas of diabetic rats and these methylation patterns could be partly reversed by minocycline, a strong neuroprotective drug and used in treatment of diabetic retinopathy. Thus, altered histone methylation appears to be important in development of diabetic retinopathy in animal models and *in vitro* conditions, however, these changes need to be replicated in diabetic patients.

Thus, in summary, hyperglycemia-induced differential histone methylation/acetylation appears to regulate expression of several genes of cellular pathways, such as endothelial activation, oxidative stress, adrenergic signaling pathway, and so on, involved in diabetes-induced vascular complications.

MODULATION OF HDACs AND HATs AS A THERAPEUTIC APPROACH

Since HDACs along with HATs have been shown to have a critical role in regulating expression of genes involved in diabetic vascular complications, modulation of these molecules is being investigated for therapeutic applications in diabetic cardiomyopathy, nephropathy, retinopathy, and endothelial dysfunction associated with diabetes (45).

For example, acetylation of 20 S proteasome subunits in the heart has been shown to mediate proteolytic activity of injured myocardium (46), it has been suggested that modulation of HDACs, the key regulators of acetylation in the cell could be used effectively in the treatment of cardiac injury (47, 48). Christensen et al. reported that HDAC inhibition could ameliorate late diabetic microvascular complications along with improving insulin resistance and β-cell function (49). HDAC inhibition was also shown to improve cardiac function and attenuated cardiac remodeling in the diabetic myocardium of the streptozotocin-treated ICR mice. Chen et al. observed that diabetic mice given 1% butyrate in drinking water resulted in HDAC inhibition in the diabetic myocardium, specifically myocardial HDAC4 was found to be significantly decreased. HDAC inhibition caused upregulation of GLUT 1 and 4, increased Caspase 3, increased myocardial superoxide dismutase, decreased cardiac interstitial fibrosis and myocyte hypertrohy resulting in improvement in cardiac performance in diabetic mice (50).

Chen et al. have also recently shown that HDAC inhibition promotes stem cell-derived myocardial repair, thereby improving cardiac function and attenuating cardiac remodeling in diabetic rats, further confirming a protective role of HDAC inhibitors against myocardial injury (50).

Peroxisome proliferator-activated receptors (PPARs) play an important role in diabetes-associated heart diseases by regulating cardiac glucose and lipid homeostasis. HDAC inhibitor, MPT0E014, was shown to decrease cardiac inflammation and dyslipidemia by modulating myocardial PPARs, and attenuated diabetic cardiomyopathy (51).

DUSP 5 is a dual-specific phosphatase which dephosphorylates and inactivates ERK1/2 MAP Kinase, a known promoter of cardiac hypertrophy (10). Xu et al. recently reported that HDAC3 inhibition with its selective inhibitor, RGFP966,

increased the expression of MAP kinase phosphatase, DUSP 5 and prevented development of diabetic cardiomyopathy in Type 1 diabetes OVE26 mice, suggesting a therapeutic potential of HDAC3 inhibition in prevention of diabetic cardiomyopathy (10).

Histone deacetylase inhibitors have been also found to be effective in preventing diabetes-induced renal damage. Gilbert et al. (52) reported that HDAC inhibitor, Vorinostat, blunted renal damage in diabetic rats by reducing renal growth and glomerular hypertrophy *via* modulating renal EGFR expression. Vorinostat has been also shown to attenuate renal damage in strptozotocin-treated mice by decreasing eNOS expression and oxidative stress (53). Valproic acid (VPA), a known HDAC inhibitor also has been shown to ameliorate diabetes-induced renal injury by inhibiting renal fibrosis (54). Increased oxidative stress is an important contributor to diabetic nephropathy; Dong et al. recently showed that sodium butyrate inhibited HDAC activity and elevated the expression of *NRF2* and its downstream targets heme oxygenase 1 and NAD(P)H dehydrogenase quinone 1. Deletion of the *NRF2* gene completely abolished sodium butyrate activation of NRF2 signaling and protection against diabetes-induced renal injury (55). Trichostatin A (TSA), an antifungal antibiotic has been shown to inhibit HDACs 1, 3, and 4. TSA suppresses redox signaling by decreasing NADPH Oxidase 4 (Nox4) expression by inhibiting p300-HAT-dependent pathway in endothelial cells (56). Cao et al. showed that TSA decreased transverse aortic constriction (TAC), induced cardiac hypertrophy and phenylephrine (PE) or ET-1, and induced cardiomyocyte hypertrophy by inhibiting autophagy, and suggested that TSA-mediated HDAC inhibition suppresses load- or agonist-induced autophagy in stressed myocardium (57).

Pancreatic duodenal homeobox 1 (PDX1) is a transcription factor associated with pancreatic β-cell function and survival. PDX1 deficiency results in defective B-cell function and diabetes. Park et al. observed that IUGR decreased fetal and postnatal PDX1 levels by histone modification of *PDX1* gene in primary islets. IUGR promoted deacetylation of histones H3 and H4 by recruiting HDAC1 and corepressor Sin3A; and histone 3 lysine 4 (H3K4) was demethylated and histone 3 lysine 9 (H3K9) was methylated, resulting in silencing of the PDX1. These authors suggested that IUGR-induced *PDX1* gene silencing in the β cell was linked with development of T2DM (58).

Johnson and Marsh recently reported that treatment of Type 2 diabetic db/db mice with a chemotherapeutic class 1 HDAC inhibitor, romidepsin (FK228), at a low dose [(0.56 mg/kg twice a week) for 8 weeks], decreased blood glucose reduction independent of plasma insulin level. These authors have suggested that these anti-diabetic effects of romidepsin were mediated through HDAC2-mediated potentiation of intracellular insulin signaling (59).

Thus, available information till date suggests that HDAC inhibition has beneficial effects in ameliorating diabetic microvascular complications by targeting multiple dysregulated pathways. However, its translation into an effective therapy requires further studies such as evaluating association between HDACs and environmental and genetic factors.

DNA METHYLATION IN DIABETES-INDUCED MICROVASCULAR COMPLICATIONS

DNA methylation involves methylation at 5′ position of cytosine residues in CpG islands, mostly in the promoter regions and is carried out by DNA methyl transferases (DNMTs). Promoter DNA methylation is an important epigenetic mechanism regulating gene expression and is known to be affected in various diseases, including cardiovascular diseases and diabetes (60). Altered DNA methylation of inflammatory genes, glucose, and lipid metabolism genes, genes involved in oxidative stress, has been reported in diabetes (61).

DNA methylation in vascular complications of diabetes have been investigated and reviewed in a recent review (62). El-Osta (63) reported that short-term exposure of aortic endothelial cells to high glucose-induced promoter DNA methylation of *NF-kB p65* subunit, an important mediator of cardiac fibrosis. These authors showed that DNA methylation was mediated by hyperglycemia-induced increased methylglyoxal generation. Pirola et al. (35) observed that hyperglycemia significantly affects human vascular chromatin resulting in differential methylation and acetylation pattern with the transcriptional upregulation of genes involved in metabolic and cardiovascular disease. A good correlation was seen between hyper-acetylation and DNA methylation and induction of genes in glucose-treated cells, and suggested that hyperglycemia-induced gene induction was mediated by distinct changes in methylation and acetylation pattern of the genes.

Distinct promoter methylation profiling has been reported in diabetic hearts too. Movassagh et al. (64) examined DNA methylation profiles in left ventricular tissues from patients with idiopathic and end stage heart failure and observed increased promoter methylation of 3 genes, *PECAM1, ARHGAP24,* and *AMOTL2,* related to angiogenesis in cardiomyopathic hearts, suggesting a role of DNA methylation-induced altered gene expression in cardiomyopathy.

However, DNA methylation pattern seen in diabetic hearts is distinct from that seen in heart failure patients (9). A specific DNA methylation CpG site of β-myosin heavy chain (β-*MYH7*) gene that was found to be extensively methylated in T2DM hearts as compared to controls and 3 CpG sites of failing human hearts. Similar DNA methylation changes were also seen T1DM hearts and in steroid induced diabetic hearts (9), suggesting altered DNA methylation of specific CpG site of β-*MHC* may be contributing to ventricular dysfunction seen in diabetic patients.

In a similar study, Mönkemann et al. (65) reported altered methylation status of P53-inducible *p21WAF1/CIP1* promoter, resulting in activation of apoptotic pathway leading to cell death of cardiomyocytes and cardiomyopathy in diabetic rats. They proposed that oxidative stress was the major trigger contributing to *de novo* methylation of p53-inducible *p21WAF1/CIP1* gene.

Diabetes-induced oxidative stress is an important mediator of diabetes-associated cardiovascular complications. Zhong et al. (66) recently reported significant hypomethylation of *KEAP1* promoter in diabetic cardiomyopathy patients, with concomitant

increase in KEAP1 protein levels in these patients. KEAP1 protein is known to bind to NF-E2-related factor 2 (NRF2), and promotes its degradation. NRF2 is known to activate several antioxidant enzymes. Studies have proposed that reduction of NRF2 antioxidant system in diabetic hearts may alter redox balance and contribute to increased oxidative stress in the heart of diabetic patients (67).

Vecellio et al. (68) have recently reported a decreased proliferation, differentiation potential, and premature cell death of cardiac mesenchymal stem cells in T2DM patients. Furthermore, they observed hypermethylation of promoter CpG islands of genes of cell cycle and DNA repair genes along with reduced acetylation of histone H3 lysine 9 (H3K9Ac) and lysine 14 (H3K14Ac) and increased trimethylation of H3K9Ac and lysine 27. They proposed that reduced HAT activity in diabetic hearts was responsible for increased DNA CpG methylation resulting in decreased cell differentiation and proliferation of cardiac mesenchymal stem cells in diabetic cardiomyopathy. However, these effects could be reversed by increasing HAT activity, suggesting a potential therapeutic application of epigenetic modulators in diabetes-associated cardiovascular complications.

Decreased promoter methylation of liver X receptor α (*LXRa*) (69) and *AT1b* angiotensin (67) receptor gene leading to their increased expression has been observed in diabetic hearts. TNF-α-mediated increased promoter methylation of sarcoplasmic reticulum Ca-ATPases (*SERCA2a*) resulting in decreased SERCA2a expression has been observed in high glucose-treated cardiomyocytes (70). These results suggest that diabetic milieu can cause increased or decreased methylation of different genes, resulting in their aberrant expression in heart.

Altered methylation of several genes dysregulated in diabetic nephropathy and diabetic retinopathy has been reported in diabetic patients and *in vitro* studies (35, 71, 72). A distinct differential promoter DNA methylation pattern has been reported in diabetic nephropathy patients with end-stage renal disease as compared to those who do not progress to this stage (73), suggesting that diabetic environment results in distinct epigenetic changes in specific genes, which could be used as prognostic biomarkers. Similarly, in diabetic retinopathy, Agardh et al. (74) reported differential DNA methylation of nearly 233 unique genes, with genes from natural killer cell-mediated cytotoxicity pathway genes to be hypomethylated in proliferative diabetic retinopathy (PDR) and suggested that this distinct methylation pattern could be used as a prospective marker of PDR. Mishra and Kowluru (75) have shown that increased DNA methylation of mitochondrial DNA (mtDNA) causes decreased transcription of mtDNA, impairing mitochondrial functions and increasing apoptosis in diabetic retinopathy. A dynamic balance between methyl cytosine and hydroxyl methylation of MMP9 was found to be important in MMP9 expression and in maintaining mitochondrial integrity and function in RECs and in preventing diabetic retinopathy (76). Diabetes-induced oxidative stress appears to be a major trigger of these epigenetic changes.

Thus, there is substantial evidence to suggest that hyperglycemia causes aberrant methylation of regulatory regions of several distinct genes resulting in their dysregulated expression. These molecular changes appear to be important in the pathogenesis of diabetes-induced microvascular changes in heart, kidney, and retina of the diabetic patients. Furthermore, diet, exercise, environment, and genetic factors, which are important contributors to risk of diabetes, are also potent modulators of epigenetic changes. Hence, their role in inducing epigenetic changes in microvascular complications in diabetic milieu needs to be explored.

NON-CODING RNAs AND DIABETES

Non-coding RNAs are non-protein coding RNAs, and include microRNAs, long non-coding RNAs (LncRNAs), circular RNAs, etc. have been identified as important regulators of gene expression. These molecules have been shown to be important in developmental, physiological, and pathological processes. Dysregulated expression of microRNAs and long non-coding RNAs (lncRNAs) has been implicated in various diseases, including vascular complications of diabetes.

MICRORNAs ASSOCIATED WITH DIABETES-INDUCED CARDIOMYOPATHY

MicroRNAs are small non-coding RNAs which regulate gene expression by mRNA degradation or translational repression of mRNAs. The role of microRNAs has been widely studied in diabetes and its vascular complications and has been reviewed recently in several articles (17, 77, 78). Dysregulated expression of several microRNAs has been reported in Diabetic cardiomyopathy, retinopathy, nephropathy, and neuropathy regulating genes involved in diabetes (**Table 1**). Most of these microRNAs are involved in fibrogenesis, hypertrophy, apoptosis, inflammation, angiogenesis, and ECM accumulation. These functions are mainly regulated by microRNAs by regulating the expression of target genes involved in these cellular processes.

Several microRNAs have been reported to contribute to pathophysiological processes of diabetic cardiomyopathy, such as myocardial fibrosis, cardiomyocyte hypertrophy, cardiomyocyte apoptosis, and mitochondrial dysfunction (**Figure 2**). For example, miR-30c, miR-133a, miR-150, and miR-373 were found to be downregulated and miR-451 was found to be upregulated in diabetes-induced cardiomyocyte hypertrophy (84). Whereas, in diabetes-induced cardiac fibrosis, the expression of miR-133a was found to be decreased and the expression of miR-21 was significantly increased (84). miR-34a, miR-1, miR-206, miR-195, and miR-30d have been implicated in diabetes-associated cardiac apoptosis and mitochondrial dysfunction (84). Raut et al. showed that the expression of putative target genes of miR-30c (*CDC42* and *PAK1*) were increased in hearts of diabetic rats and in HG treated cardiomyocytes (85, 86). miR-30c overexpression attenuated hyperglycemia-induced cardiomyocyte hypertrophy, whereas miR-30c inhibition resulted in myocyte hypertrophy in high glucose-treated cardiomyocytes, suggesting anti-hypertrophic potential of miR-30c in diabetic cardiomyopathy (85). miR-200c has been found to be pro-hypertrophic and its expression was shown to be significantly increased in diabetic hearts and in high glucose-treated cardiomyocytes. It was found to induce diabetes-associated cardiac hypertrophy by down

TABLE 1 | Dysregulated microRNAs in microvascular complications of diabetes.

MicroRNAs	Targets	Functions	Reference
Nephropathy			
miR-192	TGF-β	ECM	(53)
miR-200b/c		Collagen, fibrosis	(46)
miR-21	PTEN	Renal cell hypertrophy and Fibrosis	(8)
miR-195	Bcl-2	Podocyte apoptosis	(70)
miR-377	Fibronectin	Fibrosis	(79)
miR-29 family	Collagen I, III, IV	Fibrosis	(53)
miR-93	VEGF-A	Glomerular function	(80)
Retinopathy			
miR-146, miR-155, miR-132, miR-21 (upregulated in retina)	Nf-κβ	Pro-apoptosis of retinal pericytes	(81)
miR-17-5p, miR-18a, miR-20a, miR-21, miR-31, miR-155 (upregulated in retinal endothelial cells)	Vascular endothelial growth factor	Vascular permeability	(82)
miR-200b	VEGF-A	Vascular permeability	(83)

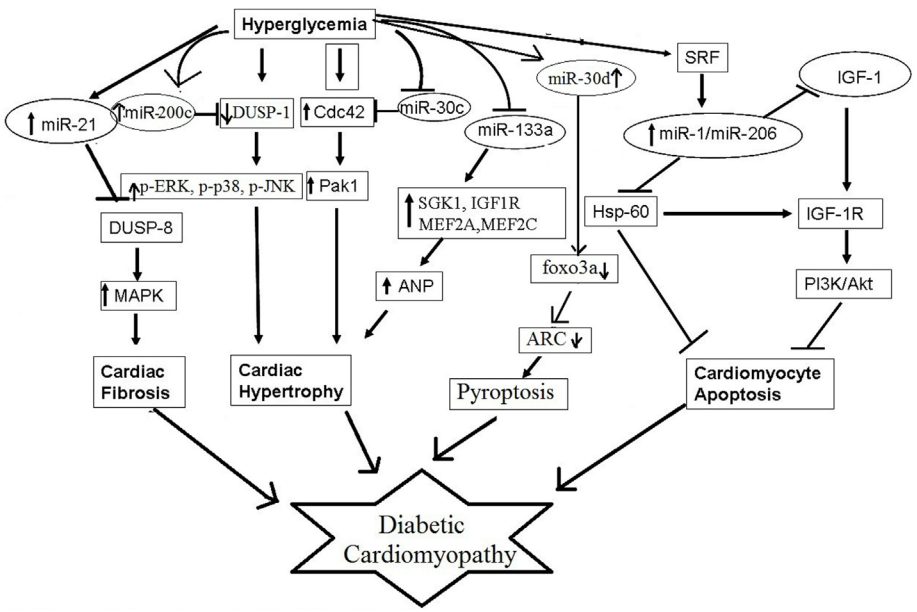

FIGURE 2 | Schematic model of epigenetic role of microRNAs in diabetic cardiomyopathy.

regulating expression of dual-specific phosphatase-1 (DUSP-1). Inhibition of miR-200c augmented the expression of the DUSP-1 causing decreased expression of phosphorylated ERK, p38, and JNK and attenuated cardiomyocyte hypertrophy in high glucose-treated neonatal rat cardiomyocytes (87). In another study, miR-133a expression was reduced in diabetic cardiomyopathy along with augmented gene expression of *MEF2A, MEF2C, SGK1*, and *IGF1R*. Over expression of this microRNA inhibited altered gene expression and hypertrophic changes, indicating that miR-133a participated in mediating glucose-induced cardiomyocyte hypertrophy in diabetes (88). Duan et al. (89) reported significantly reduced expression of miR-150 in high glucose-treated cardiomyocytes; this microRNA was shown to increase p300 expression, resulting in cardiomyocyte hypertrophy. miR-373 has also been shown to be involved in the pathogenesis of diabetes-induced cardiac hypertrophy. The expression of miR-373 was found to be markedly down regulated in STZ-induced diabetic mice, and neonatal rat cardiomyocytes in response to high glucose. Over expression of miR-373 in cardiomyocytes using synthetic miR-373 mimics resulted in decreased expression of *MEF2C* gene and attenuated cardiomyocyte hypertrophy in high glucose-treated cardiomyocytes (90). Kuwabara et al. identified calcium-binding protein 39 (CAB39), a component of AMPK signaling pathway as direct target of miR-451. They demonstrated that in miR-451 knockout mouse the protein expression of CAB39 and phosphorylated AMPK was increased significantly, indicating that miR-451 was involved in diabetic cardiomyopathy *via* suppression of the LKB1/AMPK signaling pathway (91).

Several miRNAs, such as miR-21 and miR-29, have been shown to promote cardiac fibrosis in diabetic hearts. Liu et al. demonstrated increased miR-21 expression after high glucose treatment in cardiac fibroblasts (92). miR-21 was shown to promote fibroblast survival by down regulating SPRY1 (93).

Silencing of miR-21 using synthetic mimics in mouse model of diabetic cardiomyopathy resulted in decreased interstitial fibrosis, suggesting potential role of miR-21 in cardiac fibrosis associated with diabetic cardiomyopathy. Liu et al. also showed that gain and loss of miR-21 function negatively regulated expression of DUSP8, a MAPK phosphatase, and enhanced cell proliferation and collagen synthesis *via* MAPK signaling pathway (92). Kumar et al. (94) have reported that miR-21 may also promote cardiac fibrosis by activation of AKT/PKB signaling. In addition to cardiac hypertrophy, miR-133a was found to mediate diabetes-induced cardiac fibrosis. Chen et al. observed that miR-133a expression was significantly decreased in hearts of STZ-induced diabetic mice, along with increased expression of transcriptional co-activator p300, as well as fibrosis markers (95).

miR-1 and miR-206 are cardiac-specific microRNAs (96). Increased miR-1 and miR-206 levels have been observed in high glucose-treated cardiomyocytes. Both these miRNAs were proposed to induce cardiomyopathy by inducing mitochondrial dysfunction and apoptosis (97). These microRNAs have been shown to bind to the same site in the 3′-UTR of *HSP60* mRNA and thereby could regulate HSP60 expression and glucose-mediated apoptosis in diabetic myocardium; however, this needs experimental validation (97). miR-34 too has been shown to promote HG-induced apoptotic changes in H9C2 cells (96). Pyroptosis is pro-inflammatory programmed cell death and is unlike from apoptosis or necrosis (26). Li et al. in their study showed that miR-30d expression was substantially increased in diabetic cardiomyopathy and this increased expression promoted cardiomyocyte pyroptosis; conversely, knockdown of miR-30d attenuated it (98).

It has been proposed that diabetic milieu may induce or repress the microRNAs by several different mechanisms, such as oxidative stress and ER stress, epigenetically regulating genes coding for microRNAs.

Role of microRNAs in diabetic nephropathy has been investigated widely and there are several recent reviews on this topic (99). Existing literature supports a pathogenic role for several microRNAs by promoting renal fibrosis by increased accumulation of extracellular matrix proteins related to fibrosis, glomerular hypertrophy, and renal cell apoptosis (100). Some of these microRNAs have been shown to have a potential as biomarkers as these were found to be dysregulated in early stages of nephropathy. However, more evidence is required for these data to be translated to clinical application. Furthermore, it has been observed that modulation of these microRNAs with either mimics or antagomiRs could attenuate the disease, suggesting that these microRNAs could be potential therapeutic targets.

A differential microRNA expression has been reported in patients with diabetic retinopathy as compared to controls. Animal and *in vitro* studies on RECs too showed altered retinal microRNA profile in diabetic animals and RECs treated with high glucose (35). Zampetaki et al. (101) showed that miR-27b and miR-320a increased risk of diabetic retinopathy by repressing antiangiogenic thrombospondin-1. Qin et al. have reported decreased miR-20b and correlated increase in its target genes VEGF and AKT3 in the retina and RECs in diabetic rats. These authors suggested that hyperglycemia-induced changes in retinal tissues were mediated by miR-20b *via* modulating VEGF and AKT3 in diabetic retinopathy (102). Similarly miR-15a too has been found to be protective toward developing diabetic retinopathy by inhibiting pro-inflammatory and pro-angiogenic pathways through its target genes ASM and VEGF-A (103). In a recent study, Zhou et al. (104) showed that transgenic mice over expressing let-7 show features similar to non-proliferative diabetic retinopathy suggesting its pathological role in non-proliferative diabetic retinopathy.

In addition, genetic variants in microRNA genes, such as miR-4513 rs2168518, miR-499 rs3746444, miR-196a2 rs11614913, and miR-423 rs6505162, have also been shown to be associated with the risk of cardiovascular complication of diabetes (105).

Taken together, available literature shows a definitive role of microRNAs, specifically targeting pro-angiogenic and pro-inflammatory genes in pathogenesis of diabetic retinopathy, thus providing their therapeutic potential in preventing and treatment of diabetic retinopathy.

MICRORNAs AS BIOMARKERS OF DIABETIC VASCULAR COMPLICATIONS

As serum levels of different miRNAs have been shown to be elevated in cardiovascular complication of diabetes, they could serve as sensitive and cost-effective biomarkers for these conditions. For example, the expression levels of seven diabetes-related miRNAs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) in serum were shown to be significantly elevated in T2DM subjects compared with pre-diabetes and/or normal glucose tolerance suggesting that during the pathogenesis of T2DM, the peripheral diabetes-related miRNAs have not changed significantly from susceptible individual with normal glucose tolerance at pre-diabetic stage (106). miR-1 and miR-133a have been found to be good predictors of myocardial steatosis in diabetic patients (105). The fact that miR-1 and miR-133a are poorly associated with other clinical, biochemical, metabolic, hemodynamic, and cardiac parameters, and even with verified absence of clinically evident myocardial ischemia and/or damage supports the hypothesis that these miRNAs are independent predictors of myocardial steatosis.

miR-21, miR-29a/b/c, and miR-192 could reflect DN pathogenesis and serve as biomarkers during DN progression as there levels were significantly enriched in the overt proteinuria group compared with microalbuminuria and/or overt proteinuria groups. Authors observed that miR-192 suppressed the translation of SIP1/E-box repressors ZEB2, leading to elevated collagen deposition *in vivo* indicating a role of miR-192 in the development of the matrix accumulation observed in DN. Whereas, miR-21 prevented mesangial hypertrophy by targeting the PTEN/PI3K/AKT pathway and miR-29 was negatively regulated by TGF-β1 *via* SMAD3 signaling pathway, thereby promoting collagen matrix expression (107).

Some microRNAs have been found significantly increased in blood samples of diabetic patients with retinopathy; for example, Qing et al. (108) have reported that circulating miR-21,

miR-181c, and miR-1179 together could be good biomarkers for differentiating between proliferative and non-proliferative retinopathy. Barutta et al. (109) recently reported that circulating miR-126 levels were significantly lower in diabetic patients as compared to controls and were associated with both micro- and macrovascular complications, especially with proliferative retinopathy In a large cohort of type 1 diabetic subjects. Circulating microRNAs as biomarkers of diabetes-induced cardiomyopathy have also been reviewed recently (110).

Exosomes are small extracellular vesicles present in blood and urine is rich in microRNAs and is being investigated as potential disease markers. Mohan et al. (111) showed that urinary exosomal microRNAs 451-5p levels increased and correlated with renal damage in diabetic rats and suggested these to be useful as early biomarkers of diabetic nephropathy. Thus, microRNAs show promising potential as biomarkers for vascular complications of diabetes.

MICRORNAs AS THERAPEUTICS IN MICROVASCULAR COMPLICATIONS OF DIABETES

MicroRNAs have been explored for their potential as new therapeutic targets in diabetes vascular complications; for example, Kovacs et al. (82) showed that miR-146 through its inhibition of NF-kβ activation could be a potential therapeutic target in cardiovascular complication of diabetes. miR-130a is shown to improve EPCs function by negatively regulating RUNX3 and through ERK/VEGF and AKT pathways and could have a potential use in improving endothelial function (112). Downregulation of miR-200b has been implicated in Glucose-induced augmented vascular endothelial growth factor (VEGF) production through histone H3 lysine-27 trimethylation (113). Thus, methyltransferase inhibitors like COMT inhibitor could be used to control VEGF augmentation by upregulation of miR-200b. On similar grounds, in a recent study, vitamin B_3 and nicotinic acid have been shown to have a protective effect in diabetic retinopathy by upregulating miR-126 (114). miR-34 family modulates changes in proliferation and migration of retinal pigment epithelial cells through downregulation of leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4) expressions, indicating G protein (heterotrimeric) inhibitors as potential therapeutics (115). Apart from this, miR-21 is an important miRNA frequently upregulated in T2DM and cardiovascular complication of diabetes (116). miR-21 targets SMAD7 pathway and also blocks the expression of PDCD4 and thereby, suppress activation of the TGF-β and NF-κB signaling pathways. Since miR-21 is upregulated in cells related to diabetic complications, their exclusive molecular signatures can be used as prognosis, diagnosis, and therapeutic targets. Sekar et al. (79) have also shown that targeting miR-21 by synthetic anti-miRNA oligonucleotides (AMOs) with 2-O-methylmodification effectively inhibited the miRNA 21 in cell culture and xenograft mouse models. In addition to this, antisense-RNA, miRNAs mimics, and tumor suppressor miRNAs could be also used to inhibit the expression of miR-21.

LONG NON-CODING RNAs AND VASCULAR COMPLICATIONS OF DIABETES

Long non-coding RNAs (lncRNAs) are >200-nt-long non-coding RNAs and are increasingly being recognized as important gene regulators. lncRNAs repress gene expression by binding to specific DNA/RNA or protein moieties (80). For example, they can bind to miRNAs and thereby prevent their binding to target mRNAs and, hence, gene expression (81) or they may regulate activity of regulatory proteins by altering their affinity or cellular localization for other proteins (83). Aberrant expression of lncRNAs has been implicated in pathophysiology of several diseases such as tumorigenesis and cardiovascular diseases; however, their role in vascular complications of diabetes remains largely unknown. Recent studies have identified several lncRNAs with potential role in diabetic nephropathy, retinopathy, neuropathy, and cardiomyopathy.

Data on lncRNAs in diabetic cardiomyopathy are sparse. Zhang et al. (117) recently reported increased expression of lncRNAs MALAT1 in the heart tissue of diabetic rats, and observed that its inhibition Improved left ventricular function, by attenuating cardiomyocyte apoptosis. A downregulation of lncRNA H19 has been also seen in diabetic hearts and it has been shown to increase expression of miRNA-675 and downregulation of its target VDAC1 leading to decreased cardiomyocyte apoptosis of cardiomyocytes in high glucose milieu (118). Zhuo et al. (119) have recently showed that H19 also inhibited autophagy in glucose-treated cardiomyocytes by silencing pro-autophagy DIRAS3. lnc H19 has been suggested as a potential biomarker and therapeutic target for diabetic cardiomyopathy. However, more research is needed to explore the potential role of lncRNAs in diabetic cardiomyopathy.

Wang et al. (120) reported downregulation of CYP4B1-PS1-001 in both in early stages of diabetic nephropathy and suggested its role in mesangial cell proliferation and fibrosis. Alvarez et al. (100) earlier showed that a long non-coding RNA, the plasmacytoma variant translocation 1 (PVT1), increased fibronectin 1 (FN1) ECM accumulation in the glomeruli under hyperglycemic conditions, suggesting its role in diabetic nephropathy. They recently reported that miR-1207-5p, a PVT1-derived microRNA, was also independently involved in pathogenesis of diabetic nephropathy. Similarly lncRNA ENSMUST00000147869 associated with Cyp4a12a has been shown to mediate diabetic nephropathy by increasing proliferation and fibrosis of mesangial cells (121). Several other lncRNAs, such as MALAT1 (122), myocardial infarction-associated transcript (MIAT) (123) and lnc-MGC, have been found to be dysregulated in diabetes-induced renal injury and are potential therapeutic targets for treating diabetes-induced nephropathy.

lncRNA-RNCR3 has been implicated in diabetes-induced retinopathy. Liu et al. (67) recently showed that lncRNA-RNCR3 knockdown decreased cytokine levels, retinal cell apoptosis, improved visual function, and inhibited retinal reactive gliosis in diabetic animals, indicating its role in diabetes-induced neurodegeneration. Shan et al. have increase in RNCR3 levels following high glucose stress both *in vitro* and *in vivo*. They observed

that RNCR3 knockdown inhibited RECs proliferation, and cell migration and tube formation *in vitro* and improved endothelial function *in vivo* (124) *via* RNCR3/KLF2/miR-185-5p pathway, suggesting RNCR3 inhibition as a therapeutic option in treating diabetic retinal abnormalities.

The studies done so far indicate that lncRNAs are important mediators of various vascular complications of diabetes and potential therapeutic targets and need to be explored further.

CONCLUDING REMARKS

Metabolic disorders such as diabetes are due to cumulative interactive effects of genetic and environmental factors. These effects are primarily induced by diabetes-associated factors, such as hyperglycemia, oxidative stress, inflammation, obesity, and so on, and are manifested as epigenetic changes in the genome. These epigenetic changes include DNA methylation, histone methylation and acetylation, deregulated expression of microRNAs and lncRNAs etc. and are responsible for altered gene expression of the key regulatory pathways mediating diabetes-associated vascular complications and also are major contributors to metabolic memory associated with diabetes. Thus, study of epigenetic mechanisms assumes a significant role in elucidating pathophysiology of diabetes and its complications. However, our understanding of these mechanisms is incomplete and awaits translational application. Further research focus is needed to elucidate the mechanisms especially with respect to non-coding RNAs and chromatin structure. The information being generated in microRNAs and lncRNAs shows that we are at threshold of unveiling of important biological role of these molecules in disease etiology, pathology, progression, and therapeutics, besides being non-invasive diagnostic and prognostic biomarkers of vascular complications, such as nephropathy and cardiomyopathy.

To gain a deeper understanding of T2DM and its associated microvascular complications, an incorporation of a range of novel tools and techniques, such as RNAseq, transcriptomics, metabolomics, epigenomic profiling, and chromatin 3D mapping, is needed to be integrated in diabetes research. Tissue- and cell-specific profiling of methylation levels and histone modifications of major pathophysiological genes would increase our understanding of the pathology of T2DM and associated complications. Elucidation of association between epigenetic modulations of the genome involved in microvascular complication with those of macrovascular complications of diabetes is also needed. The knowledge gained through epigenetics gene expression alteration in diabetic cardiomyopathy will provide better approaches in attenuating hyperglycemia-induced damage to the heart and other affected organs, such as kidney and brain. Thus, elucidation of epigenetic mechanisms in conjunction with environmental and genetic factors would fine tune the understanding of pathophysiology of diabetic cardiomyopathy. And, epigenetic factors could provide a wholesome picture of the role of genes and their expression in T2DM and its micro as well as macrovascular complications.

AUTHOR CONTRIBUTIONS

MK: checking, editing, re-writing, data approval, and guarantor of work. BC: data collection, writing, and reference updation. SR: checking, editing, data collection, writing, and reference updation.

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