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Brief Communication

MOLECULAR METABOLISM

Autocrine negative feedback regulation of lipolysis through sensing of NEFAs by FFAR4/GPR120 in WAT

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ABSTRACT

Objectives: Long-chain fatty acids (LCFAs) released from adipocytes inhibit lipolysis through an unclear mechanism. We hypothesized that the LCFA receptor, FFAR4 (GPR120), which is highly expressed in adipocytes, may be involved in this feedback regulation.

Methods and results: Liquid chromatography mass spectrometry (LC-MS) analysis of conditioned media from isoproterenol-stimulated primary cultures of murine and human adipocytes demonstrated that most of the released non-esterified free fatty acids (NEFAs) are known agonists for FFAR4. In agreement with this, conditioned medium from isoproterenol-treated adipocytes stimulated signaling strongly in *FFAR4* transfected COS-7 cells as opposed to non-transfected control cells. In transfected 3T3-L1 cells, FFAR4 agonism stimulated Gi- and Go-mini G protein binding more strongly than Gq, effects which were blocked by the selective FFAR4 antagonist AH7614. In primary cultures of murine white adipocytes, the synthetic, selective FFAR4 agonist CpdA inhibited isoproterenol-induced intracellular cAMP accumulation in a manner similar to the antilipolytic control agent nicotinic acid acting through another receptor, HCAR2. *In vivo*, oral gavage with the synthetic, specific FFAR4 agonist CpdB decreased the level of circulating NEFAs in fasting lean mice to a similar degree as nicotinic acid. In agreement with the identified anti-lipolytic effect of FFAR4, plasma NEFAs and glycerol were increased in FFAR4-deficient mice as compared to littermate controls despite having elevated insulin levels, and cAMP accumulation in primary adipocyte cultures was augmented by treatment with the FFAR4 antagonist conceivably by blocking the stimulatory tone of endogenous NEFAs on FFAR4.

Conclusions: In white adipocytes, FFAR4 functions as an NEFA-activated, autocrine, negative feedback regulator of lipolysis by decreasing cAMP though Gi-mediated signaling.

Keywords GPR120; FFAR4; Autocrine; Lipolysis; NEFA; GPCR

1. INTRODUCTION

For more than 40 years, nonesterified free fatty acids (NEFAs) have been known to inhibit lipolysis, but the mechanism behind this inhibition has remained unclear [1–3]. Initially, adenosine had been described to function as an autocrine inhibitory regulator of lipolysis [4]. However, in 1975, Fain and Shepherd reported that in dialyzed, conditioned medium from isoproterenol-stimulated adipocytes, an adenosine deaminase-resistant agent efficiently inhibited cAMP production in adipocytes, and they identified this to be free fatty acids as exemplified by oleic acid [1]. It was concluded that the prolonged drop in cAMP accumulation observed in adipocytes following treatment with lipolytic agents was a result of liberated free fatty acids exceeding the binding capacity of albumin in the medium and that fatty acids apparently function as physiological feedback regulators of lipolysis [1,2]. In 2012, by using chemically modified fatty acids, Kalderon et al. demonstrated that the antilipolytic effect of NEFAs was independent of their classical functions as metabolites, i.e., β-oxidation and re-esterification [3]. Because key metabolites can act as extracellular signaling molecules through selective G-protein coupled receptors

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(GPCRs) [5], the antilipolytic and cAMP-lowering effect of NEFAs in adipocytes is most likely mediated through a long-chain fatty acid (LCFA) receptor.

LCFAs are sensed by two GPCRs, FFAR1 (GPR40) and FFAR4 (GPR120) [6,7]. Of these, FFAR1 is highly expressed in pancreatic and enteroendocrine cells [8,9], where it stimulates hormone secretion in response to dietary, triglyceride-derived LCFAs [10,11]. In the gastrointestinal (GI) tract, this occurs during the absorption process, whereas in the pancreatic islet, FFAR1 most likely senses LCFAs that are liberated locally from postprandial chylomicrons by lipoprotein lipase [5,12]. FFAR1 is a Gq-coupled GPCR co-expressed with and acting in synergy with the Gs-coupled GPR119 receptor, which is a sensor of the other main triglyceride metabolite, 2-monoacyl glycerol [10,13]. Recently, FFAR1 was shown to be responsible for a major part of the glucose-induced insulin secretion through its function as an autocrine sensor of 20-HETE, an arachidonic acid metabolite produced in β-cells in response to glucose [14,15]. However, FFAR1 is very poorly expressed in adipocytes and therefore likely not involved in the autocrine sensing of NEFAs in adipocytes.

In contrast to FFAR1, FFAR4 is highly expressed in white and brown adipose tissue (WAT and BAT) [16,17]. In WAT, FFAR4 has been reported to be either upregulated or downregulated in response to high-fat diet in mice and in obese patients [18–21]. However, in BAT, FFAR4 expression is strongly upregulated upon cold exposure in mice [3,16,22]. Although FFAR4 recognizes a broad spectrum of LCFAs, the receptor is somewhat more sensitive to unsaturated fatty acids and has accordingly been advocated to be responsible for the anti-inflammatory and insulin-sensitizing effect of Ω-3 fatty acids [23]. However, it remains unclear whether the beneficial metabolic effects of Ω-3 fatty acids are in fact mediated through FFAR4, as normal effects of Ω-3 fatty acids have been reported in FFAR4-deficient mice [24,25]. Nevertheless, chronic treatment with an FFAR4 agonist has been shown to improve insulin resistance and chronic inflammation in obese mice [17,23].

Previously, we proposed that FFAR4, which is generally believed to be a sensor of dietary unsaturated fatty acids may function as an autocrine sensor of NEFAs released from adipocytes and thereby act as a feedback inhibitory brake on lipolysis [5]. This hypothesis was experimentally tested in the present study.

2. EXPERIMENTAL PROCEDURES/MATERIAL AND METHODS

2.1. Compounds

Nicotinic acid and isoproterenol were purchased from Sigma–Aldrich (Steinheim, Germany). The FFAR4-selective agonists compound A (CpdA) [23] and compound B (CpdB) [21] were provided by Merck (NJ, USA), and AZ13581837 (AZ) [26] was synthesized by Chempartner (Shanghai, China). The FFAR4-selective antagonist AH7614 [27] was synthesized according to [28]. Chemical structures of each of the FFAR4 ligands are shown in Fig. S1.

2.2. Mice

The C57BL/6J mice were purchased from Janvier Labs (France) at 8 weeks of age and either placed on a standard chow diet (Brogaarden, Denmark) or a high-fat diet with a 60% fat content (Research Diets) for 24 weeks. The mice were group-housed with up to 8 mice per cage at 24 °C on a 12:12 h light–dark cycle. The mice had free access to food and water. The FFAR4KO and WT control mice were kindly provided by Andy Howard, Merck (NJ, USA) and used for the experiment at 12 weeks of age. These mice were placed on a standard chow diet and had free access to food and water. The studies were conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture, and Fisheries. The mice were handled in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.3. Primary adipocyte cultures

Primary murine adipocytes were purified from epididymal fat pads from lean C57BL6 mice. Samples of human adipocytes were obtained during bariatric surgery of patients participating in the BARIA study [29]. The study was performed in accordance with the Declaration of Helsinki and was approved by the Academic Medical Center Ethics Committee of the Amsterdam UMC. All participants provided written informed consent. All individuals met the criteria for bariatric surgery by the International Federation of Surgery for Obesity (IFSO) [30]. Three adipose tissue compartments were analyzed in the present study, and biopsies were obtained as follows: subcutaneous adipose tissue from one of the laparoscopic incisions in the upper abdomen, mesenteric adipose tissue from the appendices of the transverse colon, and omental adipose tissue from the greater omental tissue as previously described. Both the murine and human adipose tissues were washed in phosphate-buffered saline (PBS), minced thoroughly into small pieces, and transferred into a 50-ml tube with HEPES Krebs ringer buffer (4% bovine serum albumin (BSA)) containing collagenase placed in a water bath at 37 °C with constant shaking for 1 h. The digested cells were filtered and then allowed to stand for 5 min. The infranatant was then carefully removed using a syringe with a long needle. Then, the floating layer of adipocytes was washed 3 times with 10 ml of HEPES Krebs ringer buffer (2% BSA). The adipocytes were resuspended in HEPES Krebs ringer buffer (2% BSA) supplemented with glucose, phenylisopropyl adenosine (Sigma–Aldrich), and adenosine (Roche) and then plated in non-coated transparent 96-well plates and stimulated with isoproterenol (1 μM) or vehicle for 30 min at 37 °C. NEFA content in the conditioned media was measured by a NEFA kit from WAKO (WAKO Chemicals, Germany), and the content of LCFA was determined by targeted liquid chromatography mass spectrometry (LC-MS) analysis.

2.4. Targeted LC-MS analysis

Long-chain fatty acids were compared in the samples using the semi-targeted metabolic profiling method previously described for plasma samples (method II in [31]) with minor modifications; briefly, protein in the samples was precipitated with 80% methanol:acetonitrile (50:50, both chromatography grade from Fisher Chemicals, Leicestershire, United Kingdom) and filtered on a 96-well Sirocco plate (Waters, Taastrup, Denmark), and the filtrate was evaporated to dryness. The samples were then redissolved in 96% ethanol, and 5 μL was injected into a binary ultra-high-performance liquid chromatography (UHPLC) system (Waters Acquity™) equipped with an Acquity UPLC HSS T3 1.8 mm 2.1 × 100 mm column and eluted with a gradient going from 0.4 mL/min of 0.1% formic acid in water (A) to 1.2 mL/min of 100% methanol (B) in 6.5 min and back to starting conditions for re-equilibration from 7 min (the gradient is provided in the text for Table S1). The detector was a QTof Premier (Waters) operating with a capillary probe voltage at 2.8 kV in negative ionization mode (scan time 0.08 s; interscan delay 0.02 s; ion source temperature 120 °C; desolvation gas temperature 400 °C; cone voltage 30 kV; and cone and desolvation gas flows 50 and 1,000 L/h, respectively). Leucine enkephalin was infused every 10 s for 0.1 s as a lock-mass solution for continuous mass calibration. Centroid data was generated in real time and collected for masses ranging between 100 and 500 Da during all

runs. The RT and *m/z* of all fatty acid analytes are shown in Table S1, as determined by external standards (0.5—50 ug/mL in 96% ethanol). All samples were analyzed in the same batch together with assay blanks (i.e., water processed as samples) and external standards to assure no time drift or interfering background peaks during the analysis. The resulting raw data were pre-processed using Markerlynx software (Waters), and the extracted areas for each of the targeted fatty acid peaks in all samples were used for statistical analysis. Assay blanks contained no measurable fatty acid.

2.5. IP turnover assay

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transiently transfected with pCMV-Tag (2B) (Stratagene, Ca, USA) with or without FFAR4 cDNA (short form) cloned into the vector in 96-well plates (20,000 cells per well) using the calcium phosphate precipitation method with chloroquine. Following transfection, the cells were incubated for 48 h with 0.5 μCi/ml myo [^{3}H]inositol (Perkin Elmer). After 48 h, cells were washed with Hanks Bovine Salt Solution (HBSS, Gibco, Life Technologies) and incubated for 2 h at 37 °C in 100 μl of HBSS supplemented with 10 mM of LiCl together with either conditioned media from the adipocytes, CpdA, CpdB, AZ compound, or vehicle control media. The cells were lysed with 40 μl of cold 10 mM formic acid on ice for 20 min. The lysates (30 μl) were transferred to a white 96-well plate together with 60 μl of 1:8 diluted YSi poly-D-lysine-coated beads (Perkin Elmer). After 10 min of vigorously shaking and spinning (1,500 rpm, 5 min), the plate radioactivity was counted on a top counter after 4 h to reach optimal measuring. Determinations were made in duplicates.

2.6. Mini Gα signaling in 3T3-L1 undifferentiated adipocytes

3T3-L1 cells were seeded in 96-well plates at a density of 9,000 cells/well. After 24 h, the cells were transiently transfected with FFAR4-NanoLuc and Venus = tagged mini G protein probe isoform in a 1:10 ratio, respectively, using TransfeX™ (ATCC) transfection reagent as per the manufacturer's protocol. Mini G protein probes are modified Gα subunits that recruit to active receptors in living cells. Here, they were used to assess both the receptor activation and coupling specificity of the FFAR4 [32]. On the day of imaging, approximately 48 h post-transfection, the FFAR4 antagonist AH7614 was applied to the cells for 15 min at 10 μM. The cell medium was subsequently replaced with HBSS containing furimazine/Nano-Glo® Luciferase Assay Substrate (Promega) at a final concentration of 10 μM of +/− antagonist. BRET measurements were then done at 37 °C using a PHERAstar® plate reader at dual emission wavelengths using a BRET1 430—485 nm and 505—590 nm dual filter optic module. Following four baseline reads, agonist or vehicle was administered in triplicate for each condition, and the BRET signal was measured every 2 min for 1 h. The BRET ratio, acceptor 505—590 nm emission over 430—485 nm donor emission, was calculated, and the baseline vehicle read was subtracted from the agonist-stimulated read. The area under the curve (AUC) was also calculated for ease of comparison.

2.7. In vivo lipolysis

Lean (30 ± 1.8 g) and DIO (47.8 ± 2 g) C57BL6/J mice were fasted for 17 h prior to the experiment. The mice were orally gavaged with either 30 mg/kg of CpdB, 30 mg/kg of nicotinic acid or vehicle control all dissolved in 0.5% (w/v) carboxymethylcellulose sodium salt with medium viscosity (Sigma—Aldrich). Retroorbital blood (100 μl) was collected from the mice prior to the administration and 30 min after administration. NEFA was measured by a WAKO NEFA kit (WAKO Chemicals, Germany), and glycerol was measured by a Sigma—Aldrich total glycerol kit according to the protocols of the manufacturers.

2.8. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted and purified from epididymal adipose tissue isolated from lean or diet-induced obese (DIO) C57Bl6/J mice. Reverse transcription PCR (RT-PCR) was performed using SuperScript III Reverse Transcriptase (Invitrogen). Custom designed qPCR array plates from Qiagen containing primers for FFAR4. CT values for the target genes were normalized to the CT value of the ywhaz gene.

2.9. FFAR4KO mice studies

FFAR4KO and littermate control mice were fasted for 6 h, and an oral glucose tolerance test (OGTT) was performed with 2 g/kg of glucose administered by oral gavage and glucose levels measured in tail blood at the different time points. Retro-orbital blood (200 μl) was collected after the mice were fasted for 6 h and plasma was analyzed for insulin content by MSD insulin plates (Meso Scale Discovery, USA), glycerol was measured by a Sigma—Aldrich total glycerol kit, and NEFA by the WAKO NEFA kit (WAKO Chemicals, Germany) according to protocols from the manufacturers.

2.10. Calculation and statistical analysis

Data were analyzed using Prism 7 software (GraphPad Software, San Diego) using paired t-test, Student's t-test, Tukey's multiple comparison test, and two-way analysis of variance (ANOVA) as indicated in the legend to each of the figures. Statistical significance is denoted by * - ($p < 0.05$), ** - ($p < 0.01$), *** - ($p < 0.001$), and **** - ($p < 0.0001$).

3. RESULTS

3.1. Endogenous ligands for FFAR4 are released in large amounts from adipocytes

Conditioned media were collected from primary cultures of murine and human adipocytes 30 min after induction of lipolysis by isoproterenol or vehicle control. Concentrations of NEFAs were increased to 600 μM in conditioned media from isoproterenol-stimulated adipocytes isolated from epididymal fat depots of lean mice as compared to 100 μM in media from vehicle-treated adipocytes (Figure 1A). LC-MS analysis of the conditioned media demonstrated a strong increase in several types of NEFAs, which from the literature are known to be ligands for FFAR4, with linoleic acid, oleic acid, and palmitic acid as the main species (Figure 1B—E and Table S2) [6]. Adipocytes isolated from subcutaneous, omental, and mesenteric adipose depots from obese patients undergoing bariatric surgery all released known endogenous FFAR4 NEFA ligands in a similar pattern as the murine adipocytes, with the expected difference that the basal levels were relatively high and the isoproterenol-induced increase was relatively small compared to adipocytes from lean mice (Figure 1 and Table S2).

3.2. Conditioned media from primary adipocytes activates FFAR4 signaling

Gq-mediated accumulation of inositol phosphate (IP) is a robust readout of FFAR4 signaling demonstrated here by measuring IP accumulation in COS-7 cells transiently transfected with the human *FFAR4*. Three different potent and efficient, synthetic, non-lipid FFAR4 agonists, CpdA, CpdB, and AZ13581837 were selected as positive controls, which stimulated IP accumulation 5.4—8.0-fold (Figure 2A). Conditioned media from isoproterenol-stimulated murine adipocytes stimulated IP accumulation in FFAR4-transfected cells 4.1-fold

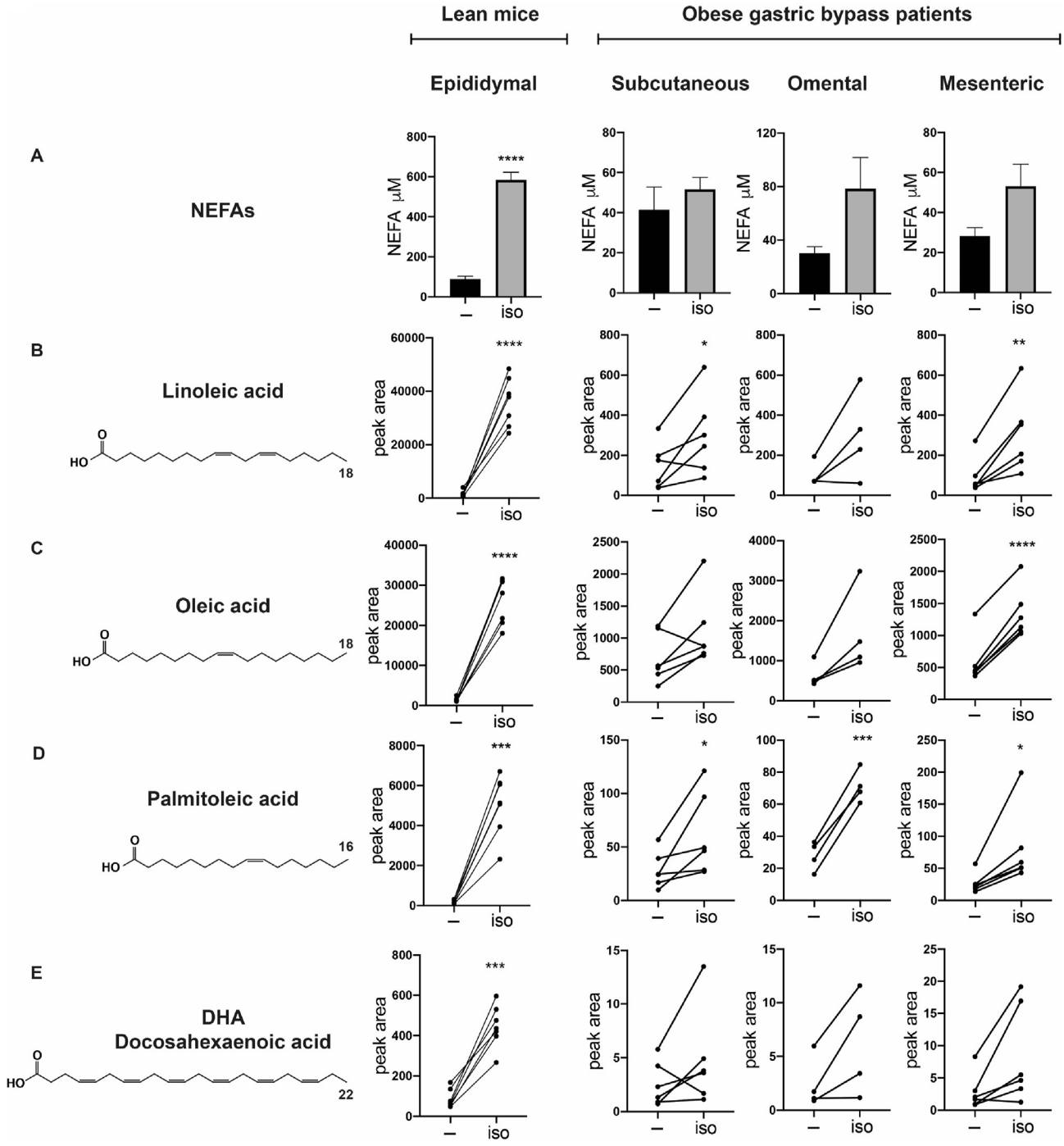

Figure 1: Unstimulated and isoproterenol-induced (1 μM) release of NEFAs and specific LCFAs from primary adipocyte cultures obtained from murine (epididymal) and human (subcutaneous, omental, and mesenteric) fat depots. Panel A - Total concentrations of NEFAs in conditioned media accumulated over 30 min. **Panels B, C, and D** — Content of the three quantitatively dominating species of NEFAs: linoleic acid (Panel B), oleic acid (Panel C), and palmitoleic acid (Panel D) as well as docosahexaenoic acid (Panel E) in conditioned media from the indicated fat depots before and after stimulation with isoproterenol as identified and quantified by LCMS analysis. Other species of NEFAs are shown in Table S2. Data are presented as mean +/− SEM for panel A. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$ using paired t-test.

(Figure 2A) an effect, which was totally blocked by the selective synthetic FFAR4 antagonist, AH7614 (Figure 2A). No effect of the conditioned medium was observed in control, empty vector-transfected COS-7 cells, and no effect was observed with unconditioned media (Figure 2A and S2). However, conditioned medium from unstimulated murine adipocytes also induced IP accumulation to a smaller degree, 1.8-fold, which was also FFAR4-dependent as it was blocked by the FFAR4 antagonist (Figure 2A). This ability of conditioned medium from nonstimulated adipocytes to activate FFAR4 is in agreement with the observation that this medium also contains NEFAs, albeit at much lower concentrations (Figure 1).

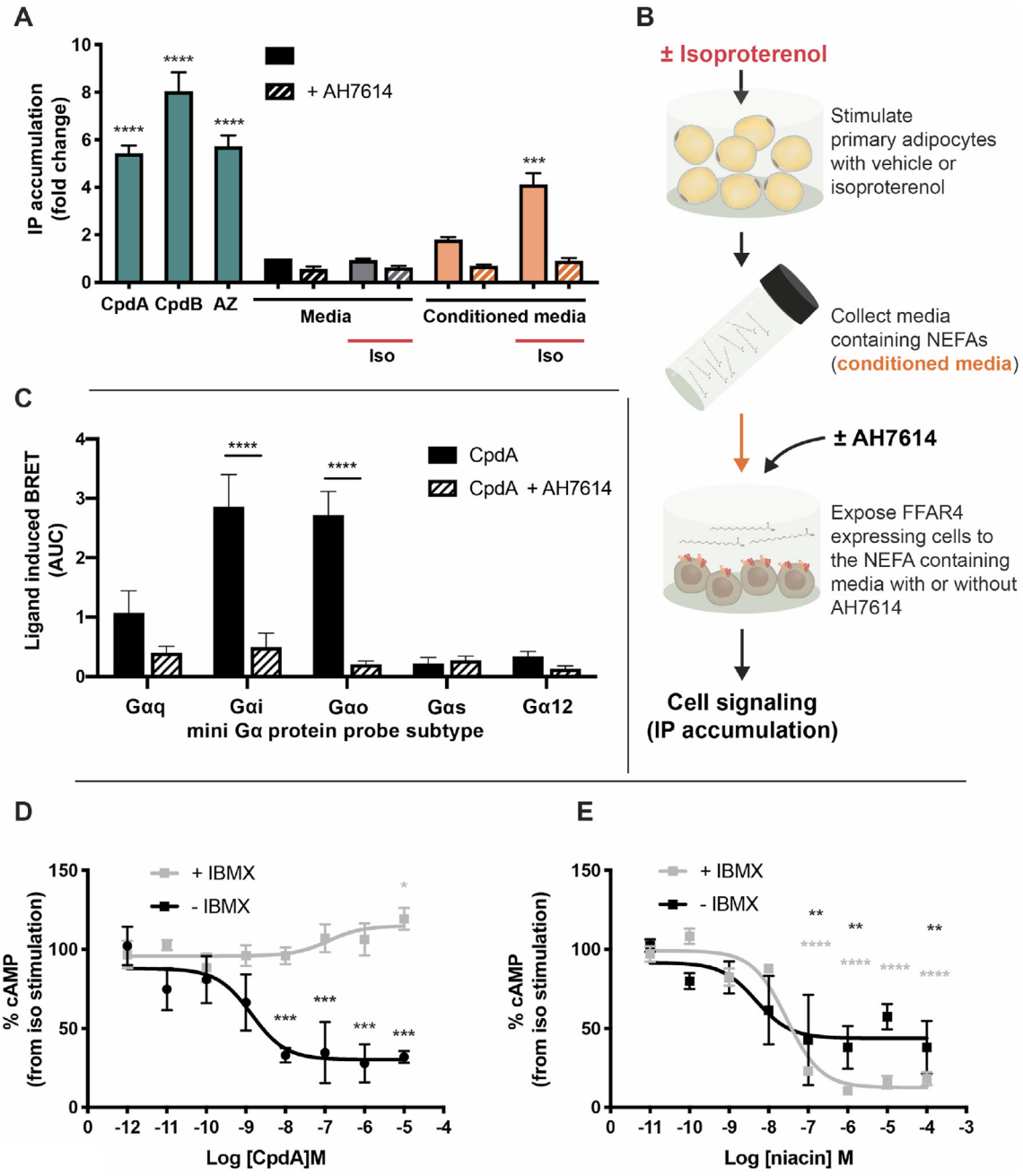

Figure 2: FFAR4/GPR120 signaling and activation by conditioned media from primary murine adipocyte cultures. **Panel A** — IP accumulation in COS-7 cells transiently transfected with human FFAR4 (n = 5). The cells were stimulated with conditioned media sampled from murine adipocytes with or without stimulation with isoproterenol and with or without the specific, synthetic FFAR4 antagonist AH7614. The selective, synthetic FFAR4 agonists CpdA (1 μM), CpdB (1 μM), and AZ (1 μM) were used as positive controls. Effects in empty vector control cells are shown in Fig. S2. **Panel B** — Setup for conditioned media stimulations of COS-7 cell in panel A. **Panel C** — Binding of different Ga subunits to 1 μM of CpdA agonist stimulated FFAR4 as determined by BRET in undifferentiated 3T3-L1 cells transfected with FFAR4-NanoLuc alongside Venus-tagged mini G-proteins (Gaq, Gai, Gao, Gas, and Ga12) with or without the FFAR4 antagonist AH7614 (n = 3). **Panel D and E** — Effects of the specific synthetic FFAR4 agonist CpdA or HCAR2 agonist niacin on isoproterenol induced cAMP accumulation in primary murine adipocytes in the absence (black curve) and the presence of IBMX (grey curve) (n = 5). Data are presented as mean +/− SEM. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$ using Tukey's multiple comparison test for panel A, two-way ANOVA with multiple comparison for panel B and Dunnet's multiple comparison for panel C.

3.3. FFAR4 signals strongly through Gi in undifferentiated 3T3-L1 cells

Although FFAR4 is considered to be a Gq-coupled receptor, it has also been reported to signal through Gi in enteroendocrine cells, such as ghrelin and somatostatin cells [11,33]. We therefore decided to study the ability of FFAR4 to bind different types of G proteins in 3T3-L1 cells transfected with mini-G proteins [32]. As shown in Figure 2C, the prototypical FFAR4 agonist CpdA induced binding of mini-Gαq, which was blocked by the FFAR4 antagonist AH7614. Importantly, however, CpdA induced 3-fold higher activation of Gi subtypes, mini-Gαi and

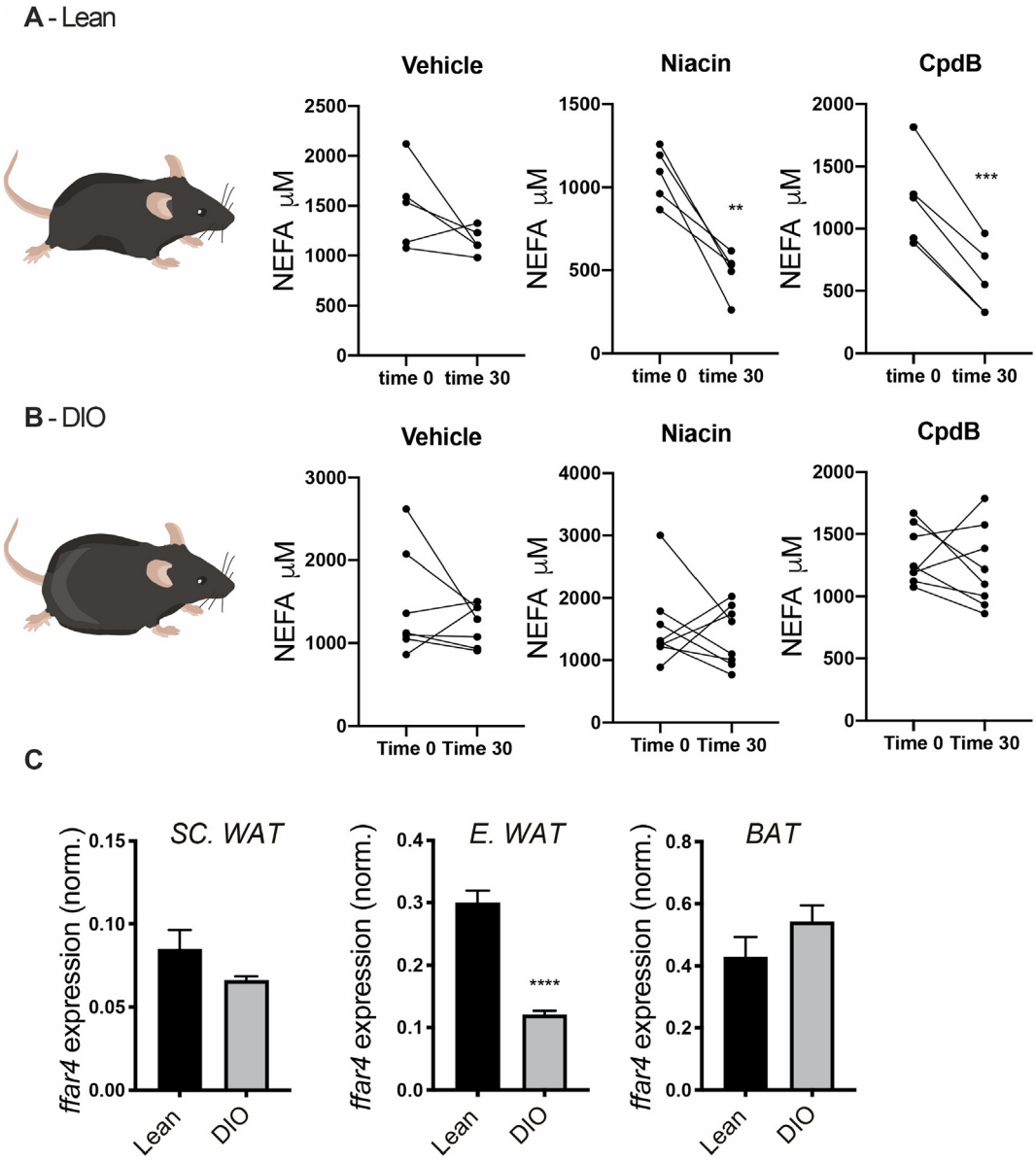

Figure 3: Effect of the FFAR4 specific agonist CpdB, the prototype anti-lipolytic agent nicotinic acid (niacin) on lipolysis *in vivo* in lean and DIO mice. Circulating NEFAs in lean (**Panel A**) or diet-induced obese (DIO) mice (**Panel B**) fasted for 18 h and given either vehicle, niacin (30 mg/kg), or CpdB (30 mg/kg) by oral gavage as measured at time zero and 30 min post gavage. **Panel C -** FFAR4 expression in subcutaneous, epididymal, or brown adipose tissues from lean (n = 8) and DIO (n = 10) mice. Data are presented as mean +/− SEM for panel C. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$ using paired t-test for panel A and B and using Student's t-test for panel C.

mini-Gαo (Figure 2C). In contrast, the binding of mini-Gαs and mini-Gα12 was negligible (Figure 2C).

3.4. Synthetic selective FFAR4 agonist decreases cAMP in adipocytes

Isoproterenol, which is the canonical stimulator of lipolysis acting through Gαs and cAMP, dose-dependently increased cAMP accumulation in primary cultures of murine adipocytes (Fig. S3). As shown in Figure 2D, the synthetic, selective FFAR4 agonist, CpdA dose-dependently inhibited the isoproterenol-induced cAMP accumulation. For methodological reasons, i.e., to increase the signal, cells are often treated with the phosphodiesterase inhibitor IBMX, which enhances the isoproterenol-induced increase in cAMP. However, in IBMX treated adipocytes the FFAR4 agonist did not inhibit the isoproterenol-stimulated cAMP accumulation but instead a trend towards a slight, increase was observed, with an at least 100-fold lower potency which indicates that this may be an unspecific effect (Figure 2D). The lack of an inhibitory effect of the synthetic FFAR4 in the IBMX-treated cells may be due to saturation of the receptor by endogenously released NEFA ligands in these massively activated cells. This notion is supported by the observation that the prototypic antilipolytic agent niacin, which acts through another Gi-coupled receptor HCAR2 was still able to inhibit the strong isoproterenol-induced increase in cAMP in the IBMX-treated adipocytes (Figure 2E).

3.5. FFAR4 activation inhibits lipolysis in lean but not obese mice

To test whether FFAR4 activation could inhibit lipolysis *in vivo*, we tested the effect of an FFAR4 agonist in parallel with the well-known antilipolytic agent niacin. Lean and DIO mice were fasted overnight and gavaged orally with either vehicle, niacin (30 mg/kg), or the synthetic, selective FFAR4 agonist CpdB (30 mg/kg). Both nicotinic acid and CpdB decreased circulating levels of NEFA in all mice on average by approximately 50% (Figure 3A). However, neither nicotinic acid nor the FFAR4 agonist decreased NEFAs in DIO mice, which are known to be relatively resistant to antilipolytic agents (Figure 4B) [21]. In fact, *Ffar4* expression was decreased in WAT, particularly in epididymal WAT as opposed to BAT from DIO mice as compared to lean mice (Figure 3C).

3.6. Lipolysis is increased upon FFAR4 blockade and in FFAR4 deficient mice

Treatment of cultures of primary murine adipocytes with the specific synthetic FFAR4 antagonist, AH7614 [28] increased isoproterenol-induced cAMP accumulation by almost 50% (Figure 4A). This agrees with the basic notion that the inhibitory, Gi-coupled FFAR4 receptor is stimulated by NEFAs liberated through the isoproterenol-stimulated lipolysis and that the FFAR4 antagonist works by blocking this autocrine inhibitory loop.

Finally, we focused on global FFAR4 KO mice. As reported in the literature [20], FFAR4-deficient mice have slightly increased body weight, are glucose intolerant (Fig. S4), and are slightly insulin-resistant, which is associated with elevated insulin levels (Figure 4B). Importantly, despite the elevated insulin levels, the FFAR4-deficient mice had increased lipolysis as determined by the borderline increased ($p = 0.054$) circulating levels of NEFAs and highly significantly elevated circulating glycerol (Figure 4B). Although several mechanisms could be responsible for this evidence of increased lipolysis, the data are at least in accordance with an inhibitory role of FFAR4 on lipolysis observed in the WT littermate control animals, which is absent in the FFAR4 KO animals.

4. DISCUSSION

This study presents evidence that FFAR4 is responsible for the autocrine inhibitory effect of NEFAs on lipolysis for which the molecular mechanism has remained elusive since this feed-back loop was first identified more than 40 years ago [1,2,4]. Our data indicates that FFAR4 inhibits lipolysis by decreasing intracellular cAMP through Gi-mediated inhibition of adenylyl cyclase (Figure 5).

4.1. FFAR4 as an autocrine sensor of NEFAs in WAT

Identification of palmitic acid, oleic acid, and linoleic acid as major species of NEFAs released from adipocytes upon stimulation of lipolysis fits well with previous reports in the literature [34–36]. Although certain unsaturated fatty acids such as omega-3 fatty acids are slightly more potent FFAR4 agonists, a broad range of saturated and unsaturated LCFAs, including the ones identified in the present study in conditioned media from human and murine adipocytes, are in fact all potent and efficacious FFAR4 agonists [6,37]. This implies that in the cell membrane of adipocytes, FFAR4 will be exposed to a broad spectrum of endogenous NEFAs and through its Gi coupling will inhibit adenylate cyclase activity and lipolysis and thereby functions as a sensor in a classical autocrine regulatory feedback loop (Figure 5). In fact, the NEFAs may not have to actually leave the adipocyte to activate FFAR4. In the closely related FFAR1 receptor, 2 binding sites for synthetic LCFA mimetics have been identified, both of which are exposed to the lipid bilayer, one for each leaflet of the bilayer [38]. Thus, LCFAs will likely access these sites by way of the lipid membrane, in which they accumulate in high concentrations [39]. Although the structure of FFAR4 remains to be characterized, it is likely that FFAR4 is activated by NEFAs through a similar 'intracrine' molecular mechanism.

The physiological role of FFAR4 in fine-tuning lipolysis and other adipocyte functions through its role as a NEFA-sensing autocrine feedback regulator remains to be characterized in detail. The main problem is that we do not yet have access to FFAR4 antagonists with appropriate bioavailability and ADME properties to be used as *in vivo* pharmacological tools, and inducible, adipose-specific FFAR4 KO mice strains are not yet available either. Nevertheless, one phenomenon for which the Gi-coupled FFAR4 likely is responsible is the elusive, prolonged *decrease* in intracellular cAMP observed in adipocytes following treatment with lipolytic agents [40,41]. Importantly, although the present study focused on Gi signaling of FFAR4, FFAR4 can still function also as a Gq-coupled receptor. However, despite the poorly understood effects of Gq signaling in adipocytes, FFAR4 is likely

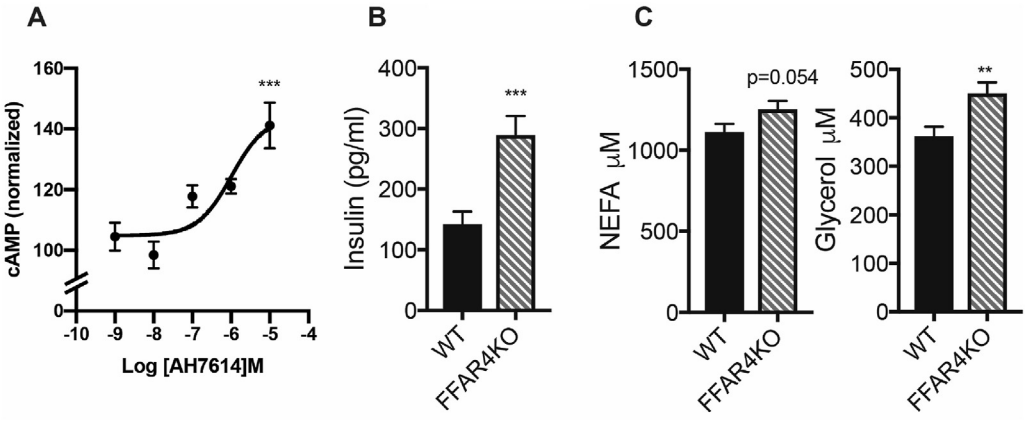

Figure 4: Stimulatory effect of FFAR4 blockade on cAMP accumulation in murine adipocytes and of FFAR4 deficiency on lipolysis *in vivo* in mice. Panel A - cAMP accumulation in primary murine adipocytes exposed to increasing concentrations of the FFAR4-selective antagonist AH7614 ($n = 5$). **Panel B** – Increased plasma insulin in globally FFAR4-deficient mice, which also are glucose intolerant and moderately obese (see Fig. S4) ($n = 30$). **Panel C** - Plasma levels of NEFAs and glycerol in globally FFAR4 KO mice ($n = 30$). Data are presented as mean+/- SEM. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, using Dunnett's multiple comparison test for panel A and Student's t-test for panels B and C.

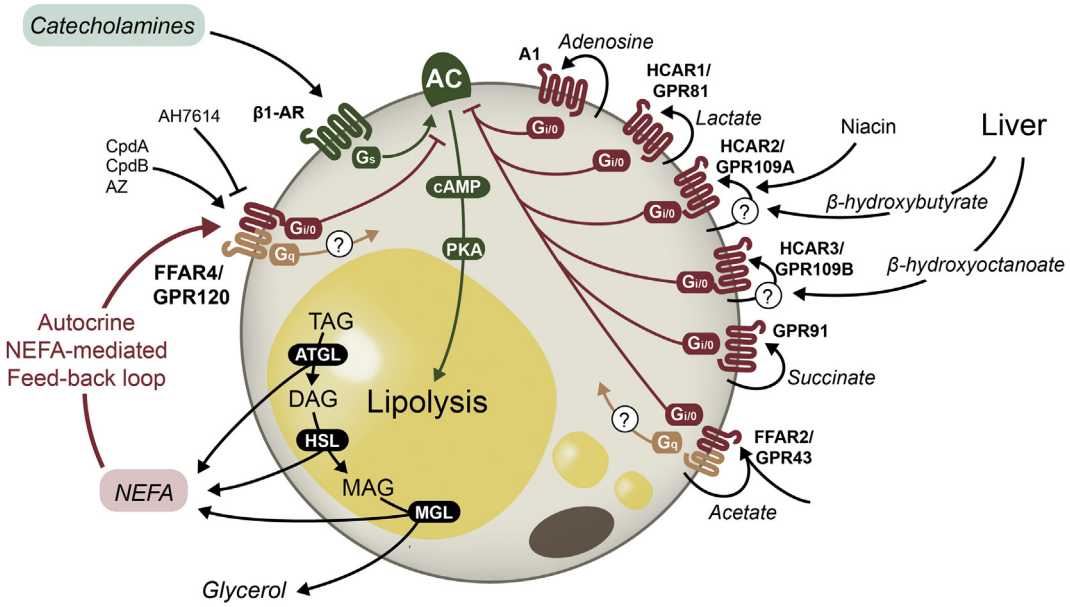

Figure 5: Simplified schematic overview of control of lipolysis by GPCRs with focus on FFAR4 in the context of the other putative autocrine feedback mechanisms. The left side indicates the autocrine NEFA and FFAR4 and Gi-mediated autocrine negative feedback loop on lipolysis characterized in the present study, including the pharmacological FFAR4 tool compounds used. The classical catecholamine, β1-receptor, Gs, and adenylate cyclase (AC)-mediated stimulatory control of lipolysis are indicated **in green**. The right side indicates six different Gi-coupled metabolite sensing GPCRs, which all act as autocrine regulators of lipolysis and other adipocyte functions under different physiological circumstances: The adenosine A1 receptor, which is responsible for the original, classical autocrine effects of adenosine [52]; the lactate receptor, HCAR1/GPR81, which is responsible for the insulin, glucose-mediated inhibition of lipolysis [47]; the HCAR2/GPR109A receptor for circulating and possibly also locally produced β-hydroxybutyrate and the prototype anti-lipolytic drug niacin [53]; the HCAR3/GPR109B receptor for β-hydroxyoctanoate [54]; the SUCNR1/GPR91 receptor sensing stress and hypoxia-induced succinate excretion [39]; and FFAR2/GPR43, which senses acetate conceivably both through autocrine and paracrine mechanisms from certain subtypes of adipocytes [55].

involved in glucose uptake as previously proposed by Olefsky [17] and could also control gene expression.

4.2. Potential role of FFAR4 in adipocyte function in obesity and diabetes

Obesity is generally associated with a high basal level of lipolysis and decreased response to catecholamines as we also observed in the present study (Figure 1). Although this so-called 'catecholamine resistance' has been attributed to decreased expression of β-adrenergic receptors [42], we propose that FFAR4 could also be involved in this phenomenon. The observation that FFAR4 is downregulated in obesity in visceral as opposed to subcutaneous adipose tissue (Figure 4) agrees with the fact that visceral adipose tissue of obese individuals has a relatively higher rate of lipolysis compared to subcutaneous fat [43,44].

4.3. Metabolite-mediated regulation of adipocyte function in general

In addition to FFAR4, a number of other Gi-coupled metabolite receptors are expressed on adipocytes, most of which also function as autocrine and/or paracrine sensors of adipocyte-generated metabolites [5] (Figure 5). These receptors include the adenosine A1 receptor, which is responsible for the inhibition of lipolysis associated with autocrine sensing of adenosine described before the similar function of NEFAs [45,46]. Through autocrine sensing of lactate, HCAR1/GPR81is responsible for the major part of the canonical insulin-induced inhibition of lipolysis [47]. The closely related HCAR2 or GPR109A sense circulating and likely also locally produced beta-hydroxybutyrate; and in humans, HCAR3/GPR109B senses beta-hydroxyoctanoate. SUCNR1/GPR91 senses succinate generated in response to metabolic, hypoxic stress and associated with reversal of succinate dehydrogenase. Finally, in BAT, thermogenesis is regulated by FFAR2/GPR43 paracrine sensing of acetate being excreted from a subpopulation of adipocytes [55] (Figure 5). As indicated, each of these metabolite sensors function under different physiological conditions to fine tune adipocyte function, particularly lipolysis. In this respect, FFAR4 is special as its function is directly coupled to lipolysis as it senses the product, NEFAs (Figure 5).

4.4. FFAR4 in BAT

In BAT, FFAR4 is highly upregulated upon cold exposure [3,16,22]. In an unbiased screen, FFAR4 was even highlighted as one of the most cold-induced genes in BAT [3]. As the increase in energy expenditure upon cold exposure involves uptake and burning of fuel, FFAR4 may serve a more sophisticated function in BAT than just inhibiting lipolysis. For example, it may be speculated that FFAR4 signals back to block the 'wasteful loss' of NEFAs, which FFAR4 senses being released and instead helps shunt the NEFAs to the burning machinery instead of out of the cell. Here, FFAR4's Gq signaling pathways could be important. Importantly, it has been reported that FFAR4 activates not only oxidative phosphorylation in BAT but also browning of WAT and that a selective FFAR4 agonist, TUG-891, increases fuel uptake and fat oxidation and reduces body weight and fat mass in mice [16,48].

4.5. FFAR4 as a potential drug target

FFAR4 agonists and dual FFAR4/FFAR1 agonists have been pursued as potential drugs for the treatment of diabetes and obesity but have not yet reached clinical development [6,37]. During preclinical development it was - in accordance with the findings of the present study - described by Satapati and coworkers that synthetic, non-metabolite FFAR4 agonists have anti-lipolytic effects *in vivo* in mice [21]. In the

academic literature, FFAR4 agonism has been strongly advocated as an anti-inflammatory mechanism for the treatment of diabetes and obesity [17,23,49]. However, the direct anti-inflammatory effect of FFAR4 agonists could unfortunately not be confirmed by the pharmaceutical industry [21]. The fact that-first generation agonists for the related FFAR1 were terminated in the late clinical phases due to liver toxicity [50] and that second generation FFAR1 agonists were reported to possess β-cell toxicity, might have dampened the enthusiasm for FFAR4 agonists also. However, FFAR4 agonists should perhaps be revisited as a novel means to treat diabetes, and in particular steatohepatitis, because chronic treatment with the synthetic FFAR4 agonist CpdA clears the liver for fat in DIO mice [23]. This effect is likely related to the FFAR4-mediated activation of thermogenic fat and the associated increased fuel consumption [16,48,51]. Time will show whether this beneficial fuel burning effect of FFAR4 agonists will carry over to humans having less BAT; however, their ability to induce browning of WAT and thereby the thermogenic fat capacity may be a favorable indication [16,48].

4.6. Concluding remarks

The present study demonstrates that FFAR4 serves an important role as a sensor of liberated NEFAs in adipocytes and thereby acts as a feedback regulator of lipolysis but conceivably also other adipocyte functions. The details of FFAR4's different role in BAT and how its Gi versus Gq and downstream signaling pathways are involved in this remain unclear. However, it appears that FFAR4's role as a drug target is being revitalized with a new focus on thermogenic fat activation.

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CONFLICT OF INTEREST

No conflict of interest is reported

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101103.

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