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Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids

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ABSTRACT

GPR41 is reportedly expressed in murine adipose tissue and mediates short chain fatty acid (SCFA)-stimulated leptin secretion by activating $G\alpha_i$. Here, we agree with a contradictory report in finding no expression of GPR41 in murine adipose tissue. Nevertheless, in the presence of adenosine deaminase to minimise $G\alpha_i$ signalling via the adenosine A1 receptor, SCFA stimulated leptin secretion by adipocytes from wild-type but not GPR41 knockout mice. Expression of GPR43 was reduced in GPR41 knockout mice. Acetate but not butyrate stimulated leptin secretion in wild-type mesenteric adipocytes, consistent with mediation of the response by GPR43 rather than GPR41. Pertussis toxin prevented stimulation of leptin secretion by propionate in epididymal adipocytes, implicating $G\alpha_i$ signalling mediated by GPR43 in SCFA-stimulated leptin secretion.

1. Introduction

GPR41 (free fatty acid receptor 3; FFA3) and GPR43 (FFA2) are related G protein-coupled receptors that are activated by short chain carboxylic acids [1,2]. Both receptors couple to $G\alpha_i/G\alpha_o$ but GPR43 also couples to $G\alpha_q$ [3,4]. Xiong et al. [5] have reported that GPR41 is expressed in mouse epididymal adipose tissue and, at a low level, in differentiated Ob-Luc cells, a mouse adipocyte cell line [6]. Propionate and butyrate stimulated leptin secretion from both minced mouse adipose tissue and differentiated Ob-Luc cells. The effect of propionate was enhanced in the presence of adenosine deaminase (ADA), which was interpreted to be due to removal of the desensitising effect of adenosine, acting through the A1 receptor, on the $G\alpha_i$-mediated response. An alternative interpretation is that the "window" available for $G\alpha_i$-mediated effects was increased in the absence of adenosine. Acetate was less potent or less effective than propionate or butyrate, consistent with the response being mediated by GPR41 rather than GPR43. Moreover, small interfering RNA (siRNA) that targeted GPR41 almost totally prevented the response to propionic acid in Ob-Luc cells [5].

These findings raise the possibility that circulating short chain fatty acids (SCFA) produced in the large intestine by bacterial fermentation of undigested carbohydrates might raise plasma leptin. Indeed, 7 h after administration of sodium propionate, plasma leptin levels were raised in mice [5]. It is premature to conclude that GPR41 mediates the effect of SCFA on leptin secretion, however. First, the Ob-Luc cells in which siRNA that targeted GPR41 blocked the effect of propionate had been transfected with GPR41 mRNA, making them more sensitive to propionate [5]. Secondly, propionate and butyrate but not acetate inhibit histone deacetylase [7,8]. EC_{50} values for these effects are higher than concentrations that stimulate leptin secretion in adipocytes, but changes in gene expression as a result of histone deacylation might explain the results of Xiong et al. [5] because they measured leptin secretion over 14 or 24 h. Thirdly, Hong et al. [9] failed to detect GPR41 mRNA in four murine adipose tissue sites. GPR43 mRNA, by contrast, was detected at all sites. They found that propionate inhibited isoproterenol-stimulated lipolysis in confluent 3T3-L1 adipocytes and that this effect was prevented by GPR43 siRNA. Others have reported that acetate and propionate inhibit basal lipolysis in epididymal adipocytes from wild-type but not GPR43 knockout mice [10].

Abbreviations: ADA, adenosine deaminase; FFA, free fatty acid; KRH, Krebs–Ringer–HEPES; QPCR, quantitative polymerise chain reaction; SCFA, short chain fatty acids; siRNA, small interfering RNA

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To investigate further the role of GPR41 in the stimulation of leptin secretion by SCFA, we have compared responses in epididymal and mesenteric adipocytes from wild-type and GPR41 knockout mice. To help interpret these findings, we have studied the expression of GPR41 and GPR43. We find that GPR41 is not expressed in murine adipose tissue; nevertheless, leptin secretion in the presence of ADA in response to SCFA is reduced in adipocytes from GPR41 knockout mice. We argue that this is due to downregulation of GPR43 expression. The stimulation of leptin secretion was prevented by pertussis toxin (PTX), consistent with GPR43 signalling via $G\alpha_i$.

2. Methods

2.1. Materials

Reagents were obtained from Sigma–Aldrich, Poole, UK, unless otherwise stated. Fatty acids were used as their sodium salts.

2.2. Generation and genotyping of GPR41 knockout mice

GPR41 knockout mice were produced by Deltagen (San Mateo, CA). The mouse GPR41 targeting vector was generated by deleting the wild-type genomic sequence and replacing it with a LacZ-Neo cassette that lacked 216 bp of the mouse GPR41 coding region (Fig. 1A). GPR41 knockout mice on a mixed C57BL/6/129 background were generated by homologous recombination in embryonic stem cells derived from the 129/OlaHsd mice to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females, and F2 homozygous mutant mice by crossing F1 heterozygous males and females. Pups were screened by PCR of genomic DNA as shown in Fig. 1B. The primers are described in Supplementary Table S1. GPR41 knockout mice were backcrossed to the C57BL/6 strain for five generations at Deltagen before heterozygotes were received by AstraZeneca (Alderley Park, Maccslefield, UK).

2.3. Animal housing and breeding at the University of Buckingham

Heterozygote mice were sent from AstraZeneca to the University of Buckingham at 10–12 weeks of age where they were bred over a number of generations, backcrossing four more times to the C57BL/6 strain (Harlan, Bicester, UK; nine backcrosses in all) to generate all three genotypes. Heterozygote mice were used for each round of breeding to obtain the experimental mice.

Mice were fed on chow (number 1) diet from Bantin and Kingman (Hull, UK) and maintained at 21° ± 1° C on a 12-h light/dark cycle. Male animals were killed at 12–18 weeks of age and 3–4 h after the beginning of the light period.

Housing and procedures were conducted in accordance with the UK Government Animal (Scientific procedures) Act 1986 and approved by the University of Buckingham Ethical review Board.

2.4. RNA isolation and real-time quantitative PCR (QPCR) analysis of gene expression

Adipose tissue was stored in RNALater^R (Ambion Inc., TX). Total RNA was isolated using Tri-Reagent followed by Rneasy RNA extraction columns (Qiagen, UK). RNA integrity and quantification was analysed using a NanoDrop ND1000 (Labtech, UK). Samples were treated with ribonuclease-free deoxyribonuclease I (Turbo DNA-free assay, Ambion, UK) to remove genomic DNA. First-strand cDNAs were synthesised using Applied Biosystems reverse transcription kit in the presence of random hexamers, 0.5 mM dNTPs, RNAse inhibitors (40 U) and MultiScribe™ Reverse Transcriptase (125 U). cDNA samples were stored at −20 °C in 96-well plates.

QPCR was performed using Assay on Demand pre-designed primer and probe sets from Applied Biosystems (Supplementary Table S2). Transcript levels for GPR41 and GPR43 were quantified in triplicate by real-time RT-PCR (ABI Prism 7900HT SDS, Applied Biosystems), using ABSOLUTE QPCR ROX PCR Mastermix (Thermo Fisher Scientific). The dye detector was set for FAM-labelled probes. Cycle threshold (Ct) values were determined using SDS 2.3 software. The Ct values for the endogenous control genes were used to generate a normalisation factor for each sample using the geNorm software (Primer Design Ltd.). The expression levels of GPR41 and GPR43 were calculated using the standard curve method.

2.5. Adipocyte isolation and incubation

Fat pads from rats and mice were cut into 2–3 mm diameter pieces. Tissue from each of at least 10 mice was divided between flasks for digestion. Adipocytes were isolated by the method of Rodbell as modified by Lincova et al. [11]. Approximately 2 g of adipose tissue was digested at 37 °C by shaking (250 cycles/min) with 4 mg of collagenase type II in 6 ml of Krebs–Ringer–HEPES (KRH) buffer (pH 7.4), containing 1% BSA, 2.5 mM $CaCl_2$, 5.5 mM glucose and 200 nM adenosine. It was filtered through 250–300 μm nylon mesh and allowed to stand for 2–3 min. The infranatant was removed and the floating layer of adipocytes was washed four times with a fresh KRH buffer. Adipocytes were concentrated to 40% of final volume of KRH buffer containing 5% BSA and 5.5 mM of glucose, and pre-incubated for 45 min in 95:5% O_2:CO_2 atmo-

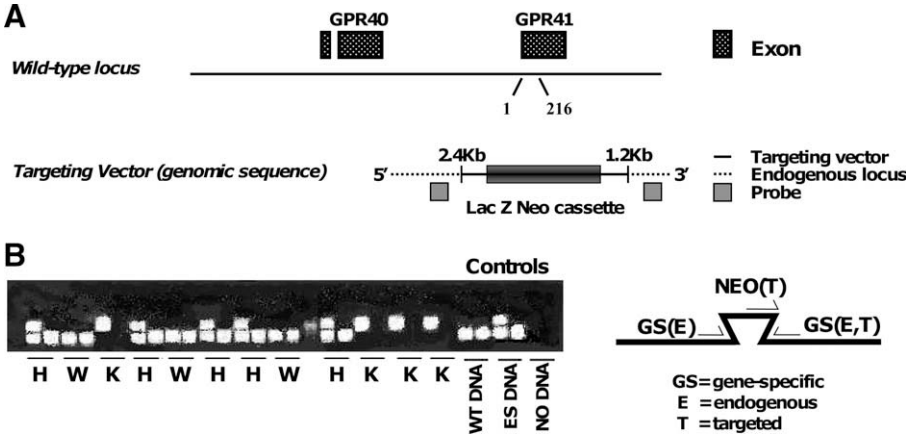

Fig. 1. Generation of GPR41 KO mice: structure of the targeting vector (A) and example of PCR analysis of tail tip genomic DNA from wild-type (W), heterozygote (H) and knockout (K) mice using (left of each pair) a multiplex PCR with endogenous (E) and vector targeted (T, ET) primers (B) and (right of each pair) endogenous primers alone. The primers for genotyping are described in Supplementary Table S1.

sphere. For the main study on the effect of PTX (Calbiochem, supplied by Merck Biosciences, Nottingham, UK), the preincubation time was 3 h in the absence or presence of 1000 ng/ml PTX. The cells were then washed so that leptin secretion could be measured in the fourth hour in the continuing absence or presence of PTX, and in the absence or presence of SCFA.

One hundred microliters of cell homogenate was added to 900 μl of KRH buffer containing 5% BSA and 5.5 mM glucose, with or without sodium salts of SCFA and 10 units/ml of ADA. After 1 h, 40 μl of medium was taken for the measurement or leptin in duplicate using mouse or rat leptin ELISA kits (Crystal Chem Inc., Downers Grove, USA) or glycerol in quadruplicate using a colorimetric glycerol assay kit (GY 105) from Randox laboratories Ltd. (Ardmore, Antrim, UK).

2.6. Statistical analysis

Statistical analysis was by one- or two-way ANOVA, except that a Mann–Whitney *U*-test was conducted for GPR43 expression in omental adipose tissue. With the exception of the studies using PTX, n values for leptin and glycerol secretion do not refer to replicate incubations from one preparation (or to separate animals) but to separate preparations of adipocytes from all the animals of one genotype. All results are presented as means ± S.E.M.

3. Results

3.1. Gene expression

GPR41 mRNA was readily detected in the murine insulin secreting cell line MIN6, but it could not be detected in various adipose tissue sites from two generations of wild-type littermates of GPR41 knockout mice (Fig. 2A and data not shown). GPR43 mRNA was found in various adipose tissue sites at higher levels than GPR41 in MIN6 cells, assuming that the efficiency of the primer and probe sets for the two genes was similar. The highest and lowest levels of GPR43 mRNA were in mesenteric white and interscapular brown adipose tissue, respectively (Fig. 2B). A 2-way ANOVA (tissue; genotype) showed significantly lower expression of GPR43 in white adipose tissue sites from the GPR41 knockout compared to control mice ($P < 0.01$), but Bonferroni post-tests did not identify a significant effect at any one site. The closest values to $P = 0.05$ were for the omental ($P = 0.076$) and mesenteric ($P = 0.096$) sites. A Mann–Whitney *U*-test was conducted on GPR43 expression for omental adipose tissue because the variances for the GPR41 knockout and wild-type mice were significantly different. This gave $P = 0.016$.

No expression of GPR41 could be detected in three batches of differentiated 3T3-L1 adipocytes (data not shown) in agreement with some [9] but not other [1] workers.

3.2. Leptin secretion

In a preliminary experiment conducted using rat epididymal adipocytes, 3 mM propionate markedly stimulated leptin secretion when ADA was present, but it had no significant effect in its absence (Supplementary Fig. S1). Therefore, ADA was included in future experiments.

Propionate at a concentration of 3 mM and 0.2 mM butyrate stimulated leptin secretion by epididymal adipocytes from wild-type littermates of GPR41 knockout mice. Propionate at a concentration of 0.2 mM and 0.2 mM acetate did not stimulate leptin secretion significantly. Baseline secretion was similar in adipocytes from wild-type and GPR41 knockout mice. However, leptin secretion in the presence of all three SCFA at 0.2 mM was significantly lower in adipocytes from the knockout compared to the wild-type

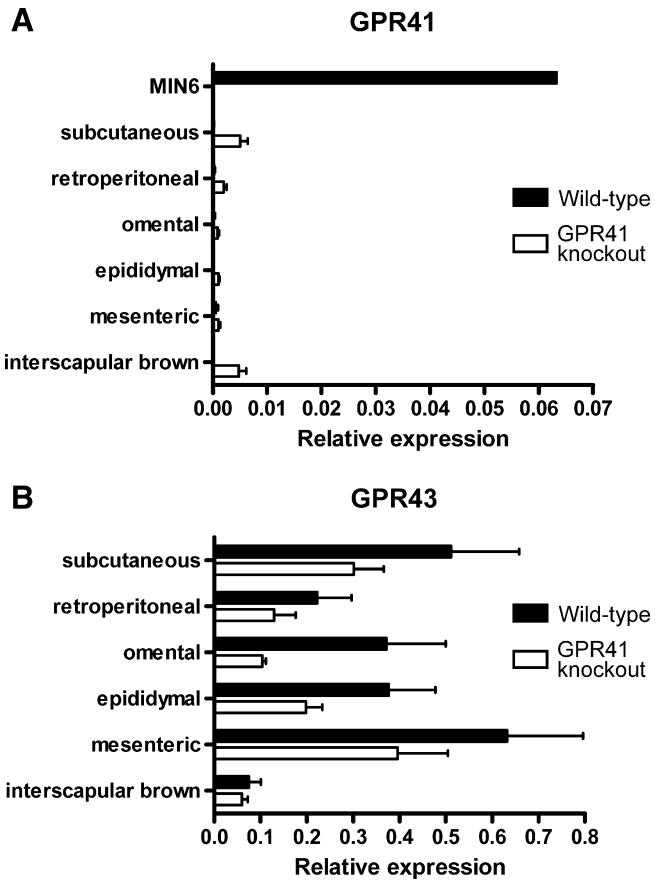

Fig. 2. Relative expression of (A) GPR41 and (B) GPR43 in adipose tissue sites from wild-type and GPR41 knockout mice. Note the differing scales for GPR41 and GPR43. Results are means of five values. Statistical analysis of GPR43 expression is described in the text. Values for GPR41 mRNA expression in adipose tissue are no higher than the background values for GPR41 knockout mice. Expression of GPR41 in MIN6 cells is shown to demonstrate the validity of the assay.

mice. The differences in mean leptin secretion between wildtype and knockout mice in the presence of 0.2 mM acetate, propionate or butyrate were 0.75, 0.66 and 0.53 ng/h/g adipocytes, respectively, compared to 0.02 ng/h/g adipocytes in controls. Acetate reduced leptin secretion by adipocytes from the knockout mice below the baseline level (Fig. 3A).

Further experiments were conducted using mesenteric adipocytes. Acetate (0.1 and 0.2 mM) increased leptin secretion by mesenteric adipocytes from wild-type littermates of GPR41 knockout mice but not by adipocytes from knockout mice. Butyrate (0.2 mM) had no effect in adipocytes from either wild-type or knockout mice (Fig. 3B).

3.3. Lipolysis

Acetate and butyrate (0.2 mM) inhibited ADA-stimulated lipolysis in mouse epididymal adipocytes but they had no effect in the absence of ADA (Fig. 4). Propionate (3 mM) did not inhibit lipolysis in rat adipocytes in the presence or absence of ADA, or in the presence of the β_3-adrenoceptor agonist BRL37344 (30 nM; Supplementary Fig. S2). Others have mentioned that SCFA have no effect on isoprenaline-stimulated lipolysis in rat adipocytes [1].

3.4. Pertussis toxin

Preliminary experiments were conducted on lipolysis in murine epididymal adipocytes in the absence of ADA using 1, 3, 10

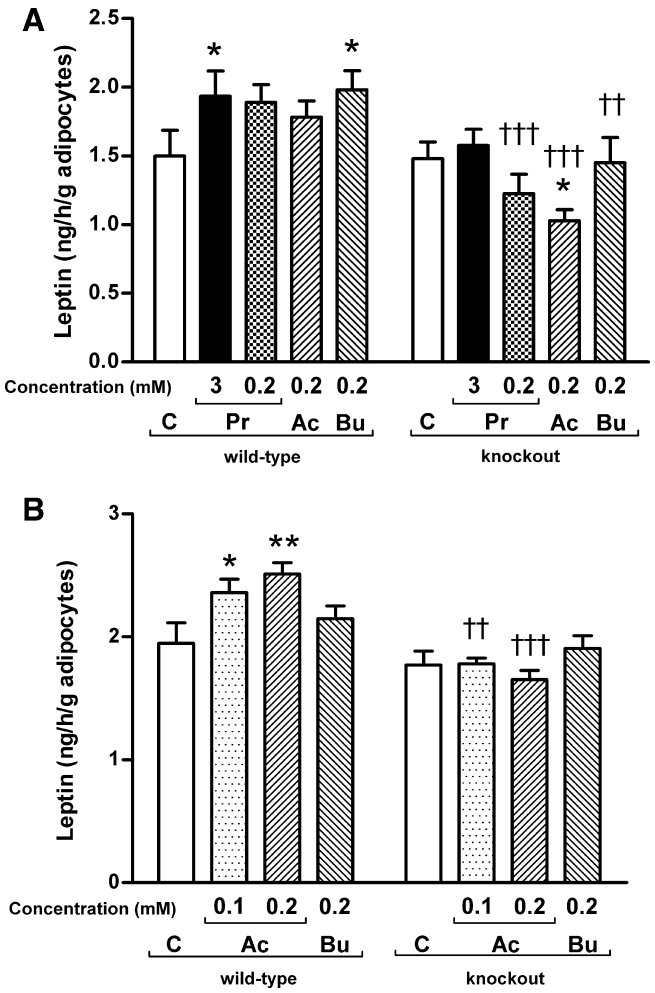

Fig. 3. Effects of propionate (Pr), acetate (Ac) and butyrate (Bu) on leptin secretion by (A) epididymal and (B) mesenteric adipocytes from wild-type and GPR41 knockout mice. Results are means of values for 5 or 6 preparations of adipocytes. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. corresponding control (C). $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$ vs. corresponding wild-type value by one-way ANOVA and Fisher's least significant difference test.

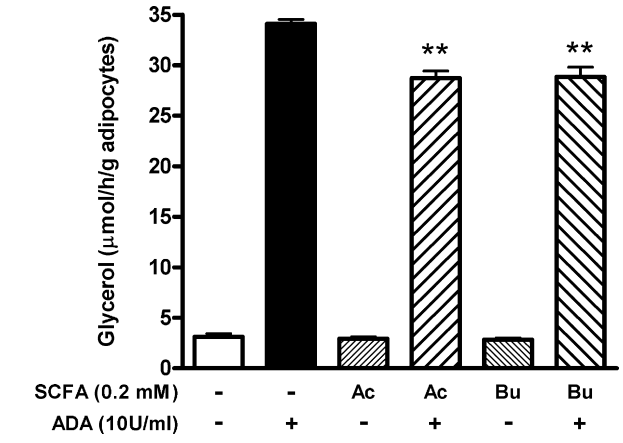

Fig. 4. Effects of acetate (Ac), butyrate (Bu) and propionate on glycerol secretion by epididymal adipocytes from wild-type mice. Results are means for 3 preparations of adipocytes. $^{**}P < 0.01$ vs. glycerol release in the presence of ADA alone.

and100 ng/ml PTX and 3 h incubation, and 200, 500 and 1000 ng/ml PTX and 2, 4 or 6 h incubation. Incubation with 1000 ng/ml PTX for 4 h was found to elicit the most stimulation of lipolysis (3.1-fold; $P < 0.0001$). 1–100 ng/ml PTX had no effect and only 1000 ng/ml PTX had any effect after the 2 h incubation (Supplementary Fig. S3). In the main experiment, 1000 ng/ml PTX reduced leptin secretion by epididymal adipocytes during the three hours of pre-incubation (Fig. 5A) but not during the fourth hour (Fig. 5B). PTX prevented the stimulatory effect of propionate (3 mM; present in the fourth hour only) on leptin secretion (Fig. 5B). PTX also prevented the inhibitory effect of propionate on lipolysis (data not shown).

4. Discussion

In common with Hong et al. [9], we have been unable to confirm the report of Xiong et al. [5] that GPR41 mRNA can be detected in mouse epididymal adipose tissue; nor could we detect it in other adipose sites. In agreement with Hong et al., we found high expression of GPR43 in adipose tissue.

Nevertheless, leptin secretion by epididymal adipocytes in the presence of 0.2 mM SCFA was lower in knockout than in wild-type

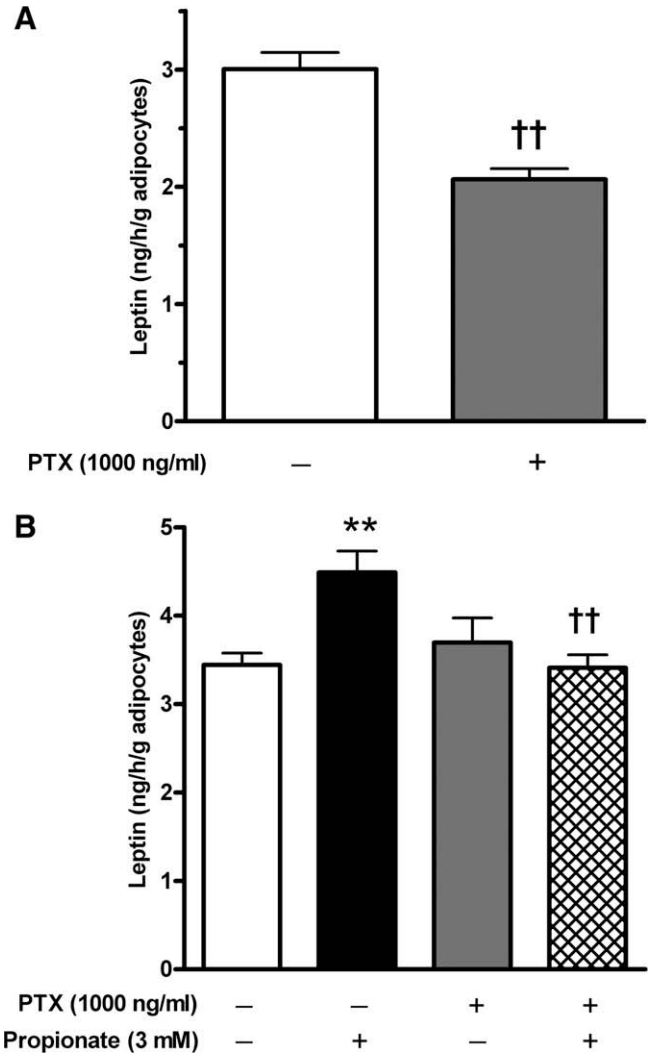

Fig. 5. Effect of PTX (1000 ng/ml) on leptin secretion by epididymal adipocytes. Pre-incubation with PTX for 3 h in the absence of ADA (A). Leptin secretion (B) in the fourth hour in response to propionate (3 mM) in the presence of ADA and in the continuing absence or presence of PTX. Results are means of 5 or 6 incubations. $^{\dagger\dagger}P < 0.01$ for effect of PTX compared to control with the same propionate treatment. $^{**}P < 0.01$ for effect of propionate compared to control with the same PTX treatment.

mice. Acetate did not stimulate leptin secretion by wild-type adipocytes but it inhibited leptin secretion by epididymal adipocytes from the knockout mice. We suggest that acetate has an inhibitory effect on leptin secretion in epididymal adipocytes that is mediated by an unknown mechanism. It is unlikely that this is due to inhibition of histone deacetylase, because acetate at a concentration of 0.2 mM does not inhibit histone deacetylase [8,12]. Moreover, the incubation period of one hour is too short to detect effects mediated by inhibition of histone deacetylase [7].

We do not believe that GPR41 mediates leptin secretion in epididymal adipocytes. The reversal of the response to acetate in GPR41 knockout mice may be due to reduced expression of GPR43 in the GPR41 knockout revealing the inhibitory effect of acetate. Like Xiong et al. [5] we found less response to acetate than to butyrate or propionate in epididymal adipocytes from wild-type mice. Acetate is tenfold less potent than butyrate as a stimulant of both Ca^{2+} elevation and GTPγS binding mediated by human or rodent GPR41, whereas it is two- to threefold more potent than butyrate as a stimulant of Ca^{2+} elevation mediated by human GPR43 [1,3]. Therefore, Xiong et al. interpreted the lower response to acetate as being consistent with the pharmacology of GPR41. However, they could not take into account our finding that acetate inhibits leptin release by epididymal adipocytes from GPR41 knockout mice.

Our experiments on mesenteric adipocytes provide further support for a possible role of GPR43 rather than GPR41, because 0.2 mM butyrate did not have a statistically significant effect and appeared less effective than 0.2 mM acetate in stimulating leptin secretion from mesenteric adipocytes from wild-type mice. The absence of an effect of acetate on leptin secretion in GPR41 knockout mice is therefore likely to be due to downregulation of the expression of GPR43 rather than to absence of GPR41. The ability of acetate to elicit a suppressive effect on leptin secretion in epididymal but not in mesenteric adipocytes may be related to the relative levels of GPR43 in epididymal and mesenteric adipocytes in GPR41 knockout mice.

GPR43 signals via both $G\alpha_i$ and $G\alpha_q$. Our finding that pre-incubation with PTX prevented stimulation of leptin secretion by propionate supports the involvement of $G\alpha_i$ and is consistent with reports that leptin secretion is stimulated by other activators of $G\alpha_i$, and is inhibited by activators of $G\alpha_s$ [13,14]. Xiong et al. [5] reported previously that PTX prevented propionate-stimulated leptin secretion by mouse epididymal adipocytes, but they used only 1 or 3 ng/ml PTX and they did not report the pre-incubation time. Although such low concentrations have been used by others [15], much higher concentrations, up to 1000 ng/ml, are more commonly used [16], and in our preliminary studies we found that at least 200 ng/ml was required to stimulate lipolysis.

Although not the main focus of this work, we found that acetate and butyrate had no effect on basal lipolysis in mouse adipocytes, but they inhibited ADA-stimulated lipolysis. This is also consistent with SCFA activating $G\alpha_i$ via GPR43, because adenosine activates $G\alpha_i$ via the A1 receptor in adipocytes and, its absence would make a greater "window" available to other activators of $G\alpha_i$. By contrast with our findings, others have reported that SCFA inhibit basal as well as isoprenaline-stimulated lipolysis in mouse adipocytes [10].

The relevance of our findings to the regulation of energy balance in mice is unclear. GPR41 knockout mice are reported to be leaner than their wild-type littermates, provided they are not germ-free so that formation of SCFA in the lower gut is prevented. The leanness of the knockout animals was linked to a lower plasma concentration of peptide YY, increased intestinal transit rate and reduced absorption of SCFA [17]. By contrast, our results might lead to the prediction that GPR41 knockout mice would have decreased plasma leptin levels owing to reduced expression of GPR43 in adipose tissue and reduced stimulation of leptin secretion by plasma SCFA (or, in the case of epididymal adipocytes, inhibition of leptin secretion by acetate). Indeed, our unpublished results (M Bellhacene et al.) show that our male GPR41 knockout mice are obese, rather than lean, compared to wild-type mice.

Regarding humans, inhibition of lipolysis might explain why infusion of acetate or propionate prevented a fasting-induced increase in plasma non-esterified fatty acid concentration [18]. The increase in the plasma acetate concentration (propionate was not measured) from 80 to 157 μM was more consistent with it suppressing lipolysis by activation of GPR43 rather than GPR41. EC50 values in the range of 35–431 μM have been reported for activation of the human cloned GPR43 by acetate, whilst values in the range 1020–1070 μM have been reported for activation of human cloned GPR41 by acetate [1,3]. However, the relevance of our findings to humans is complicated by there being disagreement as to whether GPR41 and GPR43 are expressed in human adipose tissue [1,3,5,9].

In conclusion, certain SCFA stimulate leptin secretion in wild-type murine adipocytes incubated in the presence of ADA to remove adenosine. Their relative effects appear to vary according to the source of the adipocytes. The effect is mediated by activation of $G\alpha_i$. It is absent in adipocytes from GPR41 knockout mice. However, the very low, or undetectable level of expression of GPR41 in adipocytes compared with clearly detectable expression of GPR43 suggests that GPR43, rather than GPR41, mediates the leptin response. Downregulation of GPR43 appears responsible for reduced secretion of leptin in the presence of SCFA in GPR41 knockout mice.

The relative roles of GPR43 and GPR41 in adipocytes have been deduced mainly from the potencies of SCFA for the receptors, and studies using molecular biology tools or transgenic mice. The identification of selective, small-molecule tool compounds for GPR43 and GPR41 will greatly enhance our understanding of their roles in adipose tissue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.027.

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