OBESITY AND DIABETES: ENERGY REGULATION BY FREE FATTY ACID RECEPTORS

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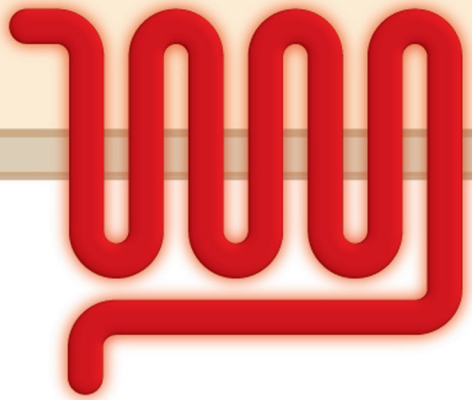

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OBESITY AND DIABETES: ENERGY REGULATION BY FREE FATTY ACID RECEPTORS

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Image by Eiri Ono

Food intake regulates energy balance and its dysregulation leads to metabolic disorder, such as obesity and diabetes. During feeding, free fatty acids (FFAs) are not only essential nutrients but also act as signaling molecules in various cellular processes. Recently, several orphan G protein-coupled receptors (GPCRs) that act as FFA receptors (FFARs) have been identified; GPR40/FFAR1, GPR119, and GPR120 are activated by medium- and long-chain FFAs. GPR84 is activated by medium-chain FFAs. GPR41/FFAR3 and GPR43/FFAR2 are activated by short-chain FFAs. These FFARs have come to be regarded as new drug targets for metabolic disorder such as obesity and type 2 diabetes, because a number of pharmacological and physiological studies have shown that these receptors are primarily involved in the energy metabolism in various tissues; insulin secretion, gastrointestinal hormone secretion, adipokine secretion, regulation of inflammation, regulation of autonomic nervous system, relation to gut microbiota, and so on. This Research Topic provides a comprehensive overview of the energy regulation by free fatty acid receptors and a new prospect for treatment of metabolic disorder such as obesity and type 2 diabetes.

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EDITORIAL
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Editorial: Obesity and Diabetes: Energy Regulation by Free Fatty Acid Receptors

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Keywords: obesity, diabetes, energy regulation, free fatty acid receptors, editorial

Food intake regulates energy balance, and its dysregulation leads to metabolic disorders, such as obesity and type 2 diabetes (T2D). During feeding, free fatty acids (FFAs) are not only essential nutrients but also act as signaling molecules in various cellular processes. Recently, several G protein-coupled receptors (GPCRs) that act as FFA receptors (FFARs) have been identified; GPR41/FFAR3 and GPR43/FFAR2 are activated by short-chain FFAs. GPR40/FFAR1, GPR119, and GPR120/FFAR4 are activated by medium- and long-chain FFAs. FFARs are widely expressed and contribute to many important physiological functions in order to maintain energy homeostasis. Hence, these FFARs have come to be regarded as new drug targets for metabolic disorder such as obesity and T2D.

All articles in this topic highlight the interconnection between FFARs and the regulation of energy homeostasis. They also focused on essential role of FFARs in the pathogenesis of metabolic syndromes, such as obesity, insulin resistance, and T2D and discussed the potential of FFARs as drug target. These articles give valuable insight into unanswered questions in relation to this topic. First, recent studies demonstrate that short-chain free fatty acids (SCFAs) produced by microbiota fermentation act as signaling molecules through SCFAs receptors (SCFARs), such as GPR41 and GPR43 and influence the host's metabolism (1–3). Hence, the gut microbiota can influence and play important roles in host physiology and pathology *via* these receptors. GPR41, which is expressed in adipose tissue, gut, and the peripheral nervous system, contributes SCFAs-dependent systemic energy regulation (1). In particular, GPR41 regulates host energy balance by modulating sympathetic activity and intestinal gluconeogenesis. GPR43, which is expressed in the adipose tissue, intestines, and immune tissues, also contributes the regulation of energy homeostasis depends on SCFAs produced by gut microbiota (2). GPR43 deficiency induced obesity in mice, while mice that overexpress GPR43 only in adipose tissue were lean under normal conditions; both of these strains did not exhibit either phenotype under germ-free conditions or after antibiotic treatment. Furthermore, SCFA-mediated GPR43 activation suppressed adipose insulin signaling, leading to inhibition of fat accumulation in the adipose tissues, while unincorporated lipids and glucose were primarily utilized in muscles. The GPR43-insulin pathway has a key role in adipose tissue acting as an important physiological mechanism through which metabolic fuels regulate body energy balance (2, 3). These studies clearly showed the importance of SCFAs produced by microbiota and their receptors (1–3). Based on the importance and dynamic roles of microbiota in host physiology, Pluznick pointed out a complex interplay between the genetics of the microbiota and that of the host organism (4). Researchers should consider the contribution of these microorganisms and their metabolites because there are many examples of phenotypes that were not easily to replicated by other groups may be due to the influence of variations of gut microbiota (4). Second, medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs) are not only essential nutrient, but also act as ligands of GPR40/FFAR1 and GPR120/FFAR4 and regulate systemic energy homeostasis (5–8). GPR40 is highly expressed in pancreatic β cells and intestine. GPR40

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augment glucose-stimulated insulin secretion after acute exposure to LCFAs by stimulation of not only insulin secretion from pancreatic β cells directly, but also incretin hormones, such as glucagon like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and cholecystokinin (CCK) from intestine (5, 8). The activation of GPR120 by ω-3 FFAs mediated anti-inflammatory effect of ω-3 FFAs as described in the articles by Oh et al. (7). This effect is associated with the suppression of Toll-like receptor via β-arrestin2 signaling pathway and transforming growth factor-β activated kinase 1 (TAK1) involved in TNF-α inflammation signaling pathway. Furthermore, both a gene deficiency in mice and non-synonymous functional-loss mutation of human GPR120 are associated with obesity, which was accompanied with decreased differentiation and lipogenesis (6). Hence, selective synthetic ligands for FFARs have consequently been developed as potential treatments for metabolic syndrome (9). Particularly, clinical studies show that TAK875/Fasiglifam, an agonist of GPR40 improved glucose metabolism with a reduced risk of hypoglycemia, although this ligand was dropped from clinical trials due to potential liver toxicity. Activation of each of GPR41, 43, and 120 has also been suggested to have potential benefits for metabolic function (9).

Overall, all the review articles provided a comprehensive overview of the energy regulation by FFARs and a new prospect for treatment of metabolic disorder such as obesity and type 2 diabetes.

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The SCFA receptor GPR43 and energy metabolism

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Free fatty acids (FFAs) are essential nutrients and act as signaling molecules in various cellular processes via binding with FFA receptors. Of these receptors, GPR43 is activated by short-chain fatty acids (SCFAs; e.g., acetate, propionate, and butyrate). During feeding, SCFAs are produced by microbial fermentation of dietary fiber in the gut, and these SCFAs become important energy sources for the host. The gut microbiota affects nutrient acquisition and energy regulation of the host and can influence the development of obesity, insulin resistance, and diabetes. Recently, GPR43 has been reported to regulate host energy homeostasis in the gastrointestinal tract and adipose tissues. Hence, GPR43 is also thought to be a potential drug target for metabolic disorders, such as obesity and diabetes. In this review, we summarize the identification, structure, and activities of GPR43, with a focus on host energy regulation, and present an essential overview of our current understanding of its physiological roles in host energy regulation that is mediated by gut microbiota. We also discuss the potential for GPR43 as a therapeutic target.

Keywords: GPR43, FFAR2, SCFA, gut microbiota, energy metabolism

INTRODUCTION

Obesity is currently one of the most serious public health problems worldwide because of its increasing prevalence and contribution to serious metabolic disorders, including type-2 diabetes (1, 2). Obesity is the result of a long-term imbalance between energy intake and expenditure, and is therefore regulated by multiple pathways involving metabolites, hormones, and neuropeptides (3). Excess food intake, especially high-fat and sugar foods, and lack of physical activity are considered as risk factors in the developing of obesity. Recent research has demonstrated that the gut microbiota is involved in obesity and metabolic disorders (4, 5). An important role of the gut microbiota is to catabolize substrates, such as dietary fiber, that are not completely hydrolyzed by host enzymes during host feeding (6). The main colonic bacterial fermentation products of dietary fiber are short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (7). SCFAs can be used for *de novo* synthesis of lipids and glucose, which are the main energy sources for the host (8).

THE SCFA RECEPTOR GPR43

In addition to functioning as an energy source, SCFAs are also essential nutrients that act as signaling molecules. Recently, two orphan G-protein coupled receptors (GPCR), GPR41 and GPR43, were reported to be activated by SCFAs. During ligand screening for bioactive compounds, researchers reported that GPR43, also known as free fatty acid receptor 2 (FFAR2), was activated by acetate using Ca^{2+} assays in transfected cells (9, 10). GPR43 can also be activated by other SCFAs, including propionate and butyrate; acetate and propionate are the most efficient for activating GPR43, followed by butyrate and then other SCFAs (9, 11).

GPR43 is a dual-coupling GPCR that binds with the pertussis toxin-sensitive $G_{i/o}$ and G_q proteins (11). Stimulation of GPR43 by SCFAs inhibits cAMP production, activates the extracellular signal-regulated kinase (ERK) cascade via interactions with the $G_{i/o}$ family of G-proteins, increases intracellular Ca^{2+} levels, and promotes activation of the mitogen-activated protein kinase (MAPK) cascade via interactions with the G_q family of G-proteins. However, the physiological significance of this GPR43-based dual-coupled signaling mechanism is still unclear. GPR43 is expressed in the adipose tissue, intestines, and immune tissues (12, 13). In the immune system, many studies have investigated the role of GPR43 in regulating inflammatory responses (13–15). These results indicate that GPR43 is important for gut immunity involving gut microbiota and food. On the other hands, GPR43 expression in adipose and gastrointestinal tissues suggests that GPR43 may be involved in energy regulation (16); moreover, reverse transcription polymerase chain reaction (RT-PCR) in mouse tissues has shown that *Gpr43* is expressed in white adipose tissue (WAT) and the intestine (12).

ADIPOSE TISSUES

Adipose tissues are very important tissues associated with energy homeostasis and energy accumulation. In adipose tissues, GPR43 may be involved in regulating obesity and energy accumulation, Similarly, *Gpr43* mRNA is expressed in WATs, including subcutaneous, perirenal, and epididymal tissues, as well as in 3T3-L1-derived adipocytes and mature adipocytes (12). Based on the observed expression of *Gpr43* in adipose tissues and adipocytes, Hong et al. performed a series of studies to elucidate the functions of GPR43 in adipocytes (12). They showed that *Gpr43* expression was significantly greater in the WAT of mice with high-fat diet (HFD)-induced obesity compared with normal chow-fed mice. Moreover, in 3T3-L1 cells, treatment with SCFAs, increased *Gpr43* and *Pparg* transcript levels, while suppression of *Gpr43* mRNA by RNA interference inhibited adipogenesis. Thus, SCFAs appear to

promote adipogenesis via GPR43. Additionally, in 3T3-L1 derived adipocytes, SCFAs suppress isoproterenol-induced lipolysis in a concentration-dependent manner (12). Ge et al. demonstrated that these effects are dependent on GPR43 using Gpr43-deficient mice (17). That is, they showed that acetate suppressed lipolysis, and release of glycerol occurred in a concentration-dependent manner in adipocytes isolated from wild-type mice *in vitro*, and the activation of GPR43 by intraperitoneal injection of sodium acetate instantly reduced plasma fatty acid *in vivo*; these effects were abrogated in *Gpr43*-knockout mice (17). In brown adipose tissues (BATs), which have a central role in the regulation of energy balance and homeostasis, Bjursell et al. reported that *Gpr43*-knockout mice fed an HFD exhibited improved insulin sensitivity in old age due to increased energy expenditure, which resulted in increased body temperature (18). As a potential explanation for this, histological observation of BAT in *Gpr43*-knockout mice revealed that these mice exhibited decreased lipid dispersion compared with wild-type mice fed an HFD. However, we could not detect *Gpr43* expression in BATs (19). Hence, further studies are needed to elucidate the role of GPR43 in energy control via BAT.

Recent evidence suggests that the gut microbiota affects host nutrient acquisition and energy regulation and is therefore related to obesity, insulin resistance, and diabetes in the host (20–22). During feeding, SCFAs, which act as ligands for GPR43, are produced by microbial fermentation of dietary fiber in the gut. Hence, we examined the relationship between gut microbiota and systemic energy regulation by GPR43 in adipose tissue using *Gpr43*-mutantand germ-free mice (19). In a series of *in vitro* and *in vivo* studies, we found that *Gpr43* deficiency induced obesity in mice, while mice that overexpress *Gpr43* only in adipose tissues were lean under normal conditions; both of these mouse strains did not exhibit either phenotype under germ-free conditions or after antibiotic treatment. Furthermore, SCFA-mediated GPR43 activation suppressed adipose insulin signaling, leading to inhibition of fat accumulation in the adipose tissue, and unincorporated lipids and glucose were primarily utilized in muscles. That is, the expression of energy expenditure-, glycolysis-, and beta-oxidation-related genes increased, while the expression of gluconeogenesis-related genes decreased in the muscles of *aP2-Gpr43* TG mice. However, the mechanism by which GPR43 mediated the suppression of insulin signaling in adipocytes is not mediated by cAMP inhibition, but instead involves the beta and gamma subunits of the $G_{i/o}$ protein, not G_q protein. Thus, GPR43 acts as a sensor for excessive dietary energy, thereby controlling body energy utilization while maintaining metabolic homeostasis. The GPR43-insulin pathway in adipose tissue may function as an important physiological mechanism through which these metabolic fuels regulate body energy balance. Hence, these previous reports in adipose tissues indicate that GPR43 has potential therapeutic relevance for the treatment of metabolic disorders, such as obesity and type-2 diabetes.

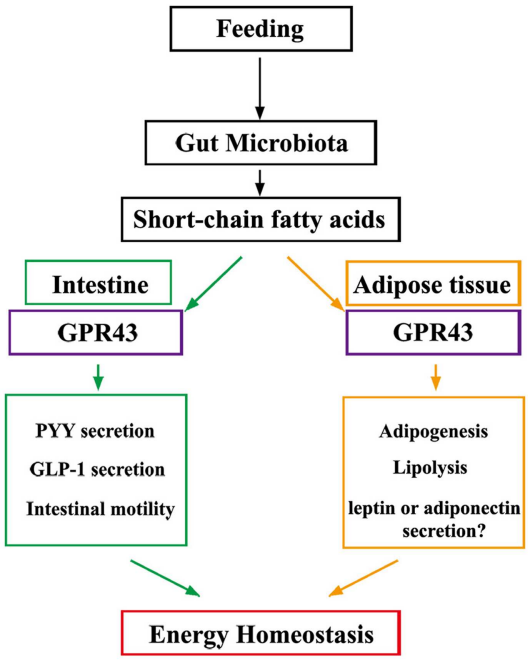

FIGURE 1 | Under "fed" conditions, SCFAs are produced in the gut by bacterial fermentation of dietary fiber. SCFAs alter the metabolic rate by stimulating GPR43 in adipocytes and promote gut hormone secretion and motility by stimulating GPR43 in the intestine, thereby increasing energy expenditure and improving glucose tolerance to increase energy utilization.

INTESTINAL TISSUES

In the intestines, GPR43 may be involved in regulating appetite and insulin signaling. Indeed, *Gpr43* mRNA has been shown to be expressed in rat and human ileum and colon, especially in enteroendocrine cells (23, 24). Like adipose tissue, the intestine is also critical for energy homeostasis, as supported by its association with secretion of appetite gut hormones and nutrients absorption (25, 26). Using immunohistochemistry analysis with GPR43 antibodies in rats, Karaki et al. reported that GPR43 is expressed in peptide YY (PYY)-containing enteroendocrine L-cells of the gastrointestinal tract (23). Enteroendocrine L-cells are also one of the major cell types that express the proglucagon genes *GLP-1* and *GLP-2*. GLP-1 and GLP-2 proteins are co-stored and co-secreted with PYY from enteroendocrine L-cells (27), and SCFAs are co-secreted with GLP-1 from mixed colonic cultures via GPR43 *in vitro* and *in vivo* (28). Quantitative RT-PCR (qRT-PCR) showed that *Gpr43* and *Gpr41* were abundantly expressed in GLP-1-secreting L-cells. Moreover, SCFAs raised cytosolic Ca^{2+} through G_q signaling pathways in L-cells in primary culture. *Gpr43*- or *Gpr41*-knockout mice exhibited reduced SCFA-mediated GLP-1 secretion both *in vitro* and *in vivo* and have impaired glucose tolerance. Additionally, *Gpr43*-knockout mice exhibited reduction of insulin secretion in accompaniment with the reduction of *in vivo* glucose-stimulated GLP-1 secretion (28). However, to determine the effects of SCFAs on the secretion of gut hormones, the expression and function of GPR41, and GPR43 in subtypes of enteroendocrine cells, such as L-cells and K-cells, must be characterized in detail using *Gpr41*- and *Gpr43*-double-knockout mice. Thus, pharmacological manipulation of appetite using a GPR43 agonist may be useful for treatment of obesity. Moreover, these types of studies may provide essential information concerning

the role of GLP-1 in insulin secretion in patients with type-2 diabetes. The anorexigenic neural circuits are subsequently activated via PYY and GLP-1, reducing food intake and increasing energy expenditure. Hence, regulation of PYY and GLP-1 secretion via GPR43 maintains energy homeostasis and may be a valid approach for treating metabolic disorders.

CONCLUSION

GPR43 regulates metabolic rate when activated by SCFAs that are produced by gut microbiota in a variety of host tissues (**Figure 1**). Future studies are expected to reveal the presence of a central mechanism that mediates the effects of diet and probiotics on human homeostasis. Additionally, GPR43 may represent a promising therapeutic target for the treatment of metabolic syndromes, such as obesity and diabetes.

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Regulation of energy homeostasis by GPR41

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Imbalances in energy regulation lead to metabolic disorders such as obesity and diabetes. Diet plays an essential role in the maintenance of body energy homeostasis by acting not only as energy source but also as a signaling modality. Excess energy increases energy expenditure, leading to a consumption of it. In addition to glucose, mammals utilize short-chain fatty acids (SCFAs), which are produced by colonic bacterial fermentation of dietary fiber, as a metabolic fuel. The roles of SCFAs in energy regulation have remained unclear, although the roles of glucose are well-studied. Recently, a G-protein-coupled receptor deorphanizing strategy successfully identified GPR41 (also called free fatty acid receptor 3 or FFAR3) as a receptor for SCFAs. GPR41 is expressed in adipose tissue, gut, and the peripheral nervous system, and it is involved in SCFA-dependent energy regulation. In this mini-review, we focus on the role of GPR41 in host energy regulation.

Keywords: GPR41, FFAR3, energy regulation, short-chain fatty acid, gut microbiota

INTRODUCTION

Dysfunctional energy regulation leads to a variety of metabolic disorders, including obesity (1, 2). Mammals utilize not only glucose as the main energy source, but also short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which are produced by colonic bacterial fermentation of dietary fiber, in a significant proportion of their daily energy requirement (3, 4). The connections between gut microbiota, energy homeostasis, and the pathogenesis of metabolic disorders are now well-established (5, 6). In 2003, several groups reported that two orphan G-protein-coupled receptors (GPCR), namely GPR41 (also called free fatty acid receptor 3 or FFAR3) and GPR43 (also called free fatty acid receptor 2 or FFAR2), are activated by SCFAs (7, 8). GPR41 is reported to couple with Gi/o protein. It is also reported that GPR41 is expressed in adipose tissue, the gut, and the peripheral nervous system. Moreover, GPR41 is reported to be involved in energy regulation in response to SCFAs produced from the gut microbiota. In the following sections, we discuss the role of GPR41 in host energy regulation.

ADIPOSE TISSUE

In adipose tissue, the role of GPR41 in the release of leptin, a polypeptide hormone with pleiotropic effects on appetite and energy metabolism, is the subject of much discussion. *Gpr41* mRNA is known to be expressed in human (7–9) and mouse (10) adipose tissue. Xiong et al. showed that propionate-stimulated activation of GPR41 increases the release of leptin (10). In mice, oral administration of propionate increased plasma leptin levels (10). Furthermore, in experiments using Ob-Luc cells, leptin secretion was increased through overexpression of exogenous *Gpr41* and was decreased by siRNA-mediated knockdown of *Gpr41* (10). Another group showed that propionate-dependent increase in *Leptin* mRNA and protein levels could be inhibited by pretreatment with the Gi/o protein inhibitor, pertussis toxin (9).

However, Hong et al. (11) were unable to detect *Gpr41* mRNA in differentiated 3T3-L1 cells or in mouse white adipose tissue (subcutaneous, perirenal, mesenteric, and epididymal fat pads) (11), even though they used the same PCR primers as Xiong et al. (10). We also previously reported that *Gpr41* expression could not be detected in mouse adipose tissue by quantitative RT-PCR or *in situ* hybridization analysis (12, 13). In contrast, *Gpr43* mRNA, rather than *Gpr41* mRNA, is expressed in mouse adipose tissues (11, 13, 14). Zaibi et al. showed that acetate, rather than butyrate, stimulates leptin secretion by mesenteric adipocytes in wild-type mice (14). GPR41 is activated equally by propionate and butyrate, whereas GPR43 is preferentially activated by propionate rather than butyrate (7). Because of the difference in ligand preference between GPR41 and GPR43, it was suggested that SCFA-stimulated leptin secretion is mediated by GPR43, rather than GPR41 (14). To clarify these discrepancies, the generation of adipose tissue-specific *Gpr41* or *Gpr43* knockout mice will be invaluable.

GUT

In the gut, GPR41 regulates host energy balance by modulating gut motility. By using *in situ* hybridization analysis, Samuel et al. showed that mouse *Gpr41* mRNA is expressed in cells with the

morphologic appearance of enteroendocrine cells (15). The body weight and fat pad weight of *Gpr41* knockout mice are significantly reduced compared to wild-type mice, and this difference is abolished in germ-free conditions (15). These results indicate that the function of GPR41 depends on the SCFA produced by the fermentation of microbiota. Tazoe and colleagues also found the human *Gpr41* is expressed in peptide YY (PYY)-containing enteroendocrine cells (16). Recently, several groups have confirmed *Gpr41* mRNA expression in mouse intestinal L cells, which secrete incretin hormones such as GLP-1 and PYY (17, 18). Samuel and colleagues (15) showed that co-colonization of human gut-derived microbiota in germ-free mice led to significantly increased circulating levels of PYY, which suppresses gut motility. Furthermore, this increase was significantly suppressed in their *Gpr41* knockout littermates, although *Gpr41* deletion did not affect the amount of chow consumption. Intestinal transit rate was significantly faster in *Gpr41* knockout mice compared with wild-type littermates; this phenotype was abolished in germ-free conditions. Moreover, the SCFA content in feces of *Gpr41* knockout mice was significantly higher than in wild-type mice. These results led the authors suggest that the decreased PYY level in *Gpr41* knockout mice increases gut motility, which leads to reduced SCFA absorption and consequently a lean phenotype (15).

In contrast, Bellahcene et al. reported a male-specific increase in body fat mass of *Gpr41* knockout mice when compared to their wild-type littermates; this was observed when mice were fed with either a low- or high-fat diet (19). Deletion of *Gpr41* had no effect on the amount of food intake by either sex, regardless of the type of diet. This included mice of the same age (10 weeks) as those used in the report by Samuel and colleagues (15). The differences in sex hormones could explain why the energy expenditure of female *Gpr41* knockout mice is similar to that of wild-type mice. Nevertheless, it is also possible that reduced SCFA absorption due to increased gut motility is responsible for the alleviation of obesity in *Gpr41* knockout mice (19). Alternatively, reduced energy expenditure in *Gpr41* knockout mice might be caused by reduced sympathetic activity (see Peripheral Nervous System below).

The altered body weight of *Gpr41* knockout mice may be due to differences in genetic backgrounds, or due to the precise constitution of gut microbiota in each animal cohort.

Tolhurst et al. suggested that SCFAs could directly enhance the release of incretin hormones such as GLP-1 and PYY from L cells in gut. In *Gpr41* knockout mice, glucose-stimulated GLP-1 secretion was lower than wild-type mice (17); this was confirmed by Nøhr et al. using the GPR41-selective agonist, AR420626 (18). Consistent with these findings, oral glucose tolerance was impaired in *Gpr41* knockout mice (17).

PERIPHERAL NERVOUS SYSTEM

GPR41 regulates host energy balance by modulating sympathetic activity and intestinal gluconeogenesis. By using *in situ* hybridization and quantitative RT-PCR analysis, we have reported that *Gpr41* mRNA is abundantly expressed in the mouse sympathetic ganglion (12). *Gpr41* knockout mice exhibit a retardation of sympathetic nerve growth. However, further studies will be required to elucidate the precise molecular mechanism by which GPR41 modulates sympathetic nerve differentiation and growth.

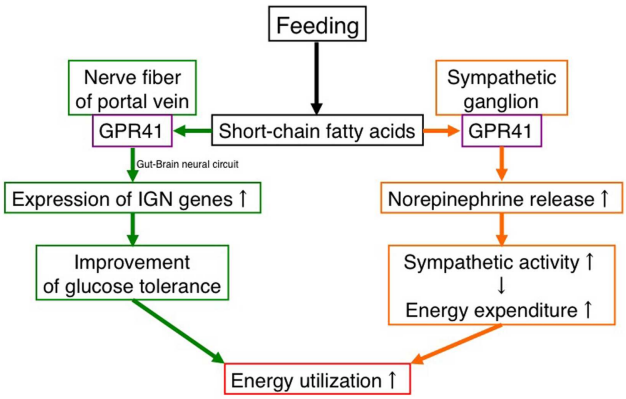

FIGURE 1 | Effects of SCFAs in energy utilization mediated by GPR41. Under "fed" conditions, SCFAs are produced in the gut by bacterial fermentation of dietary fiber. SCFAs increase energy utilization by two mechanisms. One is to activate the sympathetic nervous system by stimulating GPR41 in sympathetic ganglia, leading to an increase in energy expenditure. The other is to activate inducing intestinal gluconeogenesis by stimulating GPR41 in the nerve fibers of the portal vein, leading to an improvement of glucose tolerance. In contrast, the β-HB produced in the liver under "fasting" conditions suppresses the activation of GPR41.

In adult wild-type mice, energy expenditure and heart rate are increased by propionate administration; these effects are abolished in *Gpr41* knockout mice (12). The effect of propionate on the heart rate is inhibited by pretreatment with the β-adrenergic receptor blocker propranolol, but not by the nicotinic acetylcholine receptor blocker hexamethonium. These results indicate that propionate activates the sympathetic nervous system (SNS) via GPR41 at the ganglionic level (12). The function of GPR41 in sympathetic ganglia is consistent with the lower energy expenditure and obese phenotype of *Gpr41* knockout mice reported by Bellahcene et al. (19). Furthermore, our laboratory showed that propionate increased the release of norepinephrine from sympathetic neurons through the GPR41–Gβγ–phospholipase C (PLC) β 3-ERK1/ 2-synapsin 2 (synaptic vesicle-associated phosphoprotein) pathway (12, 20). In addition, we found that β-hydroxybutyrate (β-HB) has a potent antagonistic effect on GPR41 (12). β-HB is a ketone body that can be produced in the liver under ketogenic conditions such as starvation, low-carbohydrate dietary intake, and diabetes. β-HB suppressed propionate-induced sympathetic activation in both primary cultured sympathetic neurons and mice (12, 20). However, acetoacetate, another major ketone body, had no significant effect (12).

Recently, another group demonstrated that SCFA-mediated GPR41 activation improves glucose tolerance by inducing intestinal gluconeogenesis via a gut–brain neural circuit (21). They found *Gpr41* mRNA in the nerve fibers of the portal vein (21). The SCFA-fed rats exhibited improved glucose tolerance compared with standard-diet-fed rats. This effect of SCFA was abolished by portal denervation with capsaicin. Propionate infusion in the portal vein activated jejunum G6Pase, the rate-limiting enzyme for gluconeogenesis. On the other hand, β-HB, an antagonist of GPR41, slightly decreased G6Pase activity when infused alone and reversed propionate-mediated induction of G6Pase (21).

These findings suggest that GPR41 functions as an energy sensor in the peripheral nervous system to maintain energy homeostasis (**Figure 1**).

CONCLUDING REMARKS

It is clear that GPR41 plays a critical role in host energy regulation, although not all of the intracellular signaling cascades that are required for GPR41 function have been elucidated. We envisage that future studies of the interaction between gut microbiota and GPR41, with a particular focus on SCFAs, will provide a more complete picture of GPR41 biological function. Given the beneficial effects that SCFA-dependent GPR41 activation on regulation of metabolism, we suggest that modulating GPR41 by using synthetic ligands will be a promising therapeutic strategy for the treatment of metabolic disorders.

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Contributions of colonic short-chain fatty acid receptors in energy homeostasis

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The gastrointestinal (GI) tract is separated from the body's internal environment by a single layer of epithelial cells, through which nutrients must pass for their absorption into the bloodstream. Besides food and drink, the GI lumen is also exposed to bioactive chemicals and bacterial products including short-chain fatty acids (SCFAs). Therefore, the GI tract has to monitor the composition of its contents continuously to discriminate between necessary and unnecessary compounds. Recent molecular identification of epithelial membrane receptor proteins has revealed the sensory roles of intestinal epithelial cells in the gut chemosensory system. Malfunctioning of these receptors may be responsible for a variety of metabolic dysfunctions associated with obesity and related disorders. Recent studies suggest that SCFAs produced by microbiota fermentation act as signaling molecules and influence the host's metabolism; uncovering the sensory mechanisms of such bacterial metabolites would help us understand the interactions between the host and microbiota in host energy homeostasis. In this review, the contribution of colonic SCFA receptors in energy metabolism and our recent findings concerning the possible link between SCFA receptors and host energy homeostasis are discussed.

Keywords: luminal chemosensing, short-chain fatty acid, FFA2, FFA3, energy metabolism, gut microbiota

INTRODUCTION

Part of the gastrointestinal (GI) tract, the intestinal lumen is one-way tube where food materials from the external environment are progressively converted into molecular products. Approximately 100 trillion bacteria, which are termed the gut microbiota, are present in the intestinal lumen, especially in the colon. A single layer of epithelial cells separates this diverse bacterial community from the internal environment. Thus, host–microbiota interactions occur at the mucosal surface of the intestine. The genome of the gut microbiota contains an estimated 150 times as many genes as in the host genome, and continuously produces large amount of various chemicals, including short-chain fatty acids (SCFAs), which can be beneficial or harmful to the host (1). The intestinal lumen is therefore continuously exposed to a multitude of dietary antigens, microorganisms, and bacterial products.

It has become clear that the GI tract responds to a large array of signals in the lumen including nutrient and non-nutrient chemicals. As mentioned above, these responses occur at the level of the mucosa, which contains the epithelial cells. The recent molecular identification of membrane receptor proteins has revealed sensory roles for these epithelial cells in the gut chemosensory system. The failure of functional interactions within the gut chemosensory system between the host and microbiota may cause a spectrum of diseases beyond local GI disorders, such as obesity, diabetes, metabolic syndrome, and various neurological diseases (2). Despite the significant role of the gut microbiota in host health, the elucidation of the molecular mechanistic pathways involved has not been moving forward as expected.

SCFA RECEPTORS, FFA2 AND FFA3 IN THE COLON

Generally, the main functions of the colon are absorption of water and electrolytes and storage of fecal matter before expulsion. Most studies of the chemosensory system in the GI tract concern the small intestine in relation to nutrient absorption, incretin release, short-term energy control, appetite, satiety, and postprandial glycemic control (3). As a result, for many years chemical sensing in the colon has been considered less important compared with that in the small intestine. However, recent studies suggest that products of microbial metabolisms in the gut act as signaling molecules and influence host energy homeostasis (4).

The gut epithelium is composed of different cell types. In the colon, epithelial cells form a sheet consisting of absorptive epithelial, goblet, enteroendocrine, M, and brush (tuft, caveolae) cells (5) (**Figure 1**). Lamina propria cells and nerve fibers lie close to the epithelial cells but do not directly contact the lumen, and these act together with the cell sheet to monitor luminal contents. Among the epithelial cells, enteroendocrine cells have been proposed to possess intestinal chemosensory function because of their open-type morphology with an apical brush border surface that extends into the gut lumen that comes in contact with chemical compounds (5). Individual enteroendocrine cells are scattered throughout the mucosa, representing ~1% of all epithelial cells in the intestine and comprise a solitary chemosensory system (6). Enteroendocrine cells are subdivided into more than 15–20 different cell types based on their major secretory products and their location along the GI tract (5). A recent study has also reported that certain enteroendocrine cells

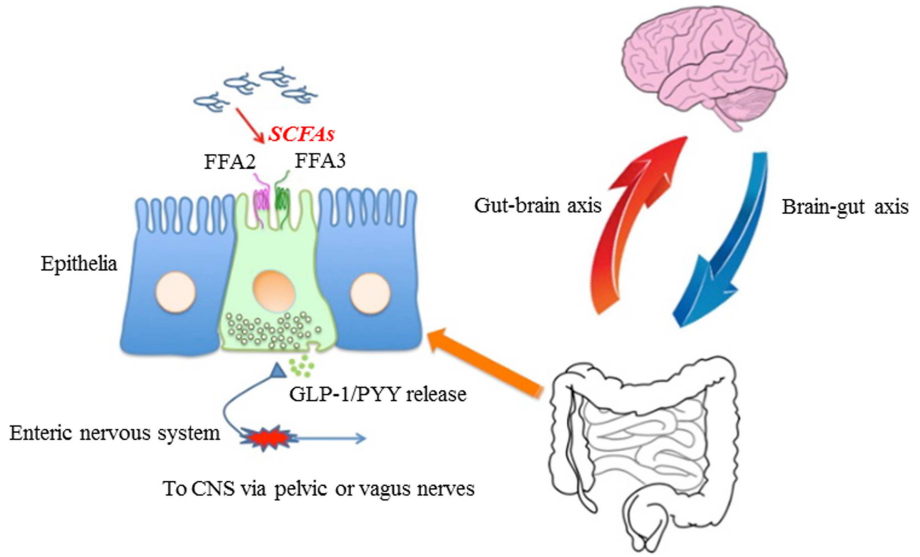

FIGURE 1 | Proposed model for roles of FFA2/FFA3 that might contribute to host energy homeostasis. In non-ruminant mammals, short-chain fatty acid (SCFA) is produced by microbiota in the distal small intestine and colon from low-digestible carbohydrates, including resistant starch and soluble oligo- and polysaccharides. These SCFAs are able to bind and activate FFA2 and/or FFA3 located on intestinal epithelia. This activation induces GLP-1 and PYY release into the basolateral side. Released GLP-1 and PYY activate enteric or primary afferent neurons in pelvic and vagal nerves in addition to humoral pathways. These information travel to the CNS, then affect the host metabolic rate to regulate energy homeostasis.

present in the GI tract express a variety of chemical receptors and gustatory signaling elements such as α-gustducin, α-transducin, and TRPM5 (7).

In 2003, FFA2 (GPR43) and FFA3 (GPR41) were deorphanized as SCFA receptors (8–10). These two receptors share ~40% amino acid sequence similarity and are conserved across several mammalian species. They differ in affinity for SCFAs, tissue distribution, and physiological roles. FFA2 has a similar affinity for acetate, propionate, and butyrate, while FFA3 has a more potent affinity for propionate than butyrate and does not have a high affinity for acetate. Thus, acetate preferentially activates FFA2, propionate mainly activates FFA3, and butyrate equally activates both FFA2 and FFA3. FFA2 and FFA3 also have distinct G proteins coupled in their intracellular signaling cascades; FFA2 couples both pertussis toxin-sensitive ($G_{i/o}$) and toxin-insensitive (G_q) G proteins, whereas FFA3 couples only to $G_{i/o}$ protein. We hypothesized that FFA2 and FFA3 function as molecular interfaces between the gut microbiota and host colon. Indeed, SCFAs produced by bacterial fermentation in the colon affects local colonic functions; luminal application of propionate and butyrate induces Cl^- secretion and muscle contraction in rat distal colons (11, 12). These local effects are not induced by serosal application of SCFAs. Based on these observations, SCFAs are likely to be detected by epithelial cells through specific receptors. We made different antisera for FFA2 and FFA3 using synthesized peptides to test this working hypothesis (13).

Using RT-PCR and western blotting analysis, mRNA and protein for FFA2 were found in the distal ileum and colon in extracts from separated rat mucosa as well as in human colon extracts (13, 14). However, FFA2 mRNA was not detected in submucosal or muscle layers in either species. FFA3 protein and mRNA were detected in human colonic mucosa at higher expression levels than in the submucosal or muscle layer (15). These results suggest the existence of FFA2 and FFA3 in colonic mucosa.

Immunohistochemistry was used to identify the cellular distribution of FFA2 and FFA3 in the colon. FFA2-immunoreactive epithelial cells were found in rat, guinea pig, and human colonic epithelia, with particularly strong expression in PYY and GLP-1-producing enteroendocrine L-cells but not 5-HT containing enterochromaffin (EC) cells (13–16). Immunoreactivity for FFA2 in laboratory animals showed a similar pattern to that of the human colon. FFA2-immunoreactive L-cells in the colon were open-type with bodies extending to the luminal surface. FFA3 was also detected in human colonic open-type L-cells, but it is unclear whether these two receptors are located in the same cells. These morphological characteristics indicate that PYY- and GLP-1-producing L-cells that express FFA2 and FFA3 are chemosensory cells. Activation of these receptors by luminal SCFAs may trigger PYY and/or GLP-1 release. Morphologically, it is still unclear whether FFA2 or FFA3 confined to the apical or basolateral membrane, although our physiological studies indicate that these receptors are located on apical side (11, 12, 16). Further study is needed to clarify the precise distribution pattern of FFA2 and FFA3 in order to understand the physiological function of these receptors.

DIRECT EVIDENCE OF GLP-1 RELEASE FROM SCFAs RECEPTOR EXPRESSING L-CELLS *IN VITRO*

The morphological data suggest that gut microbiota-derived SCFAs in the colonic lumen function as stimuli for GLP-1 and PYY release. SCFAs are known to trigger the release of gut hormones, but results have been inconsistent due to differences in the systems used. Enteroendocrine cell culture systems, such as murine STC-1

(17), GLUTag (18), and human NCI-H716 cell lines (19) are often used to study the effects of nutrients on the release gut hormones *in vitro*. The results from single cell cultures, however, are difficult to extrapolate to understand receptor physiological function because intestinal tissue contains different epithelial cell types as well as the enteric nervous system and mucosal immune system, which influence the secretion of gut hormones. In addition, even *in vivo* experiments have inconsistent results; intravenous acetate infusion in innervated and denervated loops in conscious pig did not change the concentration of GLP-1 but did for PYY (20). On the other hand, intravenous and rectal infusion of acetate raises plasma PYY and GLP-1 in hyperinsulinemic human females (21). There are a few possible reasons for such differences: (1) many results of *in vivo* experiments are obtained from an infusion system. This system cannot identify a precise stimulation or secretion site, which is a disadvantage for elucidating the function of chemical receptors in gut hormone secretion. (2) Most cultured cells in *in vitro* cell culture system experiments cannot maintain cell polarity. (3) Many studies cannot directly differentiate whether specific gut hormone-containing enteroendocrine cells are activated to secrete hormones through direct or indirect chemical sensing, particularly if non-enteroendocrine cells also express chemosensory receptors. Indeed, our morphological data suggest that enterocytes also express FFA2 and FFA3.

We used the Ussing chamber system to investigate whether SCFA stimulation induces GLP-1 secretion and to define precise stimulation and secretion sites of FFAs. This preparation maintains the polarity of epithelial cells and contains other cellular elements like intact intestine. In addition, an advantage of this system is that it allows simultaneous measurement of physiological phenomena and hormone release. In muscle-stripped mucosa–submucosal preparations, luminal application of 5 mM propionate induced GLP-1 release into the basolateral side of the rat distal colon (22). Simultaneously, 5 mM propionate induced an increase in short-circuit current, which is a parameter of ion transport in epithelial cells (22). These results show that SCFAs promote GLP-1 secretion through FFAs. It is still unclear, which type of receptor is involved in GLP-1 secretion, since both FFA2 and FFA3 are expressed in enteroendocrine L-cells containing PYY and GLP-1 (13–15). From physiological studies, FFA3 might be involved in this secretion process because acetate, which is the preferable ligand of FFA2, had no effect on local physiological responses including ion transport in the rat distal colon (22). This is further supported by observations of mice lacking FFA2 or FFA3 that had reduced SCFA-triggered GLP-1 secretion *in vitro* and *in vivo* conditions (23). Unfortunately, the molecular pathways underlying the beneficial effects of SCFAs are still largely unknown. Thus, further study is needed to identify molecular pathways of FFA-stimulated GLP-1 secretion.

DIETARY FIBER SUPPLEMENTATION AFFECTS COLONIC ENTEROENDOCRINE CELL POPULATIONS AND FFA EXPRESSION IN THE COLON

Besides the direct effects of SCFAs on gut hormone release, some studies have shown a relationship between dietary fiber intake – the substrate for SCFA production by microbiota – and GI hormone release. Indeed, non-digestible and fermentable dietary fibers, as well as SCFAs themselves, have been shown to induce GLP-1 secretion in humans (24) and rodents (25), although the underlying mechanisms are poorly understood. On the other hand, acute dietary fiber intake does not increase endogenous GLP-1 concentration in human subjects (26). To help elucidate these mechanisms, long-term ingestion of fructooligosaccharide (FOS) and its effects on density or expression patterns of FFA2, GLP-1, and 5-HT in the colon were tested using rats. Dietary supplementation with FOS for 4 weeks increased the number of L-cells expressing GLP-1 approximately twofold in the rat proximal colon, but did not affect fecal content or the density of EC cells producing 5-HT (27). These results suggest that luminal SCFAs selectively induce the proliferation of GLP-1-producing cells. This is supported by the observation that long-term ingestion of fermentable dietary fibers increases luminal concentration of SCFAs (28). FFA2 responding to supplementation with 5% FOS approximately doubled in the proximal colon. This suggests that FFA2 plays a key role in GLP-1 production and secretion in addition to FFA3. The microflora environment in the gut must have time to adapt to a new food source before the full effects of fermentation can take effect; production of SCFAs usually requires a few days in animals and humans. As a result, FOS has to be consumed during a long-enough period to allow fermentation to occur and stimulate GLP-1 and PYY production. Therefore, continuous intake of fermentable fiber in the diet is considered important for the expression of GLP-1- and PYY-secreting L-cells in long-term energy homeostasis. As colonic luminal SCFA concentrations are unlikely to be reduced markedly in response to acute food ingestion, it is possible that SCFAs produced by colonic bacterial fermentation provide chronic stimulation to L-cells via FFA2 and FFA3 under physiological condition.

The CNS plays an important role in the maintenance of body weight and energy balance within a narrow range by regulating energy intake and expenditure. A reduction in body weight requires long-term negative energy balance by reducing appetite and food intake or by increasing energy expenditure, or both. GLP-1 and PYY are postulated to be hormonal signals from the gut to the brain to inhibit food intake and appetite control because reduced food intake in part reflects increases in hormone release in conjunction with decreased secretion of ghrelin, which increases food intake through effects on hypothalamic, brainstem, and reward-related circuitry (3). Released GLP-1 is rapidly degraded by DPP-4, which results in a diminished concentration of GLP-1 in the hepatoportal vein (~50%), and an even small amount entering systemic circulation (<10%) (29). Thus, only a small amount of GLP-1 has the chance to reach the CNS. A radiolabeled GLP-1 analog has been shown to cross the blood–brain barrier by simple diffusion in mice, and gut-derived GLP-1 may enter the brain through the area postrema lacking blood–brain barrier (30). Therefore, an alternative model of GLP-1 release from colonic L-cells should be considered. For example, it is possible that continuous release of GLP-1 affects CNS activity to modulate energy homeostasis including appetite and satiety. Alternatively, FFA2/FFA3-activated GLP-1 release from colonic L-cells may activate local gut signaling events to activate the CNS through the pelvic nerve to modulate long-term regulation of appetite and satiety. However, further physiological studies are needed to prove these hypotheses.

GLP-1 is generally believed to be expressed mainly in a limited population of enteroendocrine cells in the ileum and the colon (31). However, recent study suggests that enteroendocrine cells have a much broader potential for expression than is generally believed (32). Furthermore, Li et al. (33) have shown that there are many more L-cells expressing α-gustducin and GLP-1 in the colon than in the small intestine, and that cells expressing α-gustducin increase in distribution from nearly 0% in the small intestine to ~29% in the colon (33), leading the authors to postulate that colonic L-cells might histologically and functionally differ from L-cells in the small intestine. These results suggest that FFA2/FFA3-stimulated GLP-1 secretion from colonic L-cells has a different physiological function than in the small intestine. GLP-1 release is suppressed in obese subjects, so the tonic stimulation of GLP-1 secretion induced by FFAs may chronically affect systemic energy homeostasis (34). Indeed, continuous GLP-1 receptor agonist injection for 14 days has been shown to reduce body weight (35). Therefore, the mode of action of GLP-1/PYY release in the colon stimulated by FFA2 and FFA3 is considered different from the mode of action in the small intestine. Stimulation and release in the colon might be involved in long-term regulation of energy homeostasis, while in the small intestine regulation occurs in the short-term with appetite or feeding control.

CONCLUSION

Since obesity and metabolic disorders are associated with changes in gut microbiota, the integral role of the gut microbiota in the regulation of host energy homeostasis is an important issue. SCFAs are primary metabolites of gut bacteria and are present in the colon at high concentrations. The SCFA receptors, FFA2 and FFA3 are located in rodent and human colonic L-cells containing GLP-1 and PYY, whose release are involved in the regulation of host energy homeostasis. Furthermore, luminal application of SCFA induces GLP-1 secretion and long-term ingestion of FOS influences the density of FFA2-expressing L-cells. Therefore, it seems likely that the colonic epithelia communicate with the gut microbiota through products and receptors to maintain long-term host–bacterial interactions. The studies that aimed at elucidating physiological phenomena and underlying mechanisms in the colon discussed in this review are important contributions in the race to develop new therapeutic strategies for obesity and its related disorders in future.

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Gut microbes and host physiology: what happens when you host billions of guests?

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The gut microbiota has recently emerged as an important, and previously unappreciated, player in host physiology (1). In particular, the gut microbiota contributes to a variety of physiological and pathophysiological processes in the host including immune disorders (2–4), atherosclerosis (5), irritable bowel syndrome (6, 7), blood pressure regulation (8), and chronic kidney disease (9, 10). Bacteria residing in the human gut are an important component of human physiology: the total wet weight of gut microbes in the human has been estimated to be 175 g–1.5 kg (11, 12), and the cells of the microbiota outnumber human cells by 10:1 (1). These bacteria interact with the immune system of the host (13), and secrete a variety of metabolites, which enter host circulation and can affect a variety of physiological parameters (8, 14), reviewed in Ref. (15). In fact, metabolites produced by the gut microbiota have been found to play key roles in renal disease (16), blood pressure regulation (8), and immune disorders (2–4). Therefore, just as we consider the genetic background of an animal or an individual to be an important contributing factor to their physiology, so too must we consider the genetic background of the microbiota associated with that animal.

Gut microbiota vary greatly amongst laboratory animals, and these differences result in notable differences in experimental results. Mice of the same strain from different vendors have different microbiota profiles (17), and similarly, the same mice housed at different institutions have different microbiota profiles (18, 19). Conversely, inoculating two different inbred mouse strains with the same gut bacteria leads to differences in host gene expression between the two mouse strains (20). Clearly, there is a complex interplay between the genetics of the microbiota and that of the host organism, which has only recently begun to be appreciated.

GUT MICROBIOTA AS AN EXPERIMENTAL PARAMETER

Examples in the literature have highlighted the important and unexpected ways in which gut microbiota can affect a variety of experimental parameters. In a series of studies, Vijay-Kumar et al. (13, 21) reported that although TLR5 null animals initially had a colitis phenotype, when these mice were "rederived" and their gut microbiota altered, the colitis phenotype was greatly attenuated, and instead the null animals exhibited metabolic syndrome. In addition, Lathrop et al. put forward a model by which T-cells are educated not only by self/non-self mechanisms, but also by microbiota-derived "non-self" antigens (22). Accordingly, they found that the presence or absence of microbiota determined whether T cells would induce colitis in mice. Finally, Yang et al. reported that when the same knockout mice were housed at two different institutions, they had markedly different microbiota profiles – and the mice at one institution (MIT) were quite susceptible to colitis, whereas mice at the other institution (MHH) failed to develop any significant pathology under the same conditions (19). Unequivocally, altering gut microbiota – even by housing animals at different institutions – can have dramatic effects on the phenotype observed.

GUT MICROBIOTA AND OBESITY AND DIABETES

It is important to note that not only can microbiota affect host physiology, but the gut microbiota are not necessarily stable over time. Rather, gut microbiota can change or shift as a result of experimental manipulation (in animals) or changes in lifestyle or nutrition (in humans). It is now appreciated that there are "shifts" in microbiota that occur in obesity in mice, rats, and humans (23–26). In one study, Turnbaugh et al. (25) examined human female twin pairs concordant for leanness or obesity, and found that obesity was associated with phylum-level changes in microbiota. In this study, both monozygotic and dizygotic female twin pairs (and their mothers, where available) were analyzed. Analysis of fecal samples revealed that obese individuals have reduced gut microbiota diversity, and tended to have less Bacteroidetes and more Actinobacteria. The authors suggest that conditions of "abnormal energy input" (i.e., obesity) may favor the growth of a reduced diversity community. In support of these findings, a separate study reported that ob/ob mice had a reduction in Bacteroidetes (27) as compared to ob/+ or wild-type siblings. Furthermore, in 12 obese humans who lost weight over 1 year by consuming either a fat restricted or caloric restricted diet, there was a relative increase in the abundance of Bacteroidetes over time (28). Impressively, the increase in Bacteroidetes in these individuals correlated with weight loss. Indeed, there is an increasingly convincing link between gut microbiota and obesity. These findings highlight the importance of considering and documenting gut microbiota composition in studies of obesity, as a change in gut microbiota structure has a clear tie to host pathology.

In addition, it has also been demonstrated that type 2 diabetes in humans is associated with changes in the gut microbiota (not surprising, given the

correlation between obesity and type 2 diabetes) (29–33). Specifically, it has been reported that butyrate-producing bacteria are reduced in type 2 diabetes (32), that Bifidobacterium is lowered (33), and that Firmicutes is decreased (30). Intriguingly, it was also reported that the ratio of Bacteroidetes to Firmicutes (as well as the ratio of Bacteroides–Prevotella to *C. Coccoides*–*E. rectale*) correlated with plasma glucose levels. Subsequently, it has been suggested that manipulating gut microbiota may be a therapeutic opinion for obesity and/or type 2 diabetes (34). As reviewed in depth by Kootte et al. (34), in addition to exploring possible roles for prebiotics, probiotics, and antibiotics as potential therapies, we must also better understand the changes in microbiota which appear to accompany known treatments for obesity (i.e., bariatric surgery). In addition, we must continue to explore a potential role for a variety of microbiota metabolites in order to better understand how and why changes in microbiota affect the physiology of the host.

WHAT'S A SCIENTIST TO DO?

Knowing that gut microbiota play such an important and dynamic role in host physiology, going forward we should take into account (or at least, document) the gut microbiota present in whole-animal physiology experiments. This is especially important in research focused on obesity and diabetes, as these are areas in which gut microbiota changes have been associated with host pathology. As the study of the gut microbiota requires a specialized and complex set of knowledge, it may be prudent for Universities, Companies, or other Research Entities to establish core facilities and/or collaborations to help facilitate such measurements. In addition, when differences are found in measured physiological parameters between researchers at different institutions, gut microbiota should be considered as one potential explanation. Finally, as different strains of microbiota produce different metabolites, metabolomics may also be a useful tool to help us understand the "end effect" of microbiota on host function.

CONCLUSION

The gut microbiota is a complex tangle of organisms, which easily outnumber the number of cells in the host. When considering processes in the context of whole-animal physiology, we must also consider the contribution of these microorganisms and their metabolites. The literature is rife with examples of phenotypes which were not easily replicated by other groups – even when using the "same mice" – and we should consider whether some of these examples may be, in fact, due to the influence of gut microbiota. In the future, it would be ideal for researchers to report at least a basic characterization of gut microbiota in research animals so that any "institution-specific" effects can later be examined. Understanding not only the host and the microbiota, but the host–microbiota interactions, will ultimately give us a richer and fuller understanding of host physiology.

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Therapeutic role and ligands of medium- to long-chain fatty acid receptors

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Medium- and long-chain free fatty acids (FFAs) are energy source for whole body and biological metabolites and components. In these decades, some research groups have reported that the biological functions of medium- to long-chain FFAs are exerted through G-protein coupled receptor designated free fatty acid receptor (FFAR). As the medium- to long-chain FFAs-activated FFARs, FFA1 and FFA4 are reported to be expressed widely in whole body and regulate various physiological processes. FFA1 expressed in pancreatic β-cells has been shown to be involved in insulin secretion. FFA4 expressed in intestine, adipocytes, and macrophages has been shown to be involved in incretin secretion, differentiation, and anti-inflammatory effect, respectively. These physiological functions have been focused on the treatment of metabolic disorders. In addition, these receptors have been also reported to be expressed in several other tissues such as intestine for FFA1, and tongue and stomach for FFA4. The recent functional studies indicated that they also contributed to energy homeostasis. Further, the number of synthetic compounds of FFA1 and FFA4 strongly promoted the physiological characterization of the receptors and their own therapeutic utility. In this article, we will discuss the recent progress regarding the therapeutic potential of these receptors and its ligands.

Keywords: FFA1, FFA4, medium- to long-chain fatty acid, energy metabolism

INTRODUCTION

Free fatty acids (FFAs) are known to act as the critical energy source in the whole body. In addition, FFAs also act as signal molecules in various physiological reactions (1, 2). As one of these mechanisms, G-protein-coupled receptors (GPCRs) activated by FFAs designated as free fatty acid receptor (FFAR) family have played important role especially in energy metabolism (3). Among FFAR family, FFA1 (known as GPR40) and FFA4 (known as GPR120) are classified as medium- to long-chain fatty acid-activated receptors (4, 5). On the other hand, FFA2 and FFA3 are classified as short-chain fatty acid-activated receptors (refer to each topic) (6, 7). In terms of natural ligands for FFA1 and FFA4, medium- and long-chain fatty acids that showed various physiological functions generally and contained 6–12 and more than 12 carbon chains are mainly provided by food digestion and lipolysis in adipose tissues. As unsaturated fatty acids that include double bond are not supplied in biosynthesis in human, we have to take in these unsaturated fatty acid as food intake. A number of studies for FFA1 and FFA4 revealed that these two receptors have been contributed to regulate energy metabolism and metabolic diseases such as type 2 diabetes and obesity. In this review, we summarize the therapeutic utility of medium- and long-chain fatty acid receptors, FFA1 and FFA4.

THERAPEUTIC POTENTIAL OF FFA1 AND FFA4 PHYSIOLOGICAL FUNCTIONS RELATED TO ENERGY METABOLISM

Among FFARs, FFA1 and FFA4 are classified as medium- to long-chain FFARs. The ligand property of these two receptors was similar to each other; however, the expression profile is different in several tissues (4, 5). The physiological functions of FFA1 and FFA4 related to the energy metabolism regulation are as follows.

FFA1

Free fatty acid 1 expressed in pancreatic β-cell and intestine has been considered to have therapeutic utility. FFA-induced glucose-stimulated insulin secretion (GSIS) in β-cells via FFA1 has been reported by Itoh et al. (4). Using FFA1 KO and TG mice, FFA1 exhibited protective effect against chronic and/or excess stimulation of glucose-induced toxic effect on β-cells (8–10). In addition, depolymerization of F-actin and the activation of protein kinase D1 (PKD1) are contributed to the FFA1-mediated insulin secretion from β-cells (11). Further, Flodgren et al. reported that not only in β-cells but also α-cells also expressed FFA1 and contributed to glucagon secretion (12). In intestine, FFA1 expression was also confirmed in the gastric inhibitory polypeptide (GIP) and glucagon like peptide-1 (GLP-1) incretin hormones secreting cells such as the intestinal K and L cells (13, 14). In addition, intestinal I cells that express cholecystokinin (CCK) also expressed FFA1 were reported by Liou et al. (15). Therefore, FFA1 might regulate insulin secretion not only in direct but also in indirect mechanism.

FFA4

Free fatty acid 4 expressed in intestine, adipose tissue, and taste buds is considered as therapeutic target for metabolic disorders. Similar to FFA1 expression in intestine, FFA4 is expressed in intestinal L-cell that can secrete GLP-1 (5). In adipose tissue,

FFA4 has been contributed to regulate adipose differentiation and GLUT-4 translocation that regulates glucose incorporation (16, 17). The effect of FFA4 on energy metabolism was showed in GPR120 KO mice study (18). Further, FFA4 expressed in macrophages was reported to regulate inflammatory responses (17). On the other hand, in taste buds, FFA4 expression was confirmed in type 1 and 2 taste bud cells (19, 20). Since the FFA stimulation of these cells activated taste nerve response, fat preferences might be regulated via FFA4. These findings provided us the possibility for the therapeutic utility of FFA1 and FFA4.

SYNTHETIC LIGANDS AND ITS THERAPEUTIC UTILITY

FFA1

To regulate these physiological functions, more selective ligands are expected to be developed for therapeutic candidates. For FFA1, a number of selective agonists have been reported. The expected pharmacological property of the compound is the selectivity compared to other FFARs, the potency compared to natural ligands, and efficacy not only in *in vitro* but also in *in vivo* experiments. NCG75, GW9508, and TAK-875 have been reported as selective and potent agonists. However, these compounds showed similar effect on insulin secretion compared to the endogenous FFA ligands in insulin-secreting cell line such as INS-1 and MIN6 cells (21–23). Recently, TAK-875 has been reported to be failed in clinical trial owing to the side effect such as liver toxicity. Therefore, unknown signaling mechanisms might contribute to regulate the physiological functions. To obtain the beneficial effect of FFA1, we need to understand more precise mechanisms in cell line levels.

FFA4

For FFA4, we identified a partial agonist among the natural compounds derived from fruiting bodies of *Albatrellus ovinus* (24). Further, we also identified a series of synthesized compounds based on PPARγ-agonist thiazolidinediones. Among these compounds, a selective agonist NCG21 showed selectivity for FFA4 compared to FFA1 (25, 26). TUG-891 developed by Hudson et al. showed the most potent agonistic activity that activated not only in G-protein but also in β-arrestin-dependent pathways (27, 28). Metabolex was developed as selective agonist for FFA4 and the pharmacological properties were examined not only in cultured cells but also in animal models (29).

In addition, isoindolin-1-one series and phenyl-isoxazol-3-ol series developed by Banyu Pharmaceutical Co. Ltd. showed potent selectivity for FFA4 compared to FFA1 with nanomolar order potency (30, 31).

FURTHER CHALLENGE OF THE FFA1 AND FFA4 RESEARCH FOR THERAPEUTIC APPLICATION

As described in previous section, the physiological and pharmacological functions of these two receptors have increasingly revealed. The synthetic ligands that showed the usefulness not only for *in vitro* but also for *in vivo* study have been also increased. However, to proceed these findings to develop the pharmaceutical agents, we should answer the several questions about the molecular mechanisms of these receptors. The signal transduction mechanisms of these two receptors have been reported that FFA1 coupled to G_q protein but not the $G_{i/o}$ or G_s, and FFA4 coupled to G_q family and β-arrestin pathways (4, 5, 17, 32). However, the precise mechanism that connects a signal pathway and a specific physiological function has not been well-understood yet. The dimerization of receptors has been reported to regulate the affinity of the ligand, signaling pathway, and related physiological functions (33). Since FFARs including short-chain fatty acid receptors are expressed in the same tissues such as pancreatic β-cells, intestine and immune cells, the dimerization of these receptors might be formed in cell surface, and the physiological functions of each receptor might be regulated. On the other hand, the protein expression profile should be examined more precisely because several studies reported that the receptor expression is controversial. Although the receptor expression in the tissues is evaluated by mRNA levels in some studies, mRNA levels do not always reflect the protein level. Various commercial antibodies for FFA1 and FFA4 are available, however, we should carefully validate these antibodies in terms of selectivity since the antibodies for GPCR sometimes lack its selectivity (34). Further, the specific agonist that shows the selectivity for specific signaling pathway such as biased ligand could be useful not only for the pharmacological tool but also for the therapeutic candidates. The adequate screening system for each signal pathway and structure–activity relationships of FFA1 and FFA4 ligand might be useful to develop these compounds. Further using this information, we could discriminate the beneficial effects of the FFA1 and FFA4 ligands from its adverse effects.

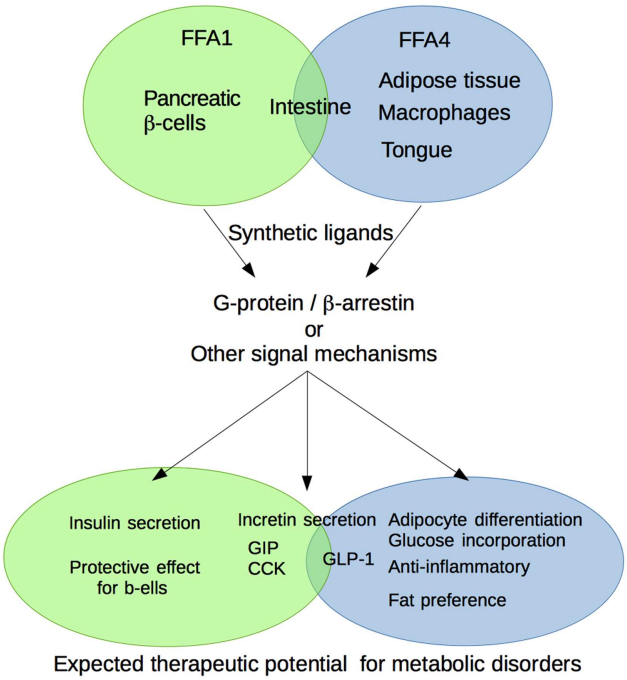

FIGURE 1 | Schematic diagram of the physiological function of medium- to long-chain fatty acid receptors FFA1 and FFA4 related to the metabolic regulation.

CONCLUSION

FFA1 and FFA4 regulate the energy metabolic mechanism by acting as sensors for FFAs mainly provided by foods and lipolysis. A great number of reports showed that FFA1 and FFA4 are regulated by various physiological processes and the possibility for the therapeutic utility for metabolic disorders (**Figure 1**). However, we should reveal more precise molecular mechanisms underling metabolic diseases regulated by these receptors. In addition, the synthetic ligands, which can show selectivity not only among these receptors but also for the specific signals in each receptor, are expected to be developed in the future. Therefore, FFA1 and FFA4 may represent a promising therapeutic utility for the treatment of metabolic syndromes, such as obesity and diabetes.

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Regulation of energy homeostasis *via* GPR120

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Free fatty acids (FFAs) are fundamental units of key nutrients. FFAs exert various biological functions, depending on the chain length and degree of desaturation. Recent studies have shown that several FFAs act as ligands of G-protein-coupled receptors (GPCRs), activate intracellular signaling and exert physiological functions *via* these GPCRs. GPR120 (also known as free fatty acid receptor 4) is activated by unsaturated medium- to long-chain FFAs and has a critical role in various physiological homeostasis mechanisms such as incretin hormone secretion, food preference, anti-inflammation, and adipogenesis. Recent studies showed that a lipid sensor GPR120 has a key role in sensing dietary fat in white adipose tissue and regulates the whole body energy homeostasis in both humans and rodents. Genetic study in human identified the loss-of-functional mutation of GPR120 associated with obesity and insulin resistance. In addition, dysfunction of GPR120 has been linked as a novel risk factor for diet-induced obesity. This review aims to provide evidence from the recent development in physiological function of GPR120 and discusses its functional roles in the regulation of energy homeostasis and its potential as drug targets.

Keywords: GPR120, FFAR4, FFAs, metabolic syndrome, diabetes mellitus

INTRODUCTION

Free fatty acids (FFAs) are basic components of biological structures, precursors of various mediators, and play important roles as essential nutrients (1). During the past decade, however, a number of studies revealed that FFAs also act as key signaling molecules to regulate a number of physiological functions through G-protein-coupled receptors (GPCRs) (1–4). The superfamily of GPCRs includes at least 800 seven-transmembrane receptors that have diverse physiological and pathological functions. GPCRs are the most successful targets of drug (5). Of interest, FFAs act as ligands of some GPCRs. FFAs can be classified depending on their chain length as short-chain fatty acids (SCFAs), which have 1–6 carbon chain length; medium-chain fatty acids (MCFAs, 7–12 carbon chain length); and long-chain fatty acids (LCFAs), which have more than 12 carbon chain length. Some of non-esterified FFAs directly regulate important biological processes such as energy homeostasis *via* their corresponding receptors (6–10). The LCFA receptor GPR40 (also known as FFAR1), SCFA receptors GPR41 (FFAR3) and GPR43 (FFAR2) were identified in 2003 (11–18). In 2005, we successfully deorphanized and identified GPR120 [also known as free fatty acid receptor 4 (FFAR4)] as a FFAs receptor (FFARs), which is activated by unsaturated MCFAs and LCFAs (19). These GPCRs are widely expressed in the body and contribute to maintain systemic energy homeostasis under changing nutritional conditions. Among these FFARs, GPR120 emerged as an important checkpoint in regulating energy homeostasis (6, 8). Previous studies also showed that GPR120 has been implicated in several key processes including the release of incretin hormone, anti-inflammation, food preference, glucose homeostasis, insulin sensitivity, and adipogenesis (6, 8, 19–24). These factors interrelate to regulate systemic metabolic energy and nutritional homeostasis under physiological and pathophysiological conditions. Hence, in this review, we attempt to summarize and discuss the recent advances in research regarding the roles of GPR120.

TISSUE DISTRIBUTION OF GPR120

GPR120 is widely expressed in various tissues and cell types including intestine, macrophages, adipose tissue, taste buds, brain, pancreas, lung, thymus, and pituitary (2, 6, 8). Hence, GPR120 has multiple functions in homeostatic regulation of systemic metabolism and inflammation depending on this diverse tissue distribution. Furthermore, GPR120 is co-localized with not only glucagon-like peptide 1 (GLP-1) in the colon and circumvallate papillae taste bud cells (19, 25, 26), but also with ghrelin (27) and α-gustducin in the duodenum and type II taste bud cells, respectively (28, 29). GPR120 was also reported to be co-expressed with other FFARs, such as GPR40 in STC-1 intestinal cells (19) and GPR43 in the proximal colon in mice (29). These characteristics of expression patterns and co-localization might reflect the physiological functions of GPR120 as described below.

INTESTINE

GPR120 is expressed in the intestines of humans as well as mice. Furthermore, the enteroendocrine cell line STC-1 also expressed GPR120 endogenously. We have previously shown that GLP-1-expressing enteroendocrine cells in the colon were expressing GPR120 in both rodents and human (19, 20, 25). Secretion of GLP-1 and cholecystokinin (CCK), both known as incretin hormones and involved in the regulation of feeding behaviors, energy metabolism and bodyweight (30–32), was induced by FFAs stimulation from enteroendocrine STC-1 cells (33). The administration of FFAs into the murine colon stimulated GLP-1 secretion and increased plasma level of insulin (19). Furthermore, we have

found that the knockdown of GPR120 expression by siRNA inhibited the FFAs-induced $[Ca^{2+}]_i$ response and incretin hormones secretion in STC-1 cells. These data highly suggested that GPR120 indeed mediate and stimulate incretin hormone secretion *in vivo*. In addition, K cells, which are found in the mucosa of the duodenum and the jejunum of the gastrointestinal tract and also synthesize gastric inhibitory peptide (GIP), also express GPR120 (34). Interestingly, recent reports indicated that GPR120 was co-localized with the orexigenic peptide, ghrelin in duodenal cells *in vivo*, and FFAs stimulation reduced ghrelin secretion in the MGN31 ghrelinoma cell line (35). Furthermore, Gong *et al.* revealed that addition of GW-9508, a GPR120 chemical agonist, inhibited the secretion of ghrelin from ghrelin-producing stomach ghrelinoma (SG-1) cells. They also showed that SG-1 cells highly expressed GPR120 endogenously and the inhibitory effect of GW-9508 on ghrelin secretion was blocked by siRNA against GPR120 in SG-1 cells. Furthermore, GW-9508 treatment reduced plasma ghrelin level *in vivo* (36). These reports indicate that the decrease of postprandial ghrelin is induced at least partially by LCFAs included in foods *via* GPR120. Given the effects on GLP-1, CCK, and ghrelin secretion, the stimulation of GPR120 might regulate appetite and systemic energy homeostasis.

MACROPHAGES

GPR120 was found to be expressed in monocytic RAW267.4 cells and primary proinflammatory M1-like macrophages (6). The activation of GPR120 by ω-3 LCFAs, such as docosahexaenoic acid (DHA) and alpha-linolenic acid (α-LA), exerts broad of anti-inflammatory effects in these cells, all of which were abolished by siRNA against GPR120. These ω-3 LCFAs are identified as anti-inflammatory fatty acids in the tissue-specific and systemic levels (9). Oh *et al.* clearly showed that ω-3 LCFAs exert anti-inflammatory effects through GPR120. *In vitro* experiments revealed the molecular mechanism underlying ω-3 FFAs-mediated anti-inflammatory effects. Stimulation of GPR120 by ω-3 LCFAs abolished lipopolysaccharide (LPS)-induced phosphorylation and activation of IκB kinase (IKK) and c-Jun N-terminal kinase (JNK) in macrophages. Recruitment of β-arrestin 2 (β-arr2) and following the GPR120–β-arr2 complex internalization is induced by the activation of GPR120. Tumor necrosis factor-α (TNF-α) and toll-like receptor 4 (TLR4) widely mediate proinflammatory cascades. In addition, tumor growth factor β (TGF-β) activated kinase 1 (TAK1) interacting with TGF-β activated kinase 1 binding protein 1 (TAB1) mediate downstream inflammatory effects *via* activation of NF-κB and JNK. The internalized GPR120–β-arr2 complex interacts with TAB1 and inhibits the interaction between TAB1 and TAK1, leading to the inhibition of the downstream proinflammatory pathways. Further *in vivo* experiments demonstrated that administration of ω-3 FFAs ameliorated tissue inflammation and thereby improved systemic insulin sensitivity in wild type (WT) mice. The gene deficiency of GPR120 abolished these effects of ω-3 FFAs (6, 9, 37). These results showed that the activation of GPR120 by ω-3 FFAs exerts potent insulin sensitizing and anti-diabetic effects *in vivo* by the repression of macrophage-induced tissue inflammation.

ADIPOSE TISSUE

GPR120 was also found to be expressing endogenously in adipocyte and adipose tissue, but not detected in pre-adipocyte (8, 22). Furthermore, GPR120 expression was increased according to the lipid accumulation in the cells during induction of adipocyte differentiation in 3T3-L1 cells (22). Knockdown and gene deficiency of GPR120 by siRNA suppressed the expression of adipogenic genes and lipid accumulation in 3T3-L1 cells and mouse embryonic fibroblast, respectively (8, 22). These data indicated that GPR120 might be an adipogenic receptor and might play important roles in adipocyte differentiation and maturation. GPR120 mRNA expression was increased in subcutaneous, epididymal, and mesenteric adipose tissue of high fat diet (HFD)-fed mice (22). Moreover, we have shown that GPR120 expression in human adipose tissue was significantly higher in obese individuals than in lean controls (8), suggesting that the expression of GPR120 could be enhanced by the accumulation of dietary lipid in both rodent and human. Our previous study revealed that GPR120-deficient mice fed HFD developed obesity, which was accompanied with decreased differentiation and lipogenesis in adipocyte. Furthermore, severe fatty liver, enhanced hepatic lipogenesis, increased fasting glucose, and impaired responses to insulin and glucose tolerance were observed in HFD-fed GPR120-deficient mice. Gene expression analysis in adipose tissue and liver revealed the molecular basis underlying obesity and insulin resistance of GPR120-deficient mice. Our data showed that HFD-fed GPR120-deficient mice showed a significantly decreased expression of adipogenic gene Fabp4 as well as the key lipogenic gene Scd1. In addition, macrophage marker genes were also increased in adipose tissue, an indication of adipose tissue inflammation. In the liver, on the other hand, the key lipogenic gene Scd1 expression was significantly increased. Insulin signaling-related genes were significantly decreased in both adipose tissue and the liver of HFD-fed GPR120-deficient mice. Furthermore, phosphorylation of IRβ and IRS1 in white adipose tissues and IRS1 and IRS2 in the liver, all of which are regulators of insulin-stimulated glucose uptake, were significantly decreased. In addition, Oh *et al.* reported that GPR120 induced a translocation of glucose transporter 4 in 3T3-L1 adipocytes and directly increased glucose uptake (6). Taking together, these data demonstrated that GPR120 acts as a lipid sensor *in vivo* and plays a critical role in sensing dietary fat to regulate glucose and lipid metabolism.

TASTE BUDS

Recent studies strongly suggested that oral perception of dietary fat was involved in the detection of taste, in addition to texture and olfaction, of LCFAs (38). GPR120 was reported to be expressed in taste bud type II cells (28). Matsumura *et al.* showed the co-localization of GPR120 with phospholipase-Cβ2 and α-gustducin in the taste buds by double immunostaining. Cartoni *et al.* further showed the expression of GPR120 in circumvallate papillae (CV) sections by immunohistochemical analysis (39). Short-access test using a lick meter showed that gene deficiency of GPR120 abolished the preference for fatty acids but not for other tastes. These data suggest that the upregulation of GPR120 in the taste buds could induce an excess intake of lipid, leading to obesity. Martin *et al.* also reported that GPR120 and GLP-1 were found to

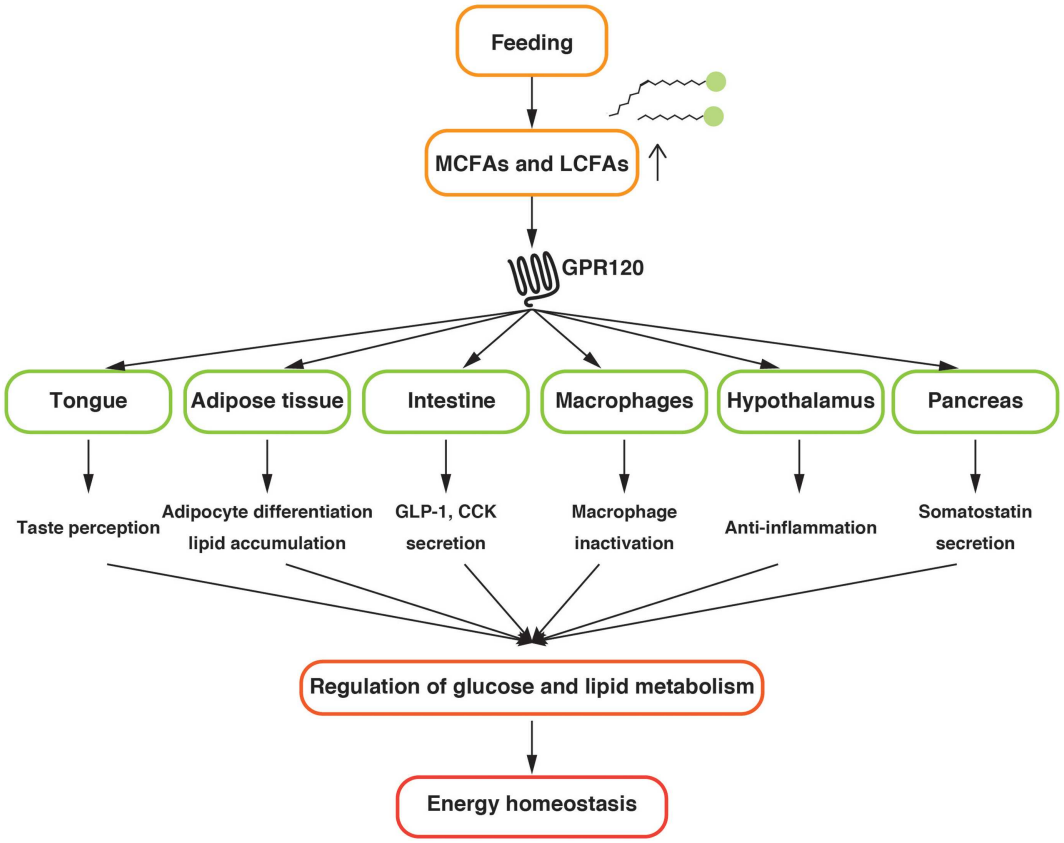

FIGURE 1 | Schematic diagram of the physiological function of GPR120 related to the energy homeostasis.

be co-localized in mouse taste cells from mouse CV (26). Studies using GPR120 selective agonist and isolated mouse CV indicated that GPR120 might be responsible for LCFAs-mediated release of GLP-1 from CVs and might thus contribute to the high palatability of foods rich in both fats and sugars. A recent study further showed that human primary taste bud cells were expressing GPR120 (40). High concentrations of linoleic acid induced $[Ca^{2+}]_i$ signaling *via* GPR120 and CD36 in human and mice primary taste bud cells. These reports strongly suggested that GPR120 expressed in taste buds plays an important role in sensing fat taste, contributing to the food intake.

OTHER TISSUES

GPR120 is also expressed in other tissues and cells. Cintra *et al.* performed immunostaining analysis and found that GPR120 co-localized with neuropeptide Y centrally in the arcuate nucleus (41). An acute injection of ω-3 and ω-9 FFAs-induced GPR120–β-arr2 complex and β-arr2–TAB1 complex as well as inhibited the interaction between TAB1 and TAK1, leading to the reduction of the downstream proinflammatory pathways in the hypothalamus. Furthermore, Wellhauser *et al.* analyzed the molecular mechanisms to modulate hypothalamic function *via* GPR120 *in vitro* using a hypothalamic neuronal model, rHypoE-7 cells, isolated from the rat. They showed that the anti-inflammatory effect of DHA was significantly reduced by siRNA against GPR120 in rHypoE-7 cells (42). Numbers of studies showed inflammatory response in the hypothalamus in reaction to excessive nutrients contributes to diet-induced obesity and type 2 diabetes mellitus (43–45). Hence, the anti-inflammatory effect mediated by GPR120 in hypothalamus might play an important role in the regulation of systemic energy homeostasis.

Recently, Xhao *et al.* showed mRNA and protein expression of GPR120 in human and rat pancreas (46). Immunohistological analysis demonstrated that GPR120 is co-localized with CD68, the specific marker of macrophages, and with CD34 and CD117, the markers of interstitial cells in the pancreas. Furthermore, Stone *et al.* generated Gpr120-knockout/β-galactosidase knock-in mice and showed the distribution of GPR120 (23). Immunofluorescence analysis demonstrated the co-localization of GPR120 with somatostatin, suggesting that GPR120 is selectively expressed in islet delta cells. They also demonstrated that treatment of GPR120 selective antagonist inhibited glucose induced somatostatin secretion from isolated islet. Additionally, GPR120-deficiency abolished this effect. Hence, GPR120 expressed in pancreatic delta cells might regulate somatostatin secretion. Further studies are required in order to reveal the functional roles of GPR120 in pancreas.

GENETIC CONTRIBUTION TO TYPE 2 DIABETES

We previously reported two non-synonymous mutation p.R270H and p.R67C by exon sequencing of GPR120 in obese and lean

European subjects. Following *in vitro* experiments revealed that the p.R270H mutant, which significantly associated with obesity, lacked the ability to transduce LCFAs signal, contrary to the p.R67C mutant, which was not associate with obesity. Taken together these human and GPR120-deficient mice, the dysfunction of GPR120 leads to obesity in both mice and human (8). In addition, the systems genomics approach to identify genes for type 2 diabetes showed that GPR120 was placed in the top 16 of the ranked list (47). Taneera *et al.* reported that GPR120 expression in human islets was positively correlated with both secretion and contents of insulin as well as lower HbA1c levels. These data suggested that GPR120 might have a protective role on human islet.

CONCLUSION

GPR120 regulates the metabolic homeostasis by sensing LCFAs provided by dietary fat in several tissues (**Figure 1**). Further investigations to uncover the precise physiological functions of GPR120 are mandatory for a better understanding of systemic nutrient metabolism and energy homeostasis. The current studies suggest that GPR120 activation might have positive outcomes on health. Hence, GPR120 might be a promising pharmaceutical target for the treatment of metabolic diseases.

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Omega-3 fatty acids and FFAR4

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The beneficial roles of omega-3 fatty acids (ω3-FAs) on obesity, type 2 diabetes, and other metabolic diseases are well known. Most of these effects can be explained by their anti-inflammatory effects triggered through their receptor, free fatty acid receptor 4 (FFAR4) activation. Although the whole mechanism of action is not fully described yet, it has been shown that stimulation of ω3-FA to FFAR4 is followed by receptor phosphorylation. This makes FFAR4 to be capable of interacting with β-arrestin-2, which in turn, results in association of β-arrestin-2 with TAB1. This stealing of an important partaker of the inflammatory cascade leads to interruption of the pathway, resulting in reduced inflammation. Besides this regulation of the anti-inflammatory response, FFAR4 signaling also has been shown to regulate glucose homeostasis, adiposity, gastrointestinal peptide secretion, and taste preference. In this review, we summarize the current knowledge about the interaction of ω3-FAs with FFAR4 and the consequent opportunities for the application of ω3-FAs and possible FFAR4 targets.

Keywords: omega-3 fatty acids, FFAR4, anti-inflammation, insulin resistance, obesity

INTRODUCTION

Free fatty acids (FFAs) serve both as a source of energy and as signaling molecules that regulate energy homeostasis and other physiological processes (1, 2). Previous studies proposed that lipotoxic stress from long-chain saturated FFAs is a major cause of JNK activation and therefore insulin resistance in obesity (3, 4). It has also been reported that long-chain saturated FFAs, but not polyunsaturated FFAs, induce inflammatory responses in macrophages (5). Among polyunsaturated FFAs, omega-3 fatty acids (ω3-FAs) have been recognized for their beneficial effects on human health (6). These beneficial effects were found in inflammatory diseases, cardiovascular diseases, and hepatic lipid metabolism, as well as glucose homeostasis and insulin sensitivity (7–10). However, the detailed mechanisms underlying the beneficial effects of ω3-FAs have not been completely defined to date.

It is known that the ω3-FAs, such as α-linolenic acid (α-LA), docosahexaenoic acid (DHA), and eicosapentaeonic acid (EPA) are endogenous ligands for the free fatty acid receptor 4 (FFAR4) (11–13). FFAR4, also known as G protein-coupled receptor 120 (GPR120), is a seven transmembrane receptor and was first reported as an orphan GPCR in 2003 (14). FFAR4 exists as two splice variants in humans (15, 16), but only the shorter variant has been found in rodents and cynomolgus monkeys (17). Consistent with the pleiotropic effects of ω3-FAs, FFAR4 has been implicated in diverse processes including anti-inflammation, insulin sensitization, release of gut peptides, and alteration of food preference (12, 13, 18–21). The wide range of processes that can be positively influenced by FFAR4 makes this receptor of potential importance in the prevention and treatment of metabolic diseases.

MECHANISMS

Consistent with roles in diverse processes, FFAR4 is expressed ubiquitously including lungs, colon, small intestine, brain, thymus, adipose tissue, taste buds, skeletal muscle, heart, and liver (12, 18, 21, 22). However, the expression pattern differs between species and also depends on the method of detection (23). Furthermore, the expression in skeletal muscle, heart, and adipose tissue is upregulated by a high fat diet (HFD) (18, 19, 22). Despite the variation of expression, FFAR4 seems to transduce ω3-FA signaling always through one of two pathways that involve either $G_{\alpha q}$ or β-arrestin-2 (12, 13) (**Figure 1**). FFAR4 coupling to $G_{\alpha q}$ induces a rise in intracellular Ca^{2+} (12, 21) without affecting the level of cyclic AMP (12). Alternatively, FFAR4 can respond to ω3-FAs by recruiting cytosolic β-arrestin-2 to the plasma membrane, leading to internalization of the FFAR4/β-arrestin-2 complex (13). After internalization, β-arrestin-2 then directly associates with TGF-β activated kinase 1 (TAK1) binding protein (TAB1), which is an adaptor molecule for the pro-inflammatory kinase TAK1 (24). In this way, β-arrestin-2 sequesters TAB1 from TAK1, leading to inactivation of TAK1 and abrogation of signaling to the inflammatory key-players IKKβ/NFκB and MKK4/JNK/AP1 (13). Besides blocking the inflammatory pathway via the TAB1/TAK1 complex, FFAR4 activation by DHA also stimulates cytosolic phospholipase A_2 ($cPLA_2$) and prostaglandin-endoperoxide synthase 2, also known as COX-2 (25). This leads to increased production of prostaglandin E_2 (PGE_2), which in turn, inhibits NFκB signaling through the prostaglandin E receptor 4 and therefore reduces inflammation in macrophages as well (25).

After activation by ligands, GPCRs are phosphorylated to provide binding opportunities for certain G proteins and arrestins (26, 27). Investigating the human short isoform of FFAR4, Sánchez-Reyes et al. (28) found that DHA and α-LA induce phosphorylation of FFAR4 by protein kinase C (PKC), leading to increase of intracellular Ca^{2+} concentration. However, recent studies of the long isoform of FFAR4 by Burns et al. (29) showed that basal as well as heterologous FFAR4 phosphorylation is mediated by PKC, while DHA-induced phosphorylation is accomplished by GPCR kinase 6 (GRK6). Interestingly, mutation of the FFAR4 C-terminal

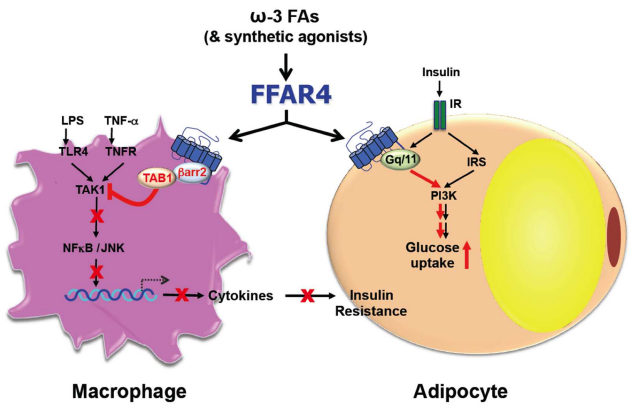

FIGURE 1 | Free fatty acid receptor 4 functions as an ω3-FA receptor/sensor to mediate broad anti-inflammatory and insulin sensitization effects.

phosphorylation sites leads to enhanced $G_{\alpha q/11}$ signaling while impairing β-arrestin-2 recruitment to the cell membrane. However, Burns et al. (15) also found that the human short and long isoforms differ in their basal levels of phosphorylation, raising the possibility that the short isoform is more constitutively active. In the activated state though, both isoforms show comparable extent of phosphorylation. While the long isoform is proposed by Watson et al. to bind with greater affinity to β-arrestin-2 (16), a partial loss of function is proposed for the long isoform by Hirasawa et al. (12) and Moore et al. (17).

TISSUE-SPECIFIC FUNCTIONS

Due to the divergence of the reported functions, FFAR4 shows tissue-specific activities, which needs to be taken into account particularly for the development of pharmaceutical intervention. Therefore, a tissue-specific knockout (KO) mouse would be of high interest to discover precisely where certain effects of FFAR4 impact. This is critical for the development of agents that target inflammation, insulin resistance, and as a result, type 2 diabetes. However, no conditional FFAR4 KO mouse is available to date and mouse models cannot be used to investigate the function of the long isoform of FFAR4, which is unique to humans. Given the known differential phosphorylation of the two isoforms (15), their functions and regulation likely differ, which will have to be addressed in human tissues and cell lines.

ADIPOGENESIS

Free fatty acid receptor 4 expression is undetectable in preadipocytes (18, 30) but increases during adipogenic differentiation (18, 30), and becomes highly abundant in mature adipocytes and adipose tissue (13, 18, 19). The expression in adipose tissue is further increased by diet-induced obesity in mice and humans (18, 19). Conversely, knockdown of FFAR4 using siRNA reduces the expression of adipogenic markers and therefore impairs the accumulation of lipids in 3T3-L1 adipocytes (18). Although these findings suggest a pro-adipogenic function of FFAR4, FFAR4-deficient mice are actually more prone to diet-induced obesity than wild type littermates (19), consistent with an anti-obesity function of FFAR4.

It should be mentioned that discordant phenotypes of FFAR4 KO mice have been reported using two different mouse models. Thus, the body weight of FFAR4 KO mice is either increased (19) or unaffected (31) on HFD, and the insulin sensitivity is either decreased (31) or unaffected (19) on chow diet between wild type and KO mice (13, 32). Being all on the same C57BL/6 background, the mice seem to be either the N or the sub-lines, which might explain the varying findings. However, all models consistently establish the key site of FFAR4 action to be the adipose tissue, where ω3-FAs clearly exert FFAR4-dependent anti-diabetic effects in adipocytes and macrophages (13, 18, 19).

INFLAMMATION

Omega-3 FAs have tissue-specific as well as systemic anti-inflammatory effects (33). FFAR4 is key to these benefits in adipose tissue macrophages, which abundantly express this receptor. Furthermore, FFAR4 expression in macrophages is induced upon obesity. It was shown that its activation by ω3-FAs in mice fed with HFD supplemented with ω3-FAs leads to the suppression of macrophage infiltration into adipose tissue (13). Additionally, ω3-FAs shift the distribution of macrophages in favor of the anti-inflammatory M2 macrophages at the expense of the pro-inflammatory M1 macrophages (13). In the brain, intracerebroventricularly injection of either ω3- or ω9-FAs induces FFAR4/β-arrestin-2 coupling followed by the release of TAK1 from TAB1, leading to attenuation of the inflammatory pathway (34). Additionally, Wellhauser et al. showed the anti-inflammatory effects of FFAR4 activation in immortalized hypothalamic neurons (35). This is of importance as on HFD that hypothalamus becomes inflamed and fails to regulate energy homeostasis through regulating glucose handling, feeding, and therefore, body weight.

Finally, FFAR4 activation also leads to improvement of non-alcoholic fatty liver disease (NAFLD) in children (36). In more detail, Nobili et al. detected FFAR4 expression in hepatocytes, liver macrophages, and liver progenitor cells. DHA treatment of children suffering from NAFLD increased the FFAR4 expression in hepatocytes and reduced nuclear NFκB translocation in hepatocytes and liver macrophages, as well as reduced hepatic progenitor cell activation and the number of inflamed macrophages in the liver (36). Accordingly, ω3-FAs also protect liver from ischemic reperfusion injury (IRI), a complication of liver surgery. Treatment with Omegaven®, a pharmaceutical ω3 formulation, reduces NFκB and JNK response and shifts the macrophage population from M1 to M2 (37).

INSULIN SIGNALING

Besides the food intake and digestive influences of FFAR4 activation by ω3-FAs, increased gut glucagon-like peptide 1 (GLP-1) secretion increases pancreatic insulin secretion, leading to enhanced glucose uptake in skeletal and cardiac muscle (38, 39). Whether FFAR4/ω3-FAs play a cell-autonomous role in muscle, the major site of insulin-stimulated glucose uptake and systemic insulin action (40, 41), has yet to be investigated in detail, as Cornall et al. (22) found FFAR4 expression increased in skeletal

and cardiac muscle of rats on HFD, while no expression was detected in L6 myocytes (13). Nevertheless, it has been shown that FFAR4 activation by ω3-FAs leads to insulin sensitization *in vivo* and also alleviates glucose intolerance in diet-induced obese mice (13, 19). Although the anti-inflammatory effects of ω3-FAs require FFAR4 coupling to β-arrestin-2, the hypoglycemic effect of insulin requires $G_{\alpha q}$ signaling that leads to translocation of the glucose transporter GLUT4 (13). Conversely, FFAR4 KO mice show reduced phosphorylation of IRβ and IRS-1 in white adipose tissue and of IRS-1 and -2 in liver, which are all important regulators for glucose uptake (19). Not surprisingly, these mice develop hyperglycemia, glucose intolerance, and insulin resistance when challenged with a HFD (19).

GASTROINTESTINAL REGULATION

Gastrointestinal peptides are known to regulate food intake, energy metabolism, and body weight (42–47). Activation of FFAR4 by ω3-FAs has been shown to either reduce or induce the secretion of several gastrointestinal peptides. For example, FFAR4 activation decreases the secretion of ghrelin, an endogenous growth hormone secretagogue that stimulates hunger (48, 49). Additionally, FFAR4 was shown to induce the secretion of GLP-1 and cholecystokinin (CCK) (12, 20, 21). GLP-1 is an insulinotropic, anorectic peptide that reduces gastric emptying and motility (43, 44). CCK is similar to GLP-1 in that it inhibits gastric motility, but in addition it inhibits gastric secretion while promoting pancreatic secretion and gallbladder contraction (50). It was shown by Stone et al. that FFAR4 activation leads to decreased somatostatin secretion (51), which in turn, can increase insulin and glucagon secretion as well as gastric emptying. This is in sharp contrast to the recent finding by Suckow et al. of increased glucagon secretion in FFAR4 KO mice (32).

Unfortunately, some of these findings are contradictory. Although several groups demonstrated FFAR4 expression in islets (52–54), there is no consensus on the site of action, like influencing secretion of GLP-1, glucagon, or somatostatin, and even the cell type in which it is expressed most, is contradictory (21, 32, 51).

TASTE PREFERENCES

Free fatty acid receptor 4 is abundantly expressed in several types of taste bud cells (55–57) and therefore can be activated directly by dietary ω3-FAs. Although detailed investigation is needed, it seems that FFAR4 might dictate spontaneous preference for specific dietary fats (31) that are abundant in energy-dense foods (58). However, Ozdener et al. (57) found that CD36 is the primary receptor for fat taste, while FFAR4 senses excess supply of FAs as in HFD.

SYNTHETIC LIGANDS OF FFAR4

Like FFAR4, FFAR1 (a.k.a. GPR40) is a receptor for long-chain ω3-FAs. Although the two receptors share endogenous as well as several synthetic agonists (11), like the PPARγ derivate GW9508 (59, 60), a few ligands are known to be partly or more selective for FFAR4 than FFAR1, namely grifolic acid, NCG21, GSK137647A, and TUG-891 (61–64). However, the relatively low efficacy of these known synthetic agonists in several measured outputs raises questions whether these can be therapeutically relevant molecules.

PERSPECTIVE

Solving the mechanism of FFAR4/ω3-FAs might lead to a more directed and therefore more potent way of fish oil supplementation. But besides that, the understanding of the mechanism hopefully will lead to the development of a more FFAR4-specific, high affinity agonist. The direct activation of FFAR4 itself is of special interest, as FFAR4 is a "druggable" GPCR. Unfortunately, to date, there are no FFAR4-specific agonists that spare other GPCRs like FFAR1, which leads to off-target effects. It might be possible to develop an FFAR4 agonist that is tissue-, pathway-, or isoform-specific and therefore provides anti-inflammatory/insulin-sensitizing effects. Taken together, finding of an FFAR4-specific agonist will be a new therapeutic approach for the treatment of both metabolic and inflammatory diseases.

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The G-protein-coupled long-chain fatty acid receptor GPR40 and glucose metabolism

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Free fatty acids (FFAs) play a pivotal role in metabolic control and cell signaling processes in various tissues. In particular, FFAs are known to augment glucose-stimulated insulin secretion by pancreatic beta cells, where fatty acid-derived metabolites, such as long-chain fatty acyl-CoAs, are believed to act as crucial effectors. Recently, G-protein-coupled receptor 40 (GPR40), a receptor for long-chain fatty acids, was reported to be highly expressed in pancreatic beta cells and involved in the regulation of insulin secretion. Hence, GPR40 is considered to be a potential therapeutic target for the treatment of diabetes. In this review, we summarize the identification and gene expression patterns of GPR40 and its role in glucose metabolism. We also discuss the potential application of GPR40 as a therapeutic target.

Keywords: GPR40, FFAR1, LCFA, insulin secretion, pancreatic beta cells

INTRODUCTION

Free fatty acids (FFAs) are essential nutrients that also act as signaling molecules in various tissues. Long-chain fatty acids (LCFAs) play a role in the augmentation of glucose-stimulated insulin secretion (GSIS) (1). GSIS was observed to be considerably decreased by FFA depletion following *in vivo* administration of nicotinic acid to rats (2) and humans (3). Thus, FFA-mediated augmentation is considered to be physiologically significant. However, the underlying mechanisms of FFA-mediated augmentation of GSIS have not been fully elucidated. Several investigators have recently demonstrated that FFAs act as ligands for membrane-bound G-protein-coupled receptors (GPCRs) such as G-protein-coupled receptor 40 (GPR40), GPR41, GPR43, and GPR120. Among these, GPR40 is preferentially expressed by pancreatic beta cells in rodents and augments GSIS after acute exposure to LCFAs, highlighting the role of GPR40 as a potential key molecule in the regulation of insulin secretion.

LCFA RECEPTOR GPR40

GPR40 consists of 300 residues and was originally reported as an orphan GPCR (4). GPR40 was deorphaned by screening using a fluorometric imaging plate reader (FLIPR) system, which detects increases in Ca^{2+} concentrations in cultured cells with transiently expressed GPR40 cDNA (5, 6). GPR40 is reportedly activated by LCFAs (C12–22) and several eicosanoids in theoretically physiological concentration ranges. The profiles of putative GPR40 ligands are well conserved among mice, rats, and humans (5).

GPR40 GENE EXPRESSION IN RODENTS

Among rat tissues, GPR40 mRNA is almost exclusively expressed in the pancreas. In pancreatic islets, GPR40 mRNA levels were found to be approximately 17-fold higher than the levels in the pancreas, suggesting selective GPR40 expression by pancreatic islets. Considerable amounts of GPR40 mRNA were detected in the pancreatic beta cell lines MIN6, betaTC-3, HIT-T15, and Rin5F but not in the pancreatic alpha cell line alphaTC1. Furthermore, *in situ* hybridization with rat pancreatic islets suggested that GPR40 mRNA is preferentially expressed in pancreatic beta cells (5).

Reports using anti-GPR40 antibodies suggest that GPR40 protein is also probably preferentially expressed in pancreatic islets (7, 8).

ROLES OF GPR40 IN REGULATION OF INSULIN SECRETION

In MIN6 cells, insulin secretion was augmented by LCFAs in a dose-dependent manner, and the augmentation was observed only under hyperglycemic conditions (11–22 mM) (5), indicating the LCFA-mediated augmentation of insulin secretion is glucose-dependent. Silencing of GPR40 gene expression using siRNA almost abolished the augmentation effects of LCFAs, indicating that GPR40 is involved in LCFA-mediated regulation of insulin secretion. GPR40 is a class A GPCR, highlighting the potential of GPR40 as a target for novel anti-diabetic oral drugs with low risk of hypoglycemia, considering that LCFA-mediated augmentation of insulin secretion is glucose-dependent.

GPR40 GENE EXPRESSION IN HUMANS

Although GPR40 is reportedly preferentially expressed by pancreatic beta cells in both rats and mice, little is known about GPR40 gene expression in humans. In this context, we assessed GPR40 mRNA expression in fresh human tissues obtained during surgery (9, 10). Analysis of 12 specimens of non-tumor pancreatic tissues revealed a considerable amount of GPR40 mRNA in each. In three pancreatic islet tissues specimens, GPR40 mRNA levels were approximately 20-fold higher than those in pancreatic tissues, comparable to the levels of sulfonylurea receptor 1, which is

known to be highly expressed in pancreatic beta cells. High levels of GPR40 mRNA were detected in insulinoma (beta cell tumor) tissues in three cases; in contrast, GPR40 mRNA was undetectable in glucagonoma (alpha cell tumor) tissues (10, 11). In human pancreas, GPR40 mRNA level is positively and significantly correlated with the insulinogenic index, an index reflecting the function of pancreatic beta cells. These results indicate that GPR40 is highly expressed in human pancreatic beta cells and possibly involved in the positive regulation of insulin secretion (10).

REGULATION OF GPR40 GENE EXPRESSION

Though the mechanisms underlying the regulation of GPR40 gene expression is not fully understood, possible mechanisms include the PDX-1/IPF1 (12), which reportedly binds to the promoter region of the GPR40 gene (13). Moreover, nutrients and therapeutic drugs such as glucose (12), palmitate, and rosiglitazone (8) are reportedly involved in the regulation of GPR40 gene expressions.

THERAPEUTIC IMPLICATIONS OF GPR40

Although an initial report of systemic GPR40 knockout (KO) mice and beta cell-specific GPR40 transgenic (Tg) mice using the PDX-1/IPF1 promoter suggested possible involvement of GPR40 in insulin resistance in the liver and beta cell failure (14), later reports using GPR40 KO mice found no link between GPR40 and beta cell dysfunction (15, 16). Studies using GPR40 KO mice suggest the implication of GPR40 in the regulation of insulin secretion, at least under some conditions including loading of intralipid (17), high-fat diet (15), hyperglycemic glucose clamp, and arginine (18). Furthermore, GPR40 Tg mice with the mouse INS2 promoter exhibited better glucose tolerance with enhanced GSIS (19), suggesting therapeutic implications of GPR40 rather than a gateway of beta cell toxicity.

Additionally, recent reports suggest that GPR40 is expressed in enteroendocrine cells and involved in the positive regulation of intestinal hormones including glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and cholecystokinin (20–22).

GPR40 AGONISTS AS ANTI-DIABETIC DRUGS

Recently, TAK-875 (Fasiglifam), a novel GPR40 selective agonist (23), was reported as a potential oral anti-diabetic drug. The potency of TAK-875 is approximately 400-fold greater than that of the endogenous ligand oleic acid (24), and it does not activate GPR120 (23), another GPCR for LCFAs. TAK-875 augmented insulin secretion under high-glucose conditions in the rat pancreatic beta cell line INS1 833/14 (24) and human pancreatic islets (25) but did not affect glucagon secretion in humans (25), in accordance with the observations in humans by our group and others (9–11). TAK-875 significantly improved glycemic control with the augmentation of insulin secretion in diabetic rat models such as Wistar fatty rats (23) and Zucker diabetic fatty rats (24).

In phase 2, randomized, double-blind, placebo-controlled trial in patients with type 2 diabetes, HbA1c was decreased in a dose-dependent manner in TAK-875 groups, and the HbA1c-lowering effect (50–200 mg, approximately −1.1% in 12 weeks) was comparable to that in glimepiride (4 mg) group, while the incidence of hypoglycemia in TAK-875 was similar to the placebo group and markedly lower than the glimepiride group (26). In Japanese patients with type 2 diabetes, 12-week treatment with TAK-875 also decreased HbA1c levels in a dose-dependent manner, and the HbA1c-lowering effect (50–200 mg, approximately −1.3%) was comparable to that in the glimepiride (1 mg) group (27).

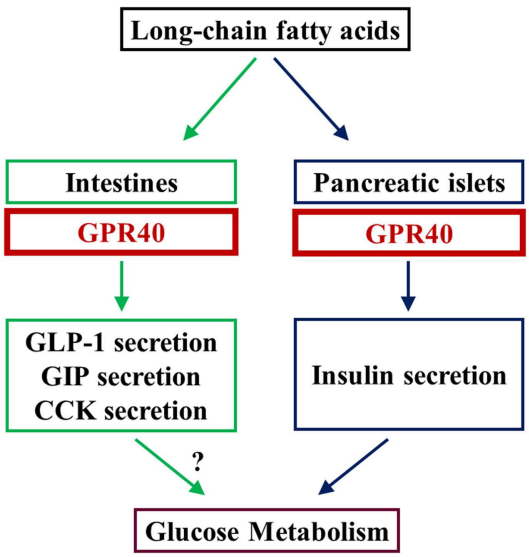

FIGURE 1 | LCFAs augment insulin secretion by stimulating GPR40 in beta cells in the pancreas. GPR40 is also reportedly involved in the positive regulation of secretion of some intestinal hormones. Studies using GPR40 agonists and analyses of beta cell-specific GPR40 transgenic mice indicate that GPR40 is involved in the regulation of glucose metabolism, at least by the augmentation of insulin secretion. Because LCFA-mediated augmentation of insulin secretion is glucose-dependent, GPR40 is thought to be a potential target for novel anti-diabetic drugs with low risk of hypoglycemia.

Though TAK-875 seemed to be a promising anti-diabetic drug, regrettably, its development was terminated in 2013 because of the risk of possible liver damage. Although the cause of the liver damage remains unclear, GPR40 is not expressed in the human liver (6, 10), suggesting that the toxicity may not be due to the GPR40 receptor itself but chemical characteristic of TAK-875 or its dose used in the clinical trials. Still, several GPR40 agonists continue to be evaluated in both preclinical (Bristol-Myers Squibb, Merck, Amgen, Johnson & Johnson, Astellas, Daiichi Sankyo, Piramal, and Connexios) and clinical (Japan Tobacco) trials, and the further development is expected in the study elucidating the significance of GPR40 in glucose and other metabolism.

CONCLUSION

Incretin mimetic-type drugs have been implicated in GPCR-mediated regulation of insulin secretion in diabetes. GPR40 is a GPCR that is highly expressed in pancreatic beta cells and involved in insulin secretion in rodents and humans. Hence, GPR40 is a potential therapeutic target in diabetes, which can lead to the development of oral drugs with fewer hypoglycemic side effects. Furthermore, GPR40 is reportedly implicated in the regulation of incretin secretion from enteroendocrine cells. GPR40 may be important to unveil the link between FFA signaling and beta

cell function as well as glucose metabolism (**Figure 1**). Hence, further studies are warranted to elucidate the physiological and pathophysiological implications of GPR40.

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Treatment of type 2 diabetes by free fatty acid receptor agonists

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Dietary free fatty acids (FFAs), such as ω-3 fatty acids, regulate metabolic and anti-inflammatory processes, with many of these effects attributed to FFAs interacting with a family of G protein-coupled receptors. Selective synthetic ligands for free fatty acid receptors (FFA1-4) have consequently been developed as potential treatments for type 2 diabetes (T2D). In particular, clinical studies show that Fasiglifam, an agonist of the long-chain FFA receptor, FFA1, improved glycemic control and reduced HbA1c levels in T2D patients, with a reduced risk of hypoglycemia. However, this ligand was removed from clinical trials due to potential liver toxicity and determining if this is a target or a ligand-specific feature is now of major importance. Pre-clinical studies also show that FFA4 agonism increases insulin sensitivity, induces weight loss, and reduces inflammation and the metabolic and anti-inflammatory effects of short chain fatty acids (SCFAs) are linked with FFA2 and FFA3 activation. In this review, we therefore show that FFA receptor agonism is a potential clinical target for T2D treatment and discuss ongoing drug development programs within industry and academia aimed at improving the safety and effectiveness of these potential treatments.

Keywords: diabetes, FFA receptor, insulin, incretin, inflammation

INTRODUCTION

In 2013, 382 million people worldwide were characterized as diabetic patients with around 90% of patients diagnosed with type 2 diabetes (T2D), a metabolic disorder intrinsically linked with obesity (1). T2D is defined by insulin resistance in peripheral tissues, such as the liver and muscle, and a loss of pancreatic beta-cell function, resulting in insufficient insulin secretion (2), and constitutes a risk factor for health issues including cardiovascular disease, impaired wound healing, blindness, and renal failure (1). Although T2D can sometimes be controlled through strict diet regulation, a large number of patients require clinical therapies. Current treatments, such as metformin, sulfonylureas, glucagon-like peptide-1 (GLP-1) receptor agonists, and dipeptidyl peptidase-4 (DPP-4) inhibitors, are deployed primarily to either improve insulin secretion, peripheral insulin sensitivity, or both (3). However, there remains a demand for distinct, safe, and effective treatments for T2D, with the current therapies often associated with side effects including hypoglycemia and weight gain. Naturally occurring free fatty acids (FFAs) found in the diet, including ω-3 fatty acids, have profound effects on metabolic and inflammatory processes associated with T2D, although the molecular basis for these effects are complex and incompletely understood (4). FFAs are classified based upon their chain length, such that short chain fatty acids (SCFAs) have 1–6 carbon atoms; medium chain fatty acids (MCFAs) contains 7–12 carbon atoms; and long-chain fatty acids (LCFAs) contain more than 12 carbon atoms (4). Many of the biological effects of FFAs have now been attributed, at least in part, to FFAs interacting with a group of G protein-coupled receptors (GPCRs) designated the FFA receptors. The most well-characterized FFA receptors are the two LCFA-specific receptors, FFA1 and FFA4, and the SCFA-specific receptors FFA2 and FFA3. FFA receptor agonism, particularly of the FFA1 receptor, has subsequently been shown to have beneficial metabolic effects (4). Consequently, a number of ongoing industrial and academic programs are focused upon developing potent and selective synthetic agonists of FFA1. Although currently less developed, activation of each of FFA2, FFA3, and FFA4 has also been suggested to have potential benefits for metabolic function. In this review, we will therefore discuss the potential of FFA receptor agonists as novel clinical treatments for T2D.

Abbreviations: αLA, α-linolenic acid; DHA, docosahexaenoic acid; CNS, central nervous system; DPP-4, dipeptidyl peptidase-4; FFA, free fatty acid; FFA1–4, free fatty acid receptors 1–4; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GPCR, G protein-coupled receptor; GSIS, glucose-stimulated insulin secretion; HbA1c, hemoglobin A1c; HFD, high-fat diet; LCFA, long-chain fatty acid; LPS, lipopolysaccharide; MCFA, medium chain fatty acid; PYY, peptide YY; SCFA, short chain fatty acid; T2D, type 2 diabetes; TNF, tumor necrosis factor.

FFA1

FFA1, activated by various saturated (e.g., palmitic acid, C16:0), mono-unsaturated (e.g., oleic acid, C18:1), and polyunsaturated long-chain FFAs (e.g., linoleic acid, C18:2) (**Table 1**), is a $G_q/_{11}$-coupled GPCR predominantly expressed in pancreatic beta cells that is associated with increased glucose-stimulated insulin secretion (GSIS) (4–6) (**Figure 1**). FFA1 is also expressed

Table 1 | FFA receptor agonists for the treatment of T2D.

FFA receptor	Agonists	Metabolic effects	Clinical trial status
FFA1	**Natural ligands:** palmitic acid, oleic acid, linoleic acid **Synthetic ligands:** GW9508, TAK-875/Fasiglifam, AMG-837, AM-1638, AM-5262, LY2881835, JTT-851, P11187, TUG-469, TUG-424, TUG-770, AS2575959, DS-1558	Improved fasting hypoglycemia and glucose tolerance in diabetic animal models Increased GSIS Increased incretin release (full agonists: AM-1638, AM-5262, LY2881835) No associated hypoglycemia in normoglycemic rats	**TAK-875/Fasiglifam (Takeda):** phase I/II trials showed reduced blood glucose levels, increased insulin levels, 1.2–1.4% reduction in HbA1c levels with no associated weight gain/hypoglycemia in T2D patients. Removed from phase III trials due to potential liver toxicity **AMG-837 (Amgen) and LY2881835 (Eli Lilly):** removed from phase I trials due to toxicity **JTT-851 (Japan Tobacco):** currently in phase II trials **P11187 (Piramal):** currently in phase I trials
FFA2	**Natural ligands:** acetate (preferred), propionate, butyrate **Synthetic ligands:** AMG7703/ 4-CMTB, Euroscreen compounds, compounds 1 and 2	Improved glucose uptake Decreased colon motility/contractility Increased GLP-1 secretion Inhibition of leukocyte activation	No agonists currently in clinical trials
FFA3	**Natural ligands:** propionate (preferred), butyrate, acetate **Synthetic ligands:** Arena Pharmaceuticals series	Increased GLP-1 secretion	No agonists currently in clinical trials
FFA4	**Natural ligands:** α-linolenic acid (αLA), docosahexanoic acid (DHA) **Synthetic ligands:** GW9508, NCG21, NCG46, TUG-891	Protection against diet-induced obesity Improved insulin sensitivity and glycemic control Increased GLP-1 release Increased insulin secretion (largely attributed to GLP-1 release) Reduced inflammation	No compounds currently in clinical trials although a number of companies have patented FFA4 agonists for the treatment of T2D (Banyu Pharmaceutical, Metabolex, Kindex Therapeutics, Pharma Frontier)

Table 1 illustrates the most commonly described natural and synthetic ligands for FFA1-4. The current clinical status of these synthetic agonists for the treatment of T2D is also described.

by various enteroendocrine cells where it regulates the release of incretin hormones such as glucagon-like peptide-1 (GLP-1), an insulinotropic, anorectic peptide that reduces gastric emptying and motility, as well as cholecystokinin (CCK), shown to regulate pancreatic secretion, inhibit gastric motility, and reduce energy intake (7–10) (**Figure 1**). FFA1 is also present within the central nervous system (CNS) (11, 12) although whether neuronal FFA1 contributes to the regulation of glucose homeostasis remains to be fully determined (**Figure 1**). FFA1 expression has also been reported in glucagon-producing alpha cells within the pancreas, although this remains controversial (13–17). FFA1 expression has also been well characterized in taste buds where it mediates, in part, taste preference for fatty acids, although the significance of this, and possible effects of pharmacological activation or blockade, remains to be fully elucidated (18, 19) (**Figure 1**).

FFA1 AND INSULIN SECRETION

Acute FFA-mediated insulin secretion from isolated human and rodent islets involves amplification of the second phase of GSIS (5, 6, 15, 20). This is reduced by approximately 50% in FFA1-null mice, with the remaining effect attributed to intracellular metabolism of FFAs (5, 6, 15, 20). In contrast, transgenic overexpression of FFA1 under the control of the mouse insulin II promoter prevents development of hyperglycemia and improves insulin secretion and glucose tolerance in diabetic mouse models (21). As anticipated from this, GW9508, a synthetic FFA1 agonist (**Table 1**) stimulated GSIS in pancreatic MIN6 cells (22). FFA1 gene expression is also reduced under glucolipotoxic conditions in rats and in islets from T2D patients while a rare mutation in the human FFA1 gene is associated with attenuated lipid-mediated enhancement of GSIS (23–25). The effects of FFA1 on pancreatic beta cell viability, however, has been controversial, with pancreatic-specific FFA1 overexpression associated with disrupted islet morphology and impaired beta cell function whereas FFA1 disruption is linked with increased beta cell viability in mice fed on a high-fat diet (HFD) (26). These observations promoted the concept that, at least in the longer term, FFA1 antagonism could be beneficial in the treatment of diabetes. However, most subsequent pre-clinical studies contradict these findings, indicating that FFA1 agonism has no detrimental effects on beta cell viability (16, 20, 21), or even protects beta cells (27–29).

THE FFA1 AGONIST FASIGLIFAM AND INSULIN SECRETION

Although there are currently no FFA1 agonists approved for clinical use, considerable interest developed around Fasiglifam

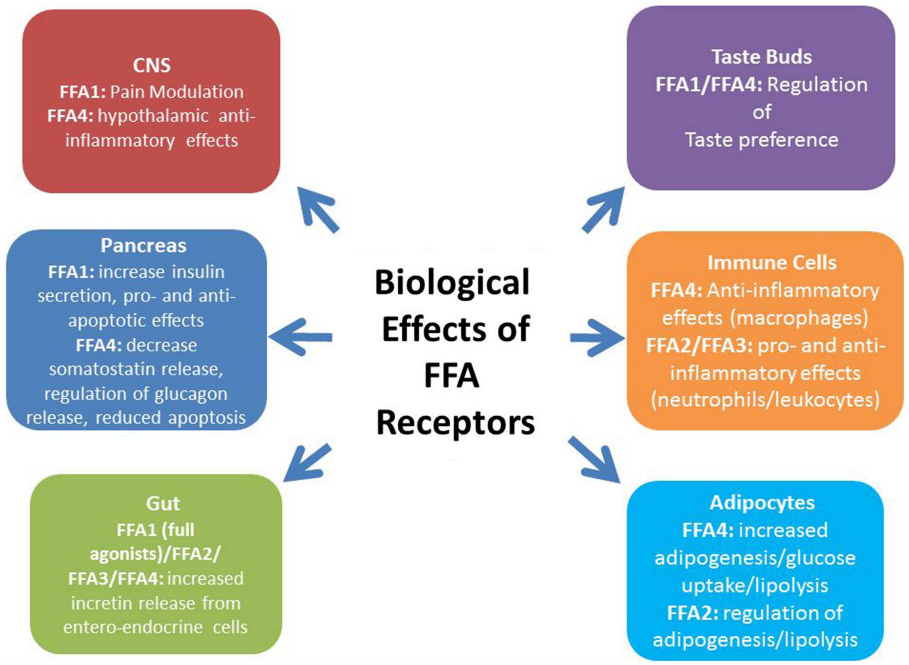

FIGURE 1 | The biological effects of FFA receptors. Dietary FFAs, such as ω-3 fatty acids from fish oils and SCFAs derived from the fermentation of dietary fiber, have profound effects on metabolic and inflammatory processes associated with obesity and T2D. These effects have, at least in part, been attributed to the activation of free fatty acid receptors (FFA1–4), leading to a great deal of interest in the development of synthetic FFA receptor agonists for the treatment of metabolic disease. Agonism of the long-chain FFA receptor FFA1, the most fully characterized of these receptors, improves glucose-stimulated insulin secretion from the pancreas. Additionally, full agonists of this receptor increase incretin release from the gut, thereby indirectly increasing pancreatic insulin secretion, as well as improving systemic insulin sensitivity and promoting satiety. Agonism of another long-chain FFA receptor, FFA4, is associated with incretin release from the gut, as well as an anti-inflammatory effect on macrophages that, in turn, may improve systemic insulin sensitivity. In the pancreas, FFA4 is associated with reduced cell apoptosis and FFA4 has recently been detected in alpha and delta cells and regulates glucagon and somatostatin release, respectively. Both FFA1 and FFA4 have been detected in taste buds although the full implications of this in relation to obesity remain to be determined. The SCFA receptors, FFA2/FFA3, have recently been linked with the beneficial metabolic effects associated probiotics within the gut. Both receptors have been linked with incretin release from enteroendocrine cells, as well as both systemic anti- and pro-inflammatory effects. However, due to conflicting results using receptor-specific knockout models and a limited selection of pharmacological tools, more work is required to elucidate the physiological effects of FFA2 and FFA3 agonism.

(designated TAK-875 in pre-clinical studies, **Figure 2**), an orally available FFA1 agonist developed by Takeda (30–32) (**Table 1**). Completed Phase II clinical trials demonstrated that T2D patients treated with Fasiglifam had reduced blood glucose levels, increased insulin levels, and resulted in a 1.2–1.4% reduction in hemoglobin A1c (HbA1c) levels (32–36) (**Table 1**). Crucially, although these effects were comparable to current sulfonylurea treatments, Fasiglifam was associated with markedly less side effects, with no significant increases in body weight and a reduced concomitant incidence of hypoglycemia (32–36). This is consistent with pre-clinical data demonstrating that Fasiglifam improved fasting hyperglycemia and glucose tolerance and augmented GSIS in diabetic rat models, with no hypoglycemia observed in normoglycemic rats (31). No changes in insulin resistance have been reported in response to Fasiglifam treatment (37, 38) and Fasiglifam had no effect on glucagon secretion in isolated human islets and did not alter glucagon levels in T2D patients (39). Importantly, prolonged Fasiglifam exposure was also not associated with beta cell dysfunction or apoptosis (31).

THE EFFECT OF PARTIAL VS. FULL FFA1 AGONISTS ON INCRETIN RELEASE FROM ENTEROENDOCRINE CELLS

The ability of synthetic FFA1 agonists to induce significant incretin release was recently shown to depend upon whether the compound was a partial or full agonist (8, 10, 40) (**Figure 1**). In this regard, TAK-875/Fasiglifam had no effect on incretin release from enteroendocrine cells with similar results reported for AMG-837 (Amgen, **Table 1**; **Figure 2**) (39). In contrast, Amgen described AM-1638 and AM-5262 (**Table 1**; **Figure 2**) as full FFA1 agonists that directly stimulate insulin secretion and promote incretin release from enteroendocrine cells (39, 41, 42) (**Figure 1**). This incretin-stimulating effect was abolished in FFA1 knockout mice and the effect of AM-1638 on glucose homeostasis was attenuated by the GLP-1R antagonist, Ex(9–39)NH2, indicating a particularly key role for GLP-1 (39). Similarly, LY2881835, a full FFA1 agonist from Eli Lilly (**Table 1**), increased GSIS, lowered blood glucose levels, and increased GLP-1 secretion in animal models (43). Amgen demonstrated that multiple ligand binding pockets exist on FFA1, comprising of up to two allosteric sites as well as the FFA binding orthosteric site (40). One allosteric site is targeted by compounds

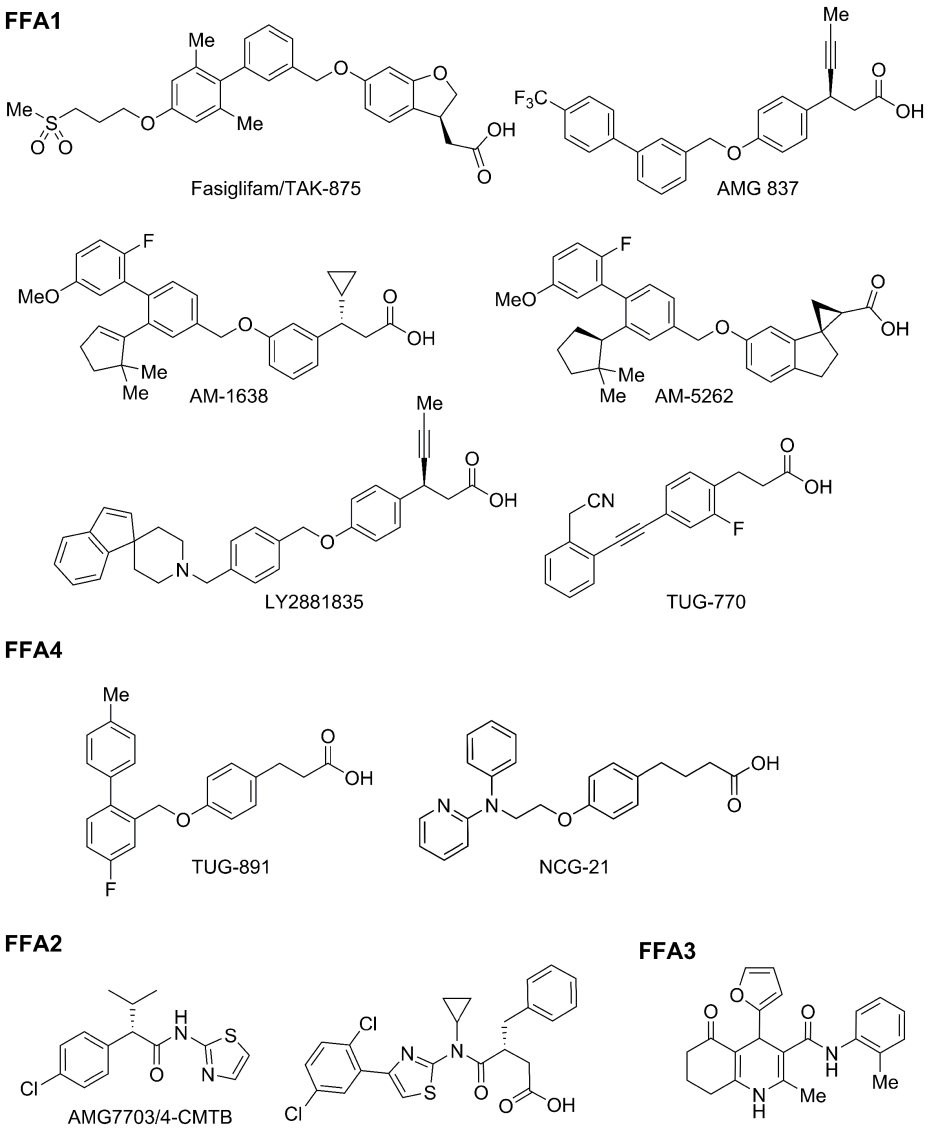

FIGURE 2 | Representative free fatty acid receptor agonists. A number of academic and industrial drug programs are aimed at developing FFA receptor agonists for the treatment of T2D. A representative selection of the current range of synthetic agonists that have so far been developed for these receptors are shown.

such as AM-837 and TAK-875 while the second allosteric site is a target for receptor agonists such as AM-1638 that act as full agonists. Consequently, positive co-operativity was shown between either AMG-837 or AM-1638 in conjunction with natural FFA ligands in cell based assays measuring second-messenger generation, as well as primary cell based assays, with positive co-operativity also reported between AMG-837 and AM-1638 during an oral glucose tolerance test in a diabetic rodent model (40).

FFA1 AGONISTS: ONGOING FFA1 DRUG PROGRAMS AND FUTURE CHALLENGES

Although no issues were raised regarding safety and tolerability during Phase I and II trials, Fasiglifam was recently withdrawn from phase III trials due to potential liver toxicity (43) (**Table 1**). Similarly, Amgen and Eli Lilly removed AMG-837 and LY2881835 (**Table 1**; **Figure 2**), respectively from Phase I clinical trials due to concerns over toxicity (43). However, the pre-clinical and clinical data generated using Fasiglifam provides a strong rationale and validation for further studies into the potential use of FFA1 agonism as a novel treatment for T2D. Currently, Japan Tobacco are conducting Phase II clinical trials with their FFA1 agonist candidate, JTT-851 and Piramal have begun Phase I clinical trials on their FFA1 agonist, P11187 (43) (**Table 1**). Daiichi Sanyko also recently described 3-aryl-3-ethoxypropanoic acids as orally active FFA1 agonists that improve insulin secretion and glucose homeostasis in rats (44). Additionally, FFA1 agonists developed by Astellas are reported to have beneficial effects on glucose homeostasis in diabetic mouse models (45, 46). Sanofi and Boehringer-Ingelheim

are also reported to have FFA1 agonist programs under development (43). In an academic context, the University of Southern Denmark have developed 4-(benzylamine)hydrocinnamic acid FFA1 agonists such as TUG-469 (47, 48) and 4-alkyne hydrocinnamic acid FFA1 agonists, including TUG-424 and TUG-770 (49–51) (**Table 1**). Within these programs, several strategies have been followed to reduce compound lipophilicity (48, 52, 53). Consequently, TUG-770 (**Figure 2**) has recently been described as a highly potent FFA1 agonist with favorable physicochemical and pharmacokinetic properties, improving glucose tolerance in diet-induced obese mice. This effect did not desensitize, being fully maintained after 29 days of chronic dosing (49).

FFA4

FFA4, a G_q-coupled GPCR activated by LCFAs, including α-linolenic acid (α-LA) and docosahexaenoic acid (DHA) (**Table 1**), is expressed in enteroendocrine cells, lung, brain, white adipose tissues, heart, and liver (4). Within adipose tissue, FFA4 gene expression is upregulated following a HFD and FFA4 activation in adipocytes is associated with increased adipogenesis and glucose uptake (54–56), suggesting that FFA4 activation may promote adiposity and obesity (**Figure 1**). However, mutation of FFA4 (p.R270H variant) is associated with an increased risk of obesity in European populations (although this variant is almost absent in a Japanese population), and young FFA4 null mice fed a HFD gained significantly more fat mass than their wild-type littermates, suggesting that FFA4 protects against diet-induced obesity (57). FFA4 agonism is also commonly associated with improved insulin sensitivity, with FFA4 null mice reported to have increased fasting glucose and impaired responses to insulin and glucose tolerance testing (56, 57) (**Figure 1**). A number of these metabolic effects, such as increased insulin secretion, satiety, and improved glycemic control, have been attributed, at least in part, to FFA4-dependent incretin release from enteroendocrine cells, particularly GLP-1 (4, 54, 55, 58) (**Figure 1**). GLP-1 secretion was demonstrated both *in vitro* and *in vivo* using aLA as an agonist (58). Similarly, TUG-891 (**Figure 2**), a potent FFA4 agonist (see below), also increased GLP-1 secretion from STC-1 and GLUTag enteroendocrine cells (55). However, a recent study has questioned the significance of FFA4-mediated GLP-1 release (59). FFA4 also co-localizes with the orexigenic peptide, ghrelin, in duodenal cells *in vivo*, with recent studies showing that FFA4 activation inhibits ghrelin secretion (60, 61). An emerging role for FFA4 within pancreatic islets has also recently developed, with the pancreatic islets of diabetic and hyperglycemic individuals shown to have decreased levels of FFA4 mRNA and knockdown of FFA4 mRNA levels within islets demonstrated to attenuate the protective effects of the ω-3 fatty acid, eicosapentaenoic acid against palmitate-induced cell apoptosis (62) (**Figure 1**). FFA4 expression has also recently been detected in delta cells and alpha cells within the pancreas and was consequently linked with the inhibition of glucose-dependent somatostatin release and the regulation of glucagon secretion, respectively (63, 64) (**Figure 1**). Similar to FFA1, FFA4 is also expressed in taste buds and is linked with the regulation of taste preference although, again, the significance of this in relation to obesity and T2D remains to be clarified (65) (**Figure 1**).

THE ANTI-INFLAMMATORY EFFECTS OF FFA4

A recent study indicated that, in addition to the previously described insulin-sensitizing effects associated with GLP-1 release, improved systemic insulin sensitivity may also be associated with FFA4-mediated anti-inflammatory effects on macrophages (56) (**Figure 1**). In this study, FFA4 expression in macrophages was elevated in response to obesity and FFA4 activation decreased pro-inflammatory gene expression in M1 macrophages and increased expression of M2 anti-inflammatory genes with reduced macrophage infiltration of adipose tissues also observed in FFA4 null mice due to decreased chemotaxis (56). These anti-inflammatory effects are largely associated with FFA4-mediated recruitment of β-arrestin 2, a scaffold protein typically associated with receptor desensitization and internalization that is also implicated in the regulation of distinct signaling pathways (56, 66, 67). In the case of FFA4, β-arrestin 2 interacts with TAB1 that, in turn, inhibits lipopolysaccharide (LPS)- and tumor necrosis factor (TNF)-alpha-induced TAK1 stimulation, thereby blocking toll-like receptor 4 (TLR4) and the TNF-alpha inflammatory pathways (56, 66, 67). Interestingly, recent studies have also reported FFA4-mediated anti-inflammatory effects within the brain. In particular, FFA4 has been associated with the anti-inflammatory effects of ω-3 and ω-9 fatty acids in the hypothalamus, thereby reducing diet-induced inflammation and reducing body adiposity (68, 69) (**Figure 1**).

SYNTHETIC FFA4 AGONISTS

Initial synthetic FFA4 agonists, including GW9508, NCG21 (**Figure 2**), and NCG46 (**Table 1**), showed significant dual agonism at FFA1 (70). However, our groups have recently reported on TUG-891, a potent and selective FFA4 agonist (55, 71) (**Table 1**, **Figure 2**), although TUG-891 is significantly less selective for murine FFA4 compared to murine FFA1, potentially limiting its use in pre-clinical *in vivo* studies in mice (71). Recent modeling and mutational efforts have, however, clearly defined how TUG-891 interacts with FFA4 (72), information that will be invaluable in developing novel ligands with improved pharmacological properties for this receptor. To date, no FFA4 agonists have entered clinical trials although a number of FFA4 agonist programs are ongoing. For example, Banyu Pharmaceutical Co. Ltd, IRM LLC USA, Metabolex, Inc., Kindex Therapeutics, and Pharma Frontier Co., Ltd have all patented FFA4 agonists for the treatment of metabolic and inflammatory disease (66) (**Table 1**). Similarly, GSK has recently described a series of diarylsulfonamides as FFA4 agonists (73) and Metabolex has reported that their series of dihydrobenzofuran-based FFA4 agonists improved glucose homeostasis in mice, with moderate glucose-lowering effects in mice shown with a separate series of FFA4 agonists (66). Additionally, Kindex Therapeutics described beneficial effects in the treatment of obesity, inflammation, and metabolic disorders with alpha acids that were reported to act both as FFA4 agonists and also as partial PPARγ agonists (66).

METABOLIC REGULATION BY FFA2 AND FFA3

High fiber intake protects against obesity and T2D via SCFA production, particularly butyrate, acetate, and propionate, from bacterial fermentation of dietary fiber in the large intestine (74).

Moreover, modulation of gut microbiota using pre- and probiotics in both mice and humans regulates body weight, appetite, and glucose homeostasis (74). These SCFA-mediated beneficial effects on body weight and glucose homeostasis in HFD-fed mice are due, at least in part, to FFA2/FFA3-dependent mechanisms, including for example increased secretion of incretins, such as GLP-1, glucose-dependent insulinotropic polypeptide (GIP), and peptide YY (PYY) (74, 75). These receptors are activated by the SCFAs produced by fiber fermentation in the gut, with the human FFA2 ortholog preferentially activated by shorter SCFAs, such as acetate, whereas human FFA3 is activated preferentially by the longer SCFAs, with propionate being the most potent SCFA for both receptors, at least in human (4, 74–80) (**Table 1**; **Figure 1**). However, the relative potency and preference for various SCFAs appears to vary significantly across species (81, 82). SCFA-triggered secretion of GLP-1 was almost completely abolished in primary colonic cultures from FFA2 null mice and reduced, to a lesser extent, in mice lacking FFA3 (76). FFA2 is expressed in adipose tissue, intestine, islet cells, enteroendocrine cells, and immune cells while FFA3 is highly expressed in the small intestine, colon, and pancreas (4). FFA2 expression levels are also elevated in the skeletal muscle, liver, and adipose tissue of HFD-fed rodents, with FFA2 shown to regulate adipogenesis and adipocyte differentiation and inhibit lipolysis (83) (**Figure 1**).

Complete elucidation of the metabolic effects of FFA2 and FFA3 has, however, been complicated by conflicting results using FFA2 and FFA3 null mice. For example, in one study, HFD-fed FFA2 null mice display lower body fat mass and improved glucose control compared to wild-type mice, indicating a role for FFA2 antagonists in the treatment of T2D (84). Contrastingly, FFA2 null mice were also shown to be obese on a normal diet, with reduced insulin sensitivity and marked insulin resistance whereas adipocyte-specific overexpression of FFA2 resulted in lower body weight in a HFD study (85). Similarly, the loss of FFA3 either resulted in weight loss, obesity, or had no effect in different studies (86–88). Hence, the development of more potent and selective FFA2 and FFA3 agonists will hopefully facilitate the elucidation of the metabolic effects of FFA2 and FFA3 and ultimately provide future treatments for T2D. Several selective compound series are already known, especially for FFA2 (89) (**Table 1**). Small carboxylic acids derived from the natural SCFA ligands have shown appreciable and predictable selectivity but have low potency (80). Selective allosteric agonists of FFA2 were reported by Amgen to regulate lipolysis (e.g., AMG7703/4-CMTB, **Table 1**; **Figure 2**). However, the clinical use of these drugs was deemed to be limited due to low solubility and poor pharmacokinetics (90). Orthosteric FFA2 agonists and antagonists have also now been reported (82, 91) and used to demonstrate a role for this receptor in improved glucose uptake, decreased colon motility and contractility, increased GLP-1 secretion, and inhibiting leukocyte activation (81, 82, 89, 92). FFA3 agonists are even less developed although Arena Pharmaceuticals has reported a series of FFA3-selective compounds (89) (**Table 1**). Pharmacological characterization of compounds from this series demonstrated that individual members have diverse pharmacological properties, acting as intrinsic agonists and/or allosterically modulating the potency or efficacy of the response to SCFA propionate (93). Hence, although recent microbiota studies highlight quite elegantly the role that gut-derived SCFAs can play in the regulation of metabolism, there still remains a great demand for improved FFA2 and FFA3 agonists to fully unravel and define the consequences of activation of these receptors for metabolic health.

FUTURE PERSPECTIVES

The withdrawal of FFA1 agonists from clinical trials, particularly Fasiglifam, highlights the critical importance of establishing whether the adverse effects reported during these clinical trials are due to FFA1 agonism or the chemical structures of the particular FFA1 agonists. Additionally, as FFA2, FFA3, and FFA4 agonists further develop and hopefully enter clinical trials, it will be interesting to see if the same issues highlighted during FFA1 trials will also arise. Future research should also fully address the relative effects of partial and full FFA1 agonists, particularly in relation to allosterism in conjunction with natural FFA ligands. Additionally, dual agonists of FFA1 and FFA4 may have enhanced effects on insulin secretion and insulin sensitivity compared to selective FFA1 or FFA4 agonists alone. Similarly, co-therapeutic approaches involving FFA receptor agonists and current T2D therapies should be examined. For example, the FFA1 agonist, AS2575959 (**Table 1**), acts synergistically with a DPP-IV inhibitor to improve glucose homeostasis (45) and combination therapy with Fasiglifam and metformin displayed enhanced anti-diabetic effects in a diabetic rat model (94). Similarly, the FFA1 agonist, DS-1558 (**Table 1**), acts synergistically with exendin-4, a GLP-1 receptor agonist, to improve glucose homeostasis in diabetic mice (95). Clearly, there are a number of significant challenges ahead in the development of clinical treatments based on FFA receptor agonism. However, should these challenges be met, FFA receptor agonism may provide a novel and effective way to treat T2D.

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