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Activation of β3-adrenoceptors increases in vivo free fatty acid uptake and utilization in brown but not white fat depots in high-fat-fed rats

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1Cardiovascular and Metabolic Diseases Innovative Medicines and Early Development Biotech Unit, AstraZeneca Gothenburg, Sweden; 2Discovery Sciences, Transgenics, AstraZeneca Gothenburg, Sweden; 3Pathology, Drug Safety and Metabolism, AstraZeneca Gothenburg, Sweden; and 4University of Pennsylvania, Philadelphia, Pennsylvania

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Warner A, Kjellstedt A, Carreras A, Böttcher G, Peng X, Seale P, Oakes N, Lindén D. Activation of β3-adrenoceptors increases in vivo free fatty acid uptake and utilization in brown but not white fat depots in high-fat-fed rats. Am J Physiol Endocrinol Metab 311: E901–E910, 2016. First Published October 25, 2016; doi:10.1152/ajpendo.00204.2016.—Activation of brown adipose tissue (BAT) and browning of white adipose tissue (WAT) present potential new therapies for obesity and type 2 diabetes. Here, we examined the effects of β3-adrenergic stimulation on tissue-specific uptake and storage of free fatty acids (FFA) and its implications for whole body FFA metabolism in diet-induced obese rats using a multi-radiotracer technique. Male Wistar rats were high fat-fed for 12 wk and administered β3-agonist CL316,243 (CL, 1 mg·kg−1·day−1) or saline via osmotic minipumps during the last 3 wk. The rats were then fasted and acutely infused with a tracer mixture ([14C]palmitate and the partially metabolized R-[3H]bromopalmitate) under anesthesia. CL infusion decreased body weight gain and fasting plasma glucose levels. While core body temperature was unaffected, infrared thermography showed an increase in tail heat dissipation following CL infusion. Interestingly, CL markedly increased both FFA storage and utilization in interscapular and perirenal BAT, whereas the flux of FFA to skeletal muscle was decreased. In this rat model of obesity, only sporadic populations of beige adipocytes were detected in the epididymal WAT depot of CL-infused rats, and there was no change in FFA uptake or utilization in WAT following CL infusion. In summary, β3-agonism robustly increased FFA flux to BAT coupled with enhanced utilization. Increased BAT activation most likely drove the increased tail heat dissipation to maintain thermogenesis. Our results emphasize the quantitative role of brown fat as the functional target of β3-agonism in obesity.

Beige (or brite) adipocytes, such as those contained in inguinal fat pads, also harbor thermogenic potential (37). Both brown and beige adipocytes can be activated and recruited by cold exposure or by activating the β3-adrenergic receptor (β3-AR), expressed primarily in BAT and white adipose tissue (WAT) (1, 16, 31). Administration of CL316,243 (CL), a highly specific β3-AR agonist, increases energy expenditure, reduces fat mass (8, 19, 46, 47), and improves insulin action in various rodent models of obesity and type 2 diabetes (8, 24, 46).

Uncoupling protein-1 (UCP1) drives mitochondrial uncoupling and the production of heat rather than ATP in brown and beige adipocytes. Positron emission tomography–computed tomography has been used to study glucose uptake in tissues using the tracer fluorodeoxyglucose (FDG) in both rodents and humans (6, 28, 35). A few studies have started to scrutinize the tissue-specific contribution of BAT and beige adipose tissue to the uptake of lipids by using radioactive tracers (2, 20). However, elucidating the fate of these lipid tracers once taken up by tissues remains an open question and needs to be addressed. When discerning the fate of free fatty acids (FFA), the use of (R)-2-[9,10-3H]bromopalmitate allows measurement of FFA utilization (i.e., uptake and partial metabolism) in specific tissues. When combined with [14C]palmitate, this tracer mixture provides a unique opportunity to determine the tissue-specific fate of FFA after uptake, being either oxidized, or stored as triglycerides (10, 18, 32).

In this study, we performed a comprehensive investigation of tissue-specific FFA metabolism in high fat-fed rats, a rodent model of diet-induced obesity. We show that β3-agonism promoted both storage and metabolism of FFAs in classical BAT depots of these obese rats; β3-agonism also decreased the FFA flux to skeletal muscle while it did not affect FFA metabolism in WAT depots, in which very few beige adipocytes were detected by immunohistochemistry. In addition, the elevated BAT activity drove heat dissipation via the tail while the core body temperature was unchanged.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Gothenburg Ethics Committee for Experimental Animals in Sweden.

Pre-acute radiotracer protocol. Male Wistar rats were randomized on body weight, pair-housed and kept at 22 ± 1°C on a 12:12-h
light-dark lighting regimen and fed a 45% (kcal) high fat diet (HFD, D12451; Research Diets, New Brunswick, NJ) for 12 wk in a staggered approach. At the start of the final 3 wk, osmotic minipumps (model 2ML4; Alzet, Cupertino, CA) containing either CL316,243 (CL, 1 mg·kg\(^{-1}\)·day\(^{-1}\); n = 6, Bio-techne/Tocris, Abingdon, UK) or saline (n = 9) were implanted subcutaneously under isoflurane anesthesia (Forene; Abbot Scandinavia, Sweden). Minipump infusion of CL has been shown to be comparable to daily CL injections (17). All animals were given analgesia postoperatively (2 mg/kg, Comforion; Orion Pharma, Sollentuna, Sweden). Body weight and food intake were measured weekly throughout the 12 wk.

**Acute Radiotracer Protocol**

*Infrared thermography.* Still pictures were taken with an infrared camera (Thermotracer TH9100; NEC, Tokyo, Japan) immediately before anesthesia. Two pictures per rat were taken, one covering the tail and lower body and one covering the head for inner ear and eye temperature, all at ~35 cm from the rat. Crosshairs were marked in a standardized landmark on the tail on each image ~10 mm from the tail base and in the center of eye and inner ear ( tympanic temperature).

**Tracer preparation.** Tracer infusates were freshly prepared each day. For each rat, the two tracers, R-[9,10-\(^3\)H]palmitomaltate (\(^3\)H-BrP, ~100 \times 10^6 dpm) and \(14^C\)-palmitate \((14C-P, 35 \times 10^6 dpm\)) were mixed together with 8 \(\mu\)l of Na-palmitic acid (5 mg/ml in ethanol; Sigma-Aldrich, St. Louis, MO) and evaporated (under N\(_2\)), Acute Radiotracer Protocol

**Tracer administration and blood sampling.** Blood sampling. Blood samples were collected into vials containing potassium-EDTA (Microvette® CB300; Sarstedt, Nümbrecht, Germany). The samples were centrifuged immediately (1 min, 16,100 g, 4°C), and plasma was removed for biochemical analysis as before. For \(^3\)H-BrP and \(14^C\)-P tracer plasma concentrations, a 25-\(\mu\)l plasma aliquot was placed directly into 2 ml of lipid extraction mixture.

**Lipid extraction.** To discriminate \(^3\)H-BrP and \(14^C\)-P from total plasma \(^3\)H and \(14^C\) activity, a lipid extraction and separation procedure was performed essentially as previously described (43). This involved an initial acid lipid extraction using a mixture of isopropanol-hexane-1 mol/l acetic acid (40:10:1 vol) followed by solid-phase separation of FFAs (including \(^3\)H-BrP and \(14^C\)-P) and neutral lipids.

**Measurement of tissue-specific \(^3\)H and \(14^C\) activity.** \(^3\)H and \(14^C\) activities were determined using an automated sample preparation unit (Packard System 387; Packard Instrument, Meriden, CT), which completely oxidized the sample and collected the resulting \(^3\)H\(_2\)O and \(14^CO_2\) into scintillation cocktails (water/Monophase S and Carbosorb/Permafluor; Packard Bioscience, Groningen, The Netherlands) for \(^3\)H and \(14^C\) counting using liquid scintillation spectrometry (Wallac 1409 counter; Wallac OY, Turku, Finland).

**Whole body FFA clearance and appearance rates.** Estimates of whole body \(14^C\)-P clearance (MCR\(_{PP}\)) were calculated as previously described (32). Estimates of appearance rate (R\(_a\)) of FFA into plasma were calculated as

\[
R_a = C_p \times MCR_{PP}
\]

where \(C_p\) is arterial plasma FFA concentration (\(\mu\)mol/ml).

**Tissue-specific FFA utilization rate indexes.** \(^3\)H-BrP undergoes the same initial transport steps and activation reaction as the native FFAs. Beyond the activation reaction mediated by the enzyme acyl CoA synthetase, the metabolic handling of the analog diverges from the native FFAs. It is assumed that tritium from \(^3\)H-BrP is effectively trapped in the cells. An index of FFA utilization rate (R\(_{\text{BrP}}\)) can be calculated as previously described (32):

\[
R_{\text{BrP}} = \frac{C_p \cdot m_B}{\int_0^T c_B(t) \, dt}
\]

where \(T\) is time of tissue collection (16 min), \(m_B\) is total tissue \(^3\)H content (at \(t = T\), \(c_B\) is arterial plasma concentration of \(^3\)H-BrP, and \(C_p\) is arterial plasma FFA concentration.

Given several assumptions (32), it can be shown that the true FFA utilization rate (R\(_f\)) is theoretically proportional to R\(_{\text{BrP}}\), i.e.,

\[
R_f = \frac{R_{\text{BrP}}}{LC^*}
\]

where LC\(^*\) (or the lumped constant for R-BrP) represents the ratio of the probabilities that a molecule of arterial \(^3\)H-R-BrP will undergo metabolic sequestration (activation to the CoA derivative) to the probability that a molecule of arterial palmitate will undergo metabolic sequestration. LC\(^*\) is a function of several mass transfer coefficients that would equal unity if \(^3\)H-R-BrP kinetics were identical to the native compound up to and including the activation step. It is assumed that, following sequestration, the bulk of native fatty acid is directed either into storage or into oxidation, i.e.,

\[
R_f = R_{j_s} + R_{jox}
\]

After the metabolic sequestration step, \(14^C\)-P is directed into either: 1) oxidation, in which case the radiolabeled products, notably \(14^CO_2\) are very rapidly cleared from the cells and do not contribute to tissue \(^14^C\)-activity at the time of tissue sampling or; 2) storage (e.g., esterification) where all \(14^C\)-products are effectively trapped over the time period of the tracer experiment. An index of the rate of FFA incorporation into storage (R\(_{j_s}\)) can be calculated as
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\[ R_{fs} = \frac{C_p - m_p}{T} \int_0^T c_p(t) \, dt \]  

where \( m_p \) is total tissue \(^{14}\)C content (at \( t = T \)) and \( c_p \) is arterial plasma \(^{14}\)C-P concentration. Note that under conditions of suppressed tissue FA oxidation (\( R_{fox} = 0 \)) Eqs 2 and 3 can be combined to obtain \( LC^* \) i.e.,

\[ LC^* = \frac{R_f^*}{R_{fs}} . \]

We have previously estimated values of \( LC^* \) during fatty acid oxidation blockade with etomoxir or hyperinsulinemia in insulin-sensitive animals (10). \( LC^* \) varies in a tissue-specific manner, with estimates for the current study taken from Ref. 10, e.g., the value for BAT assumed to be 0.44.

Finally, an index of the rate of plasma FFA oxidation (\( R_{fox} \)) can be estimated based on Eqs 2 and 3 as follows:

\[ R_{fox} = R_f^*/LC^* - R_{fs} . \]

**Quantitative PCR**

RNA was isolated from snap-frozen tissues using an RNasy Mini Kit (Qiagen). cDNA was generated using reverse transcription with a cDNA kit (cat. no. 4368814; ThermoFisher Scientific, Stockholm, Sweden) and used for real-time PCR with the QuantStudio 7 Flex (Applied Biosystems), using a Ucp1 gene expression assay (Taqman, Life Technologies Europe, Stockholm, Sweden). The results were normalized using TATA-binding protein (TBP) as a reference gene.

**Generation and Characterization of a UCP1 Polyclonal Antibody**

Two rabbits were immunized using an UCP1 peptide covering amino acids 142–159 in the human UCP1 protein sequence (QAQSHLHGBKRYTGTYN) by four rounds of injections over an 80-day period. An aliquot of serum was obtained before the immunization and served as negative control (preimmune), and the final serum was collected 14 days after the final immunization (postimmune). Serum from the rabbit showing the strongest reactivity toward the UCP1 protein was selected for further purification. The antibody was purified using an affinity column generated using the same peptide as for immunization.

To test antibody specificity and sensitivity, protein extracts from rat epididymal adipose tissue (WAT) and brown adipose tissue (BAT) were separated on a 4–20% Bis-Tris gel, transferred, and blotted with the human UCP1 primary antibody (1:3,000, Dako Sweden, Stockholm, Sweden). After more washing, then incubated with a goat anti-rabbit HRP secondary antibody (1:2,000) for 1 h, washed repeatedly in TBS-0.1% Tween, and powder, the membrane was incubated with the UCP1 primary antibody (1:2,000, same antibody as for Western blotting). The staining was visualized using an HRP developer (Pierce ECL Western Blotting Substrate, Thermo Scientific, Gothenburg, Sweden). A prominent band corresponding to a ∼33-kDa protein was detected in postimmune serum in BAT and confirmed as UCP1 by gel extraction and sequencing.

A Western blot with lysates from HEK293 cells transfected with control lentivirus (Fig. 1B, lane 1), lentivirus expressing human UCP1 (Fig. 1B, lane 2), or a plasmid expressing human UCP1 (Fig. 1B, lane 3), and recombinant his-tagged UCP1 (Alpha Diagnostic International, San Antonio, TX; Fig. 1B, lane 4) was performed. A strong UCP1 signal was detected from extracts of cells overexpressing UCP1 and against recombinant UCP1.

**Histopathology and Immunohistochemistry**

Adipose tissue samples (\( n = 3 \)) were fixed in a phosphate-buffered 4% formaldehyde solution for 24–48 h and then rinsed and dehydrated, embedded in paraffin, sectioned, and stained for hematoxylin-eosin for histopathological analysis. In addition, sections were rehydrated and immunostained for UCP1 on an automated staining platform, Ventana Discovery XT, using a primary UCP1 antibody in dilution 1:2,000 (same antibody as for Western blotting). The staining followed the no. 912 Ventana staining protocol (Ventana Medical Systems/Roche Diagnostics, Tucson, AZ), with antigen retrieval at pH 8, and including HRP/DAB visualization and hematoxylin counterstain. After completion of the staining, sections were finally dehydrated and mounted for microscopic examination.
MCRP the whole body level, shown by a marked enhancement in significantly different from saline controls.

of insulin resistance (Fig. 3; mM glc·nM ins; saline 0.98 ± 0.13 nM, CL 0.74 ± 0.11 nM, P = 0.20), or comparative index of insulin resistance (Fig. 3C; mM glc-nM ins; saline 4.99 ± 0.59, CL 3.37 ± 0.56, P = 0.08) were significantly different from saline controls.

The effect of CL316,243 on FFA metabolism was evident at the whole body level, shown by a marked enhancement in MCRp (Table 1), an index of the combined ability of the body’s tissues to take up plasma FFA. On its own this would tend to lower plasma FFA levels, however, the difference between means in the Saline and CL groups did not achieve statistical significance (Table 1). In vitro rat adipocytes, CL increases lipolysis (11), but there was no significant difference in FFA mobilization (Rfo) between the groups. However, CL infusion decreased plasma triglyceride levels compared with saline infusion (Table 1). Importantly, there was no significant emergence of esterified fatty acids (EFA) derived from the labeled FFA tracers during the 16 min time period for the experiments (Fig. 4). A comparison of 14C-P curves for groups revealed a tendency toward lower plateau levels in the CL compared with the saline group indicating a treatment induced enhancement in whole body FFA clearance (Fig. 4, A and B).

When quantifying FFA utilization (using 3H-R-BrP; Rfu), significant increases in CL-infused rats were observed for both perirenal and interscapular BAT (Fig. 5A). Likewise, FFA storage (14C-P; Rfs) was also increased in CL-infused rats in perirenal and interscapular BAT (Fig. 5B). Interestingly, white quadriceps and soleus muscles both had significantly lowered FFA storage under CL infusion (Fig. 5B). The similar relative CL-induced increases in BAT Rfu and Rfs indicated that both FFA oxidation and storage were similarly increased. This was confirmed by estimation of the rate of FFA oxidation (Rfox); thus, CL infusion resulted in an approximately threefold increase in interscapular BAT FFA oxidation (Fig. 5C). There was a tendency for oxidative muscles to have higher Rfox after CL infusion; however, the differences did not achieve statistical significance. In contrast to BAT, there was no significant effect on utilization (Fig. 5A), storage (Fig. 5B), or estimated oxidation (Fig. 5C) in the WAT depots.

Infrared thermography was performed in the rats’ conscious state before the tracer studies to determine the effect of CL on
heat dissipation in the rats (Fig. 6). CL infusion significantly increased heat dissipation from the tail (Fig. 6, A and B), without affecting inner ear or eye temperature as approximations of core body temperature (Fig. 6B, middle and right).

Three of the adipose tissue depots were analyzed for mRNA and protein expression of UCP1 (Fig. 7). CL infusion did not significantly affect Ucp1 mRNA expression in interscapular BAT (Fig. 7A) but did cause a 1.7-fold increase in UCP1 protein expression (Fig. 7A, bottom; saline = 7.7 \times 10^6 \pm 2.3 \times 10^5, CL = 1.6 \times 10^6 \pm 1.3 \times 10^5 normalized band volume, \( P < 0.05 \)). CL infusion did not affect Ucp1 mRNA expression in the inguinal depot (Fig. 7B, top), and UCP1 protein was undetectable (Fig. 7B, bottom). In epididymal WAT, CL infusion lead to a 15-fold elevation in UCP1 mRNA expression levels (Fig. 7C, top), but this did not translate to detectable UCP1 protein levels in Western blot analyses (Fig. 7C, bottom).

Histopathological analysis confirmed the morphological phenotype of the adipose tissue depots in the saline-infused rats, with a typical appearance in the interscapular BAT and an archetypal WAT appearance in the inguinal and epididymal WAT (Fig. 8). In sections from the CL-infused rats, the interscapular BAT displayed a BAT-like phenotype but with very interspersed single BAT-like beige adipocytes with multilocular small droplet appearance, and a less marked multimlocular small droplet appearance in the majority of adipose cells. These interscapular BAT adipocytes showed strong UCP1 immunostaining (Fig. 8). Sections from the epididymal WAT of CL-infused rats displayed a WAT-like phenotype but with very interspersed single BAT-like beige adipocytes with multilocular small droplet appearance, showing distinct UCP1 immunostaining (Fig. 8). The CL inguinal WAT did not show any UCP1-immunostained beige adipocytes (Fig. 8).

**DISCUSSION**

This study provides a comprehensive overview of tissue-specific FFA metabolism during \( \beta_3 \)-adrenergic agonism in HFD-induced obese Wistar rats. Moreover, it demonstrates that BAT is the main functional target of \( \beta_3 \)-adrenergic agonism in this obesity model, as shown by increased FFA utilization and storage. This is despite evidence of sporadic browning in the epididymal WAT depot.

Body weight gain decreased upon CL infusion, coupled to a significant glucose-lowering effect. This indicates that CL infusion had systemic actions in increasing energy expenditure and improving glucose homeostasis, which is in line with previous studies (8, 19, 40). BAT simultaneously takes up, oxidizes, and stores fatty acid. The methodology applied in the current study based on the combined use of \(^3\)H-BrP and \(^{14}\)C-P tracers provides a unique opportunity for the in vivo study of plasma FFA utilization, storage, and oxidation in this tissue. This follows since \(^3\)H-BrP retention by BAT reflects both oxidative and storage FFA metabolism, whereas \(^{14}\)C-P retention reflects storage metabolism only (10). By contrast, methods based on retention of native FFA tracers alone reflect the flux of FFA into storage metabolism and are insensitive to changes in oxidative metabolism, as the final oxidation products (CO2 and H2O) are very rapidly lost from their site of production. Application of the \(^3\)H-BrP/\(^{14}\)C-P method in the current study revealed that BAT FFA utilization, oxidation, and storage were

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**Table 1. Plasma FFA, TG, and whole body \(^{14}\)C-P clearance rate (MCR\(_P\)) and rate of appearance of FFA (Ra\(_a\)) in saline- and CL316,243-treated rats fed a HFD**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>CL316,243</th>
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<tbody>
<tr>
<td>Plasma FFA, mM</td>
<td>0.84 ± 0.08</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>MCR(_P), mg kg(^{-1}) min(^{-1})</td>
<td>37.7 ± 2.6</td>
<td>49.2 ± 3.5*</td>
</tr>
<tr>
<td>Ra(_a), (\mu)mol kg(^{-1}) min(^{-1})</td>
<td>31.8 ± 3.2</td>
<td>36.1 ± 3.0</td>
</tr>
<tr>
<td>Plasma TG, mM</td>
<td>0.81 ± 0.13</td>
<td>0.23 ± 0.06**</td>
</tr>
</tbody>
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Data are means ± SE. *\( P < 0.05 \), **\( P < 0.01 \) vs. Saline.
Upon CL stimulation, BAT far exceeded the FFA utilization rates (per gram of tissue) of the other major metabolic tissues, with values for utilization and storage ~40-fold greater than in WAT. Our data indicate that CL increased the direct oxidation of FFA as well as the storage of FFA in BAT. The subsequent fate of the stored FA is unknown; however, the intracellular multilocular lipid pool in BAT has a high turnover, providing fuel for local oxidation (30). Circulating glucose (24, 38) and lipids derived from FFA (21), triglycerides (2), or triglyceride-rich lipoproteins (20) are quantitatively important circulating fuels for BAT. In addition, a recent study in humans indicated that FFA utilization in cold-activated BAT was significant (34). However, a key question is the quantitative contribution of plasma FFA utilization by BAT to whole body energy expenditure. Specifically, could the observed enhancement in utilization of circulating FFA by BAT significantly contribute to the CL-induced reduction in body weight gain? Using an indirect approach, i.e., BAT flux data, an estimation of the total BAT weight needed to produce the observed body weight difference was performed. It was assumed that 1) the flux difference was sustained throughout the 3-wk treatment period; 2) the body weight difference was due to a difference in fat mass; and 3) FFA directed into immediate storage is, over the longer term, used for local oxidation. Based on the CL-induced increase in FFA utilization by interscapular BAT (ΔRf + ΔRfox = 70.4 μmol·100 g·min⁻¹), the amount of BAT required to oxidize an amount of fat equal to the body weight difference (17.2 g over 3 wk) is 3.2 g. This corresponds to only 0.4% of body weight, which seems reasonable based on the fact that interscapular BAT mass alone has been measured to be 1.6 g in similar-size rats (27) and that this depot comprises ~1/3 of the macroscopically dissectable BAT from interscapular, cervical, axillary, thoracic, and perirenal depots (29, 39). We also observed the FFA flux of at least one other BAT depot (perirenal BAT) to match that of interscapular BAT. Furthermore, on its own, the effect of CL on FFA utilization by BAT would be expected to indirectly reduce systemic FFA partitioning into other tissues. Thus, the selective enhancement of FFA clearance into this tissue, would tend to divert systemic FFA away from other key metabolic tissues. That BAT had a substantial impact on systemic FFA metabolism is evidenced by the CL induced enhancement in whole body plasma FFA clearance which based on BAT FFA clearance rate estimates (from Rf, Rfox and plasma FFA concentration data) can be accounted for by the equivalent of 6.2 g of BAT, not unreasonable given the calculations above of BAT size. It thus appears that circulating FFA is a highly significant fuel for activated BAT at least during fasting. However, these are ballpark calculations, and we do not want to imply that other fuels are not quantitatively important as well. In particular, circulating TG is likely to be a highly significant source of FA for this tissue (2). Indeed, in our hands, in healthy control rats in the fasting state, we observe approximately equal fluxes of FA into BAT from circulating VLDL TG and FFA (N. D. Oakes, unpublished observations). Circulating TG levels were markedly lowered by CL treatment, potentially lowering the opportunity of BAT to take up TG-FA. Furthermore, we have not studied fuel fluxes under conditions of absorption of the high fat diet, chronically consumed by the rats during the study, a situation where the contribution of TG-FA may dominate over FFA.

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**Fig. 5.** Free fatty acid (FFA) metabolism in CL-infused HFD-fed rats. FFA utilization (A; Rf), storage (B; Rfs), and oxidation (C; Rfox) in a range of tissues in HFD-fed rats infused for 3 wk with CL (1 mg·kg⁻¹·day⁻¹; closed bars; n = 6) or saline (open bars; n = 9). WQ, white quadriceps; RQ, red quadriceps; epiWAT, epididymal WAT; ingWAT, inguinal WAT; periBAT, perirenal BAT; interBAT, interscapular BAT. *Statistical significance by multiple t-tests (*P < 0.05; **P < 0.01, ****P < 0.0001). Data represent means ± SE.

All markedly enhanced by CL. BrP is an inhibitor of FFA metabolism, and to avoid this effect we make use of high-specific-activity [³H]-BrP; ~2 × 10¹² Bq/mm mol. Based on measured doses of [³H] activity only ~250 pmol of the BrP was administered to each rat in the current study. These tiny amounts of BrP are not expected to have any significant impact on native FFA metabolism. In contrast, pharmacological doses of this substance (µmol/mmol per rat) inhibit FFA metabolism. Based on the specific activity, during the tracer infusion peak concentrations (at the 4-min time point) of ~2.5 nM are reached, again orders of magnitude below the concentrations needed to inhibit FFA metabolizing enzymes, e.g., acyl-CoA synthetase (5, 26).
At the tissue level, effects on FFA metabolism were not restricted to BAT. CL also decreased FFA flux into skeletal muscle storage. A reduced storage flux could prevent lipid intermediates, which can interfere with insulin signaling (7) from accumulating in the muscle and thereby contribute to the CL-induced enhancement in skeletal muscle insulin sensitivity (24). The mechanism of this effect deserves attention in future studies.

Rats and mice control their core body temperature mainly via vasoregulation in the tail (14), where dilation of the ventral artery increases blood flow to the lateral veins to aid heat dissipation when the threshold core temperature is exceeded.

![Image](image1.png)

**Fig. 6.** Heat dissipation in the tail base in HFD-fed CL-infused rats. Representative images (A) showing the standard tail base landmark in saline- (left) and CL- (right) infused rats. Tail base, inner ear, and eye temperature (B) in CL (1 mg kg⁻¹·day⁻¹; closed bars n = 4) or saline (open bars n = 5). *Statistical significance by t-test (***P < 0.001). Data represent means ± SE.
Infusion of CL significantly increased the temperature of the tail base compared with saline-infused controls, suggesting that vasodilation occurred to dispel heat generated by active BAT, in order to defend core body temperature. Indeed, inner ear or eye temperature by infrared thermography, or rectal temperature monitoring during the acute tracer protocol, did not differ significantly between the groups. This demonstrates that the triggering of compensatory mechanisms while activating thermogenic adipose tissue will need attention when developing a therapeutic approach based on this principle.

Increasing importance is being put on the need for translatability of metabolic studies in rodents, where possible housing and experiments should be done at thermoneutrality (4). Recently, Xiao et al. (45) showed that CL treatment increased energy expenditure, activated BAT, and improved glucose tolerance at both standard housing temperature (22°C) and thermoneutrality (30°C) in mice. However, CL treatment reduced adiposity only at thermoneutrality, due to disproportional effects on energy expenditure and food intake (45). Due to the complex logistics of the acute tracer protocol, it was necessary to house the animals at room temperature to avoid transient cold stress during the movement and preparation of the animals for the tracer protocol, as an acute drop to room temperature would be unavoidable. However, soon after onset of anesthesia, the rats were very close to thermoneutral conditions due to the core body temperature-responsive heating system, which maintained thermostasis. We can only speculate that the effects of CL infusion on FFA uptake and utilization in BAT might have been even more pronounced if the entire study could have been performed at thermoneutrality.

The simplest explanation for the lack of effect of CL on FFA uptake and utilization in WAT is insufficient induction of beige adipocytes. CL did increase Ucp1 mRNA levels in epididymal WAT by a factor of 15; however, this is still far below the levels seen in true brown fat. The histology analyses also showed that beige adipocytes were induced only very sparsely in the same depot. On the other hand, a recently published mouse study (22), also failed to show increased FFA metabolism in WAT despite UCP1 levels being upregulated to an apparently greater extent than seen in the current study. It cannot be completely excluded, therefore, that there is a fundamental difference in the ability of beige fat to deliver increased energy expenditure; e.g., enhanced adipocyte function probably also requires a substantial increase in local perfusion capacity to be able to match the gas exchange and nutrient supply demands of the enhanced oxidation capacity. Interestingly, also in mice, CL induced Ucp1 mRNA levels to a greater extent in epididymal WAT compared with inguinal WAT (45), indicating that the epididymal WAT depot is more responsive to β3-agonism treatment.

HFD feeding has been shown to stimulate thermogenesis and BAT activity and to elevate BAT UCP1 levels in rats (12, 13, 23, 33, 36). One report in rats showed markedly increased Ucp1 mRNA levels in “whole abdominal WAT” (combined retro- and intraperitoneal, omental and mesenteric depots) following HFD feeding (25). There are, however, several reports on mice showing undetectable, unchanged, increased, or downregulated WAT Ucp1 mRNA levels by HFD feeding, reviewed in Ref. 9. It is possible that β3-agonism could induce higher Ucp1 mRNA levels in WAT in lean animals fed a low-fat diet that potentially could influence the FFA uptake and utilization. However, our aim with the present study was to evaluate the potential therapeutic effects of β3-agonism in the context of HFD-induced obesity.

In summary, our study demonstrates for the first time that β3-agonism markedly increases both FFA utilization and stor-
age in vivo in BAT in a diet-induced obese rat model. These data hold promise that activation of BAT could increase FFA utilization in obesity and its comorbidities.

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DISCLOSURES


AUTHOR CONTRIBUTIONS


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