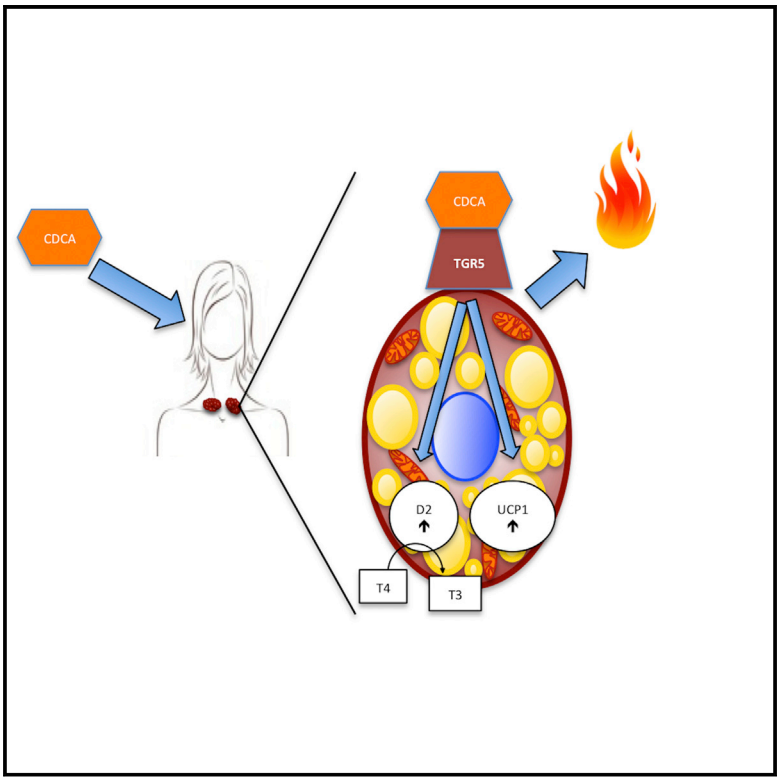
Clinical and Translational Report

Cell Metabolism

The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity

Graphical Abstract



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In Brief

While cold is an effective strategy to activate brown fat, alternative approaches are warranted. Broeders et al. show that the bile acid chenodeoxycholic acid (CDCA) promotes mitochondrial uncoupling via TGR5 in human brown adipocytes and increases brown fat activity and energy expenditure in women.

Highlights

- The bile acid CDCA increases human BAT activity
- CDCA increases mitochondrial uncoupling in human primary adipocytes derived from BAT
- CDCA promotes mitochondrial uncoupling via TGR5

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The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity

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SUMMARY

The interest in brown adipose tissue (BAT) as a target to combat metabolic disease has recently been renewed with the discovery of functional BAT in humans. In rodents, BAT can be activated by bile acids, which activate type 2 iodothyronine deiodinase (D2) in BAT via the G-coupled protein receptor TGR5, resulting in increased oxygen consumption and energy expenditure. Here we examined the effects of oral supplementation of the bile acid chenodeoxycholic acid (CDCA) on human BAT activity. Treatment of 12 healthy female subjects with CDCA for 2 days resulted in increased BAT activity. Whole-body energy expenditure was also increased upon CDCA treatment. In vitro treatment of primary human brown adipocytes derived with CDCA or specific TGR5 agonists increased mitochondrial uncoupling and *D2* expression, an effect that was absent in human primary white adipocytes. These findings identify bile acids as a target to activate BAT in humans.

INTRODUCTION

Since the discovery in 2009 that humans possess functional depots of brown adipose tissue (BAT) (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009), many studies have been initiated to identify novel molecular targets for the activation of BAT. We have previously shown that acute cold exposure is a very potent activator of human BAT (van Marken Lichtenbelt et al., 2009), whereas cold acclimation is able to recruit BAT in human volunteers (van der Lans et al., 2013; Yoneshiro et al., 2013). Cold may activate BAT via activation of the SNS, and some studies have suggested that beta-agonist treatment may be able to activate BAT in humans (Cannon and Nedergaard, 2004), although this has not been confirmed by others (Vosselman et al., 2012). However, due to unwanted side effects, these interventions are not easily applicable in humans, urging the search for novel activators of human BAT.

Bile acids (BAs) are essential factors in lipid metabolism and end products of cholesterol catabolism (Lefebvre et al., 2009; Watanabe et al., 2006), the latter being the most important route for elimination of surplus cholesterol from the circulation (Chen et al., 2011). BAs are natural ligands for the nuclear farnesoid X receptor (FXR) (Kawamata et al., 2003; Maruyama et al., 2002), which controls the synthesis and enterohepatic circulation of BAs by adjusting the expression of essential gene components involved in BA synthesis, transportation, conjugation, and detoxification (Houten and Auwerx, 2004; Russell, 2003). More recently, several studies have also implicated a role for BAs in the regulation of energy metabolism (Fiorucci et al., 2009; Hofmann, 2009; Lefebvre et al., 2009), and FXR agonists as well as the FXR-target FGF19 have been suggested for the treatment of the metabolic syndrome (Fu et al., 2004; Kir et al., 2011; Miyata et al., 2012; Prawitt and Staels, 2010; Staels and Prawitt, 2013; Tomlinson et al., 2002). Next to activation of FXR, BAs signal through another pathway involving TGR5. In BAT, BAs activate the TGR5 G protein-coupled receptor, resulting in increased concentrations of the second messenger cAMP. Elevated concentrations of cAMP activate type 2 deiodinase (D2), which converts the inactive thyroid hormone thyroxine to active 3-5-3′-triiodothyronine, thereby resulting in increased BAT activity and enhanced energy expenditure in murine BAT and human

Table 1. Skin Perfusion, Blood Pressure, and Body Temperatures and Fasting Plasma Levels under Thermoneutral Conditions after Placebo and CDCA Administration

	Placebo	CDCA
Mean skin temperature (°C)	33.8 ± 0.7	33.5 ± 1.1
Core temperature (°C)	37.4 ± 0.1	37.3 ± 0.3
Gradient core-mean skin (°C)	3.3 ± 1.3	3.3 ± 1.4
Insulin (μU/ml)	7.6 ± 2.3	7.2 ± 3.5
Glucose (mM)	4.7 ± 0.3	4.7 ± 0.3
Free FAs (μM)	746 ± 268	586 ± 214[a]
Triglycerides (μM)	1,228 ± 575	1,227 ± 612
TSH (U/l)	1.4 ± 0.8	1.7 ± 1.3
FT4 (pmol/l)	14.1 ± 1.3	13.7 ± 1.1
FT3 (pmol/l)	1.6 ± 0.4	1.6 ± 0.4
CRP	5.2 ± 9.0	2.2 ± 2.3
Total bile acids (μM)	1.24 ± 0.81	12.47 ± 17.82[a]
Total CDCA (μM)	0.45 ± 0.38	10.30 ± 5.41[a]
Total CA (μM)	0.27 ± 0.33	0.15 ± 0.13
Total DCA (μM)	0.30 ± 0.23	0.42 ± 0.28
Total LCA (μM)	0.06 ± 0.00	0.08 ± 0.02
Total UDCA (μM)	0.08 ± 0.08	0.14 ± 0.12[a]
Total HCA (μM)	0.07 ± 0.09	0.15 ± 0.06[a]

Results are expressed as means ± SD. The effect of CDCA administration was tested with a paired-samples t test.
[a]$p < 0.05$, n = 12.

skeletal muscle (Watanabe et al., 2006). Indeed, the primary BA cholic acid increases whole-body energy expenditure in mice (Teodoro et al., 2014; Watanabe et al., 2006). Also in humans, an effect of BAs on energy expenditure has been proposed. Thus, the changes in circulating BAs have been correlated with changes in energy and substrate metabolism upon Roux-en-Y gastric bypass surgery (Ahmad et al., 2013; De Giorgi et al., 2014; Kohli et al., 2013; Patti et al., 2009; Simonen et al., 2012). In a study performed in healthy volunteers and patients with liver cirrhosis, a positive correlation between circulating BAs and energy expenditure was reported (Ockenga et al., 2012). However, the use of the BA sequestrant colesevelam was without effect on resting energy expenditure (Brufau et al., 2010). So far, no effects of BA on BAT activity in humans have been demonstrated, but a recent study in mice showed enhanced BAT thermogenesis when using chenodeoxycholic acid (CDCA) (Teodoro et al., 2014).

Here we investigated if increasing circulating BA levels upon treatment of humans with CDCA would lead to an activation of BAT activity and energy expenditure in human volunteers. To investigate if the effect of CDCA on BAT activity can be attributed to direct activation of human BAT, we cultured adipocytes from the human "BAT" region and examined if CDCA could enhance mitochondrial uncoupling and whether these effects are TGR5- or FXR-specific. We show that the BA CDCA increases BAT activity in humans via a TGR5-dependent mechanism.

RESULTS AND DISCUSSION

Effect of CDCA Supplementation on Circulating BA Levels

To investigate the effect of CDCA supplementation on energy expenditure and BAT activity, we performed a placebo-controlled crossover design study, in which healthy young female volunteers ingested for 2 subsequent days 15 mg/kg CDCA or placebo in the morning after an overnight fast. None of the subjects reported nausea or diarrhea during the course of the study. Fasting total BA levels increased from 1.24 ± 0.81 μM in the placebo intervention to 12.47 ± 17.82 μM in the CDCA intervention group. Plasma levels of CDCA increased approximately 23-fold after CDCA treatment (from 0.45 ± 0.38 μM to 10.30 ± 5.41 μM, $p < 0.05$, Table 1). In contrast, no or only minor changes in absolute levels of the other BA were seen upon CDCA treatment (Figures 1A and 1B, Table 1). CDCA treatment resulted in a significant decline in fasting FFA levels, whereas other blood parameters were unaffected, as summarized in Table 1. These results thus confirm that the intervention was successful in raising circulating CDCA levels.

Effect of CDCA Supplementation on Whole-Body Energy Expenditure

Next, we investigated the effect of 24-hr CDCA treatment on whole-body energy expenditure. Therefore, subjects stayed in the respiration chamber for 24 hr. CDCA treatment did not increase 24-hr energy expenditure (5.7 ± 0.7 kJ/min during placebo intervention versus 5.9 ± 0.9 kJ/min during CDCA intervention, $p = 0.735$). Since 24-hr energy expenditure can be confounded by small changes in, for example, physical activity, we also examined basal metabolic rate the next morning after

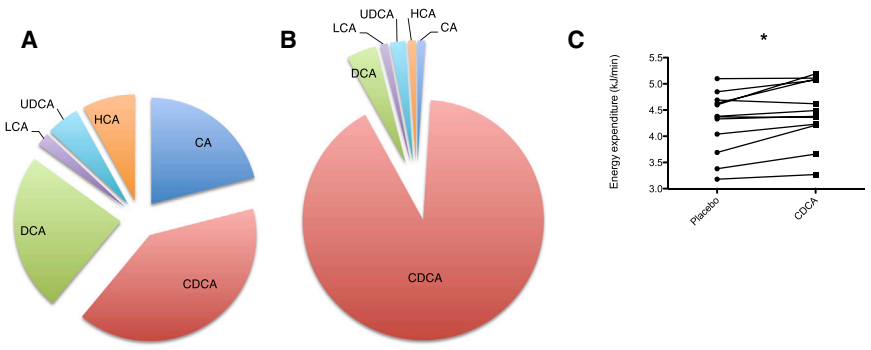

Figure 1. Composition of Bile Acid Pool and Energy Expenditure Following CDCA Intervention

(A and B) Composition of the total bile acid pools in the placebo intervention (A) and CDCA intervention (B) measured by standard LC-MS/MS. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, litocholic acid; UDCA, ursodeoxycholic acid; HCA, hyocholic acid (n = 12).

(C) Basal metabolic rate after 24-hr intervention with bile acid CDCA. *$p < 0.05$ (n = 12).

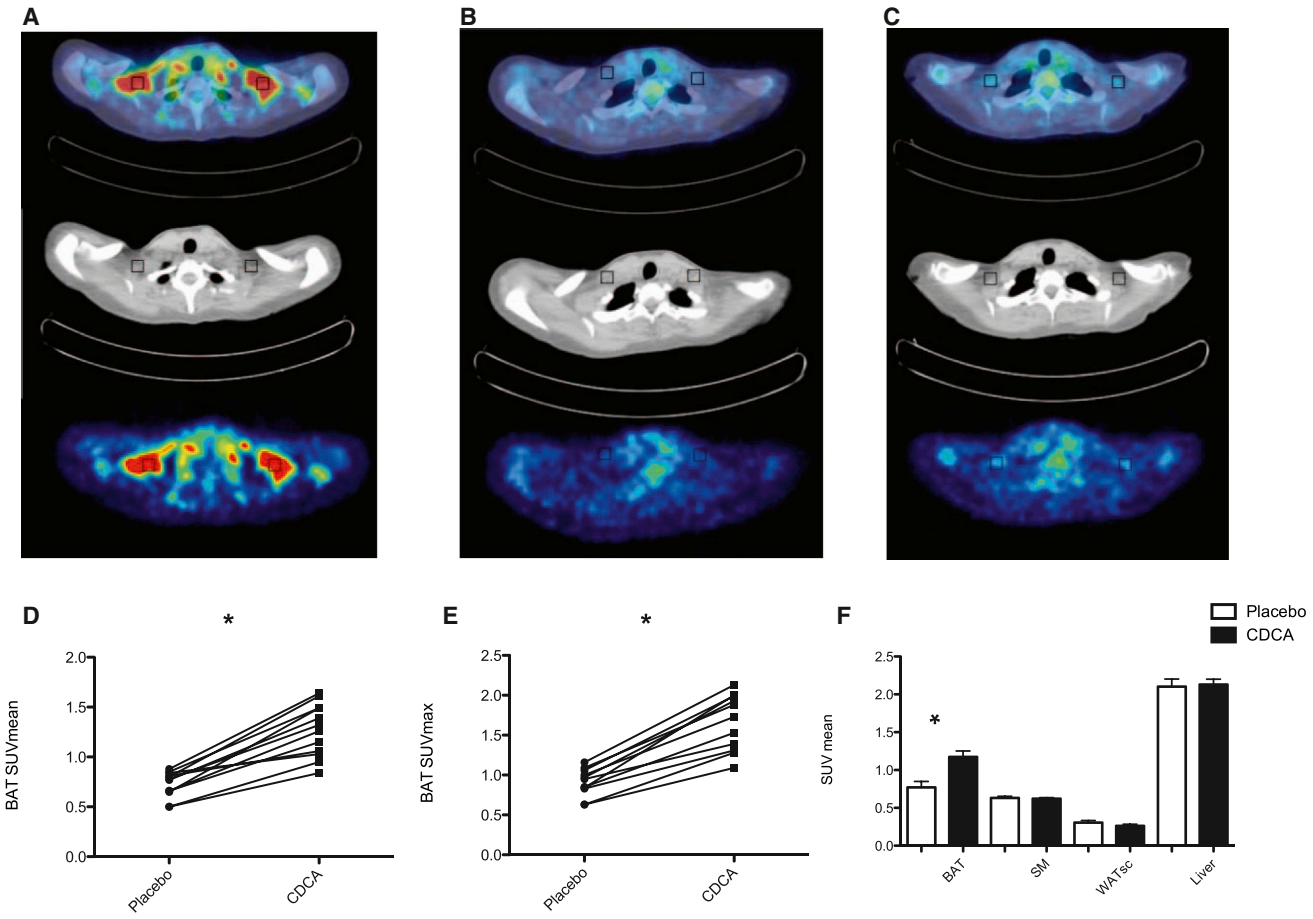

Figure 2. BAT Activity Following CDCA Intervention

(A–C) Representative PET/CT (upper pictures), CT (middle pictures), and PET (lower pictures) scans of one subject after mild cold exposure (A), placebo (B), and CDCA (C) intervention. The cubes indicate the VOIs with maximum [^{18}F]FDG uptake.

(D and E) BAT SUV mean (D) and BAT SUV max (E) were measured under thermoneutral conditions.

(F) [^{18}F]FDG uptake in BAT, WAT, muscle, and liver after placebo and CDCA intervention. *$p < 0.05$ ($n = 12$). Results are expressed as mean ± SEM.

ingestion of the second dose of 15 mg/kg CDCA or placebo. Basal metabolic rate was significantly higher by approximately 5% after CDCA administration compared to the placebo intervention (4.5 ± 0.6 kJ/min versus 4.3 ± 0.6 kJ/min, $p < 0.05$, Figure 1C). Although no long-term effects of CDCA on body weight have been described so far, theoretically a permanent 5% increase in resting metabolic rate could have major effects on body weight regulation and metabolic health if it would not be compensated by increased energy intake.

Effect of CDCA Supplementation on BAT Activity

Recently, functional brown adipose tissue was discovered in adult humans (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). However, the role of BAT activation in the battle against the negative metabolic health effects of obesity still needs to be established. Moreover, alternative ways to activate BAT in humans are needed, as cold exposure may not be the strategy of first choice to combat obesity and its complications. We therefore next examined if CDCA treatment increases BAT activity under thermoneutral conditions (i.e., in the basal state). Therefore, we performed PET-CT scanning 3 hr after the last ingestion of placebo and CDCA, under thermoneutral conditions (e.g., Figures 2B and 2C). We found that both BAT mean standardized uptake values (SUVmean) (from 0.8 ± 0.3 to 1.2 ± 0.3, $p < 0.05$, Figure 2D) and BAT SUV max (from 1.0 ± 0.4 to 1.6 ± 0.4, $p < 0.05$, Figure 2E) were increased after CDCA ingestion, whereas ^{18}-Fluoro-Deoxy-Glucose ([^{18}F]FDG) uptake in skeletal muscle, subcutaneous white adipose tissue (WAT), and the liver did not change (Figure 2F). Although a strong relation between BA and BAT activity and NST has been confirmed in rodents (Watanabe et al., 2006), human evidence for such relation is still lacking. In the current study, we show in adult human subjects a highly significant activation of BAT upon administration of 15 mg/kg body weight CDCA compared to placebo, which was accompanied by a significant ~5%–6% increase in energy expenditure in the basal, resting state. However, it should be noted that it cannot be excluded that BAs increase energy expenditure via BAT-independent mechanisms. Furthermore, apart from its thermogenic potential, BAT has also been associated with improved glucose

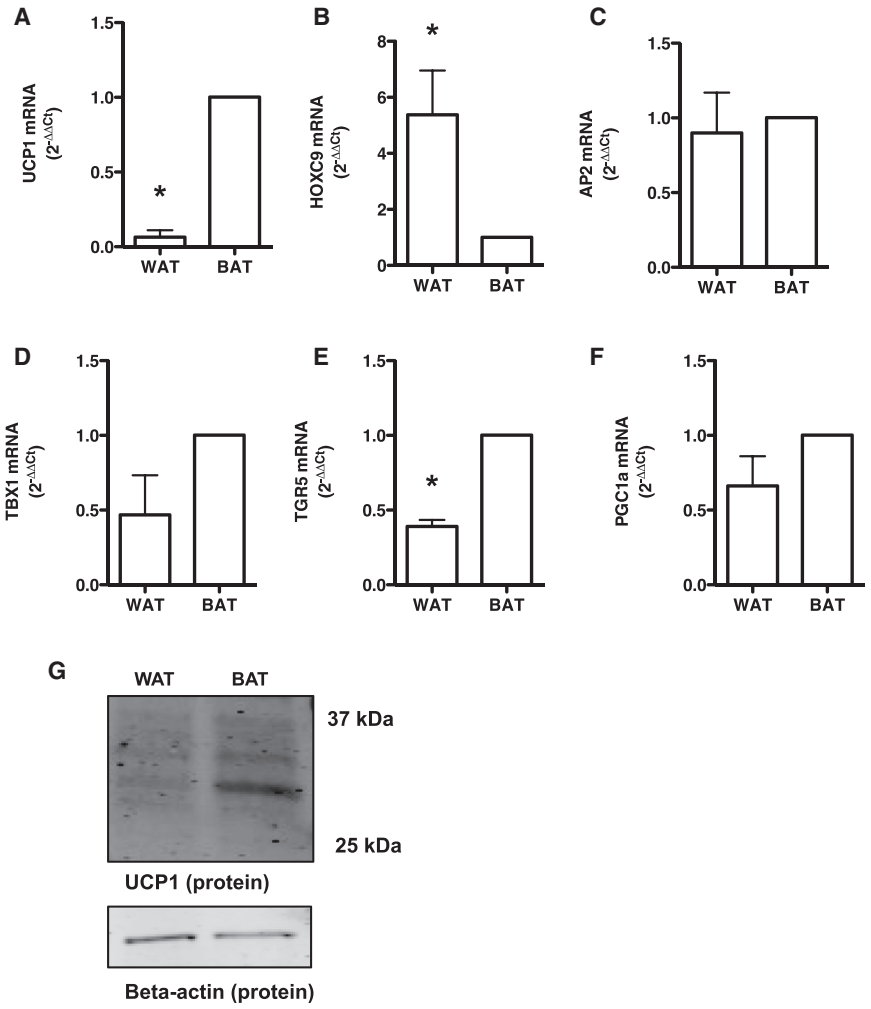

Figure 3. Gene and Protein Expression in Adipocytes Derived from WAT and BAT Biopsies

(A–G) mRNA expression of *UCP1* (A), *HOXC9* (B), *AP2* (C), *TBX1* (D), *TGR5* (E), and *PGC1α* (F) was determined in adipocytes differentiated from WAT and BAT biopsies. UCP1 protein was detected using western blot techniques in adipocytes derived from BAT biopsies (G). mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Results are expressed as mean ± SEM (n = 3–4). *p < 0.05

and lipid handling (Bartelt et al., 2011; Muzik et al., 2012; Orava et al., 2011), but the effects of CDCA on substrate handling could not be tested in this short-term proof-of-concept study. Clearly, long-term human clinical intervention follow-up studies are needed to examine if a sustained activation of BAT activity—probably by other means than CDCA (see below)—is translated into beneficial metabolic health effects.

Although BAT activity increased significantly upon CDCA administration under thermoneutral conditions, the activation of BAT upon CDCA was still markedly less than what could be achieved by an acute cold exposure. Indeed, as a positive control, we measured in all subjects, on a separate test day, cold-activated BAT activity, which revealed that in all subjects BAT could be activated by mild cold exposure (e.g., Figure 2A). As expected, cold exposure had more marked effects on BAT activity as shown by the SUV mean (5.2 ± 3.6) and SUV max (7.2 ± 5.4) values. In fact, acute cold increases BAT activity by ~400% compared to thermoneutral conditions, indicating that the maximal achievable activation in these subjects far exceeds the effects of CDCA. To allow the measurement of low BAT activity under thermoneutral conditions upon CDCA or placebo, we used the fixed volume method (van der Lans et al., 2014), and as a result total BAT volume could not be determined. Therefore, the effect of CDCA on total BAT activity is difficult to estimate. It is important to note that CDCA may not be the most potent BA to activate BAT nor to activate TGR5; the choice to use CDCA in the current study was solely based on the fact that this compound is registered as a drug and could therefore be safely administered to our human volunteers as a proof of concept that BA are able to activate BAT in humans. Therefore, other BA or TGR5 agonist could be tested for the effects on BAT activity in humans.

CDCA Has Direct Effects on Mitochondrial Uncoupling in Human Brown Adipocytes

From rodent and cell experiments, it is suggested that BAs may have a direct effect on BAT metabolism via interaction with the TGR5 receptor. Since CDCA may not be the most potent BA to activate BAT and more potent TGR5 agonists are in clinical development, we next wanted to establish if the effects of CDCA ingestion on BAT activity observed in our human intervention could be due to direct effects on human brown adipocytes via TGR5 activation. To this end, we employed a human in vitro model and prepared human adipose tissue samples from subcutaneous fat and from the prevertebral BAT region. Samples were obtained during thyroid surgery and both white and BAT samples were taken from the same subject. Mature adipocytes were differentiated from the isolated stromal vascular fractions. To characterize the differentiated cells, we measured mRNA expression levels of brown/beige and white adipose tissue markers. mRNA expression levels of the brown/beige markers *UCP1*, *TBX1*, and *TGR5* were significantly higher in adipocytes derived from BAT biopsies (Figures 3A, 3D, and 3E), whereas the white adipocyte marker *HOXC9* was significantly higher in adipocytes derived from WAT biopsies (Figure 3B). Furthermore, UCP1 protein abundance was also higher in adipocytes derived from BAT biopsies (Figure 3G). We next measured oxygen consumption in these in vitro differentiated cultured adipocytes while ATP synthase was blocked using oligomycin (state 4o), thereby focusing

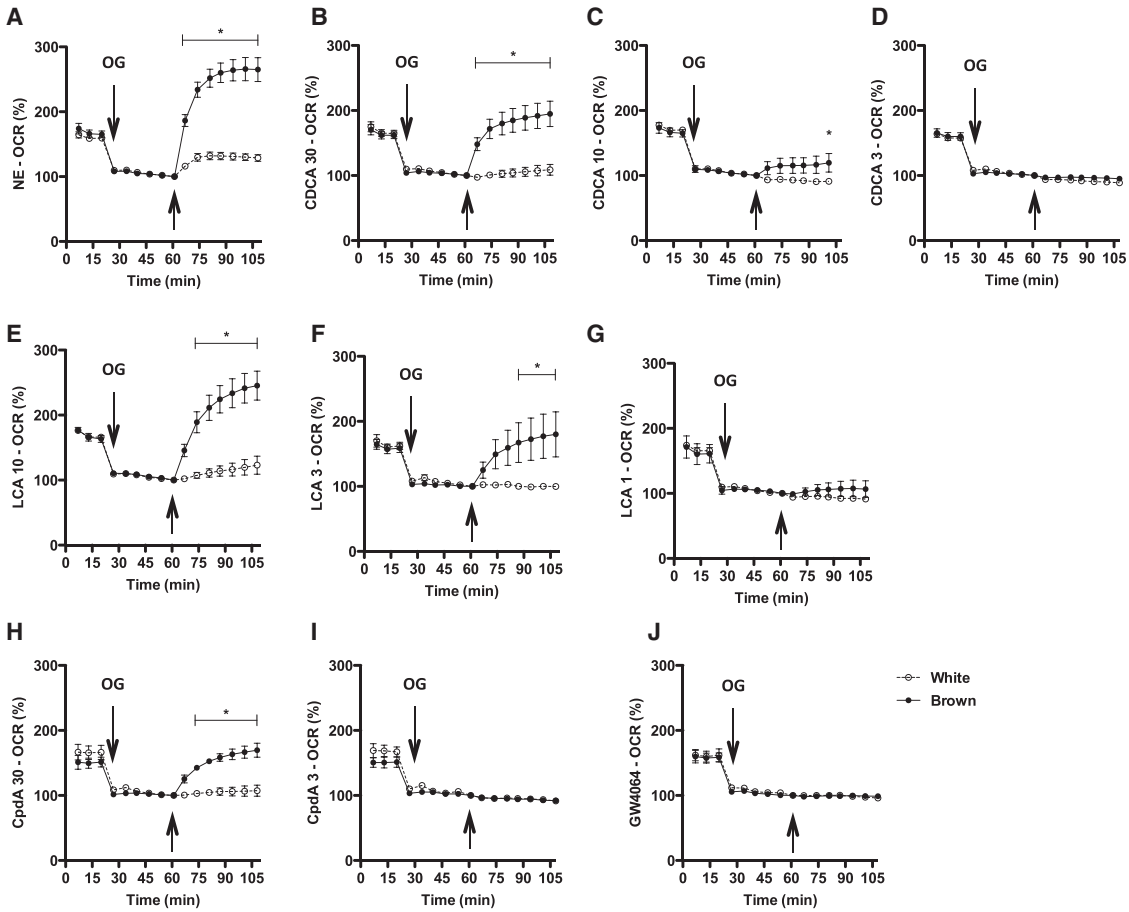

Figure 4. CDCA Increases Mitochondrial Respiration in BAT In Vitro in a TGR5-Dependent Manner

(A–J) Respiration was measured in oligomycin (OG)-treated white adipocytes (white circles) and brown adipocytes (black circles) following 1 μM norepinephrine (A), 30 μM CDCA (B), 10 μM CDCA (C), 3 μM CDCA (D), 10 μM LCA (E), 3 μM LCA (F), 1 μM LCA (G), 30 μM TGR5 agonist CpdA (H), 3 μM TGR5 agonist CpdA (I), or 3 μM FXR agonist GW4064 (J) at t = 61 min, indicated by the upward pointing arrow. Results are expressed as mean ± SEM. *$p < 0.05$ (n = 3–4). See also Figures S1 and S2.

on the capacity for mitochondrial uncoupling. As a proof of concept that human primary brown adipocytes have capacity for mitochondrial uncoupling, we administered NE while ATP synthase was blocked using oligomycin (state 4o) and showed that NE only increased mitochondrial uncoupling in brown adipocytes (from 60.5 ± 4.7 to 187.5 ± 21.4 pmol/min) and not in white adipocytes (from 72.4 ± 1.8 to 85.3 ± 6.9 pmol/min) (Figure 4A), illustrating the characteristic uncoupling properties of "brown" (but not white) adipocytes. Thereafter we examined if CDCA could directly activate BAT. CDCA at 10 μM and 30 μM, but not at 3 μM, increased oxygen consumption under oligomycin-treated conditions in brown adipocytes, whereas such an effect was absent in white adipocytes, demonstrating that CDCA activates BAT (Figures 4B–4D). Of note, the absolute increase in mitochondrial uncoupling upon 30 μM CDCA was 2-fold (from 62.8 ± 4.1 to 125.9 ± 1.7 pmol/min) and thus lower when compared to the 3-fold increase upon NE. We also evaluated the effect of the BA LCA on mitochondrial uncoupling in the white and brown adipocytes. LCA increased mitochondrial uncoupling in brown but not in white adipocytes at 3 and 10 μM (Figures 4E–4G). BAs have been shown to be able to activate the nuclear receptor FXR and G-coupled protein receptor TGR5. To investigate the receptors implicated in the uncoupling effects of CDCA, we used specific FXR and TGR5 agonists. The TGR5 agonist Compound A (CpdA) increased mitochondrial uncoupling at 30 μM, specifically in brown adipocytes (Figures 4H and 4I), without an effect in white adipocytes. CpdA displays high affinity toward TGR5 as measured in a TGR5 activation assay (Figure S1) and FXR activation assay (Figure S2). In contrast, the FXR agonist GW4064 did not increase oxygen consumption in either white or brown adipocytes (Figure 4J). Consistent with a lack of the FXR agonist on mitochondrial uncoupling, we were unable to detect FXR mRNA expression in brown adipocytes (data not shown). These in vitro results clearly show that specific TGR5 activating compounds have the potential for mitochondrial uncoupling in human brown adipocytes, thereby confirming the results of CDCA administration on BAT activity that were observed in vivo. Interestingly, the in vitro effect was only observed in primary adipocytes derived from the human neck region. Although the debate is

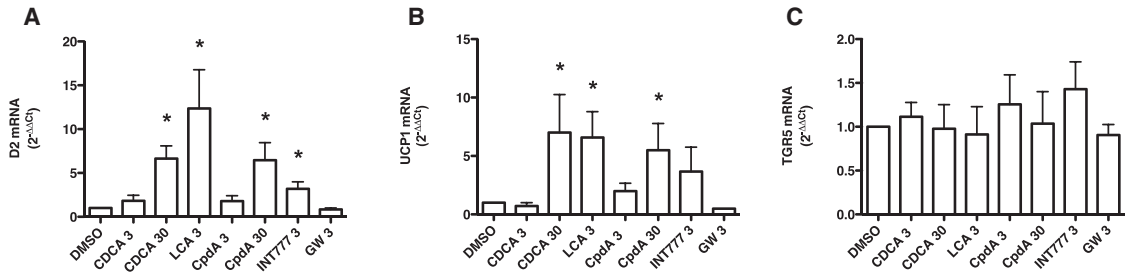

Figure 5. CDCA Stimulates the TGR5 Signaling Pathway in BAT In Vitro

(A–C) White and brown adipocytes were stimulated for 24 hr with 3 μM CDCA, 30 μM CDCA, 3 μM LCA, 3 μM TGR5 agonist CpdA, 30 μM TGR5 agonist CpdA, 3 μM INT777, and 3 μM GW4064. Gene expression for *D2* (A), *UCP1* (B), and *TGR5* (C) was determined. *$p < 0.05$ ($n = 3$). Results are expressed as mean ± SEM. See also Figures S1 and S2.

still ongoing if these adipocytes belong to the classical BAT or are an intermediate "beige" adipocyte (Waldén et al., 2012; Wu et al., 2012), we clearly demonstrate that the adipocytes cultured from the adipose tissue of the human BAT region have a marked different response to CDCA, LCA, TGR5 agonists, and/or NE when compared to "white" adipocytes cultured from the adipose tissue of subcutaneous fat of the same donors. Again, this is consistent with our in vivo finding that CDCA administration only increased glucose uptake in the BAT region, and not in any other tissue including white adipose tissue.

CDCA Activates the TGR5 Pathway in Human Brown Adipocytes

Next, we aimed to provide further evidence that CDCA acts via activation of TGR5 in human brown adipocytes. Activation of the TGR5 signaling pathway increases cAMP, leading to activation of D2. Since the amount of human BAT that can be obtained to culture primary human brown adipocytes is too limited to perform extensive characterization of the underlying mechanism, we examined whether BA and TGR5 agonists can stimulate *D2* expression as a proxy of TGR5 stimulation. A 24-hr treatment with 30 μM CDCA increased *D2* expression in human primary brown adipocytes, whereas 3 μM CDCA was inactive, in line with its EC_{50} value for TGR5 activation (Figure 5A); similar observations were made when brown adipocytes were incubated with the TGR5 agonist CpdA, INT777, or LCA. Again, GW4064 had no effect on *D2* mRNA expression (Figure 5A). A characteristic of BAT is the ability to uncouple ATP production via UCP1. Therefore, we next examined if CDCA can alter *UCP1* expression in brown adipocytes. A 24-hr treatment with 30 μM CDCA increased *UCP1* expression, while again no effect was detected upon 3 μM CDCA (Figure 5B). Similarly, *UCP1* expression increased following treatment with the TGR5 agonists CpdA or LCA in brown adipocytes (Figure 5B). The FXR agonist GW4064 was without effect on *UCP1* expression in brown adipocytes (Figure 5B). *TGR5* mRNA expression remained similar following a 24-hr treatment with BA (CDCA, LCA), TGR5 agonists (CpdA, INT777), or the FXR agonist GW4064 (Figure 5C). Taken together, these results show that human primary brown adipocytes increase mitochondrial uncoupling following CDCA treatment, which is likely dependent on TGR5 and not FXR. These findings suggest that BAs or pharmacological activators of TGR5 may be attractive targets to activate BAT in humans.

In conclusion, the present study shows that administration of 15 mg/kg body weight CDCA activated BAT under thermoneutral conditions; furthermore, the increase in BA was accompanied by increased energy expenditure. This effect was specific for human brown adipocytes and not observed in white adipocytes. Although more and prolonged intervention studies are warranted, the BA CDCA seems to be a potential activator for BAT. Administration of BA supplements may be an effective method for counteracting obesity and related metabolic diseases, by increasing BAT activity and potentially energy expenditure.

Table 2. Subject Characteristics

Age (year)	22 ± 3
BMI (kg)	21.9 ± 1.6
Body mass (kg)	62.2 ± 8.3
Body fat (%)	27.5 ± 6.1
Fat mass (kg)	17.2 ± 4.2
Fat free mass (kg)	43.0 ± 6.6

Values are expressed as means ± SD ($n = 12$).

EXPERIMENTAL PROCEDURES

Subjects

The study was reviewed and approved by the ethics committee of Maastricht University Medical Center (METC 11-3-029, NL44774.068.13). Written informed consent was obtained from 13 healthy female subjects. All subjects were treated according to the standards of the Declaration of Helsinki of 1975, as revised in 1983. All subjects were studied between February 2014 and June 2014.

A total of 13 healthy female subjects were enrolled in the study. Subject characteristics are shown in Table 2. All subjects were screened for medical history and status. Subjects had stable physical activity for at least six months. All subjects used a specific oral contraceptive pill (levonorgestrel/ethinylestradiol) for at least one month prior to participation in the study. Subjects who were psychologically unstable and subjects with mental retardation or severe behavior disorders were excluded from the study. Other exclusion criteria were pregnancy and/or lactation; the use of beta-blockers; the use of ursodeoxycholic acid, antacids, and/or BA sequestrants; participation in an intensive weight-loss program or vigorous exercise program during the last year

before the start of the study, and drugs and/or alcohol abuse. Furthermore, participation in earlier research or medical examinations that include PET/CT scanning was an exclusion criterion. One subject showed pathological [^{18}F]FDG uptake in the brown fat regions on the PET/CT scans during both thermoneutral conditions. Also, this subject reported sweating and showed inadequate thermoregulatory response during cold exposure. This may indicate that the subject suffered from an autonomous dysregulation, and therefore the subject was referred to her GP for further investigation and excluded from further analyses. Study registration: EudraCT number 2013-002512-27.

Study Design

We used a randomized, double-blinded, placebo-controlled crossover design, with BAT activity and energy expenditure as primary outcome parameters. Subjects came to the university for two 2-day intervention periods. On each occasion, subjects came to the laboratory after an overnight fast. Upon arrival, first an intravenous catheter was inserted in the antecubital vein, after which baseline blood samples were taken. Then subjects entered the respiration room and ingested 15 mg/kg body weight of CDCA or placebo alongside a standardized breakfast. In the respiration room, energy expenditure was constantly monitored. During the measurements subjects wore standardized clothes (socks 0.02 clo, shirt with short sleeves 0.19 clo, sweatpants 0.28 clo, sweatshirt with long sleeves 0.34 clo, underwear 0.04 clo, total clo factor 0.88 clo) (Bligh and Johnson, 1973). The temperature inside the respiration room was kept constantly at 23°C to ensure thermoneutrality. Subjects received three standardized meals during their 24-hr stay in the respiration chamber, and subjects remained fasted from 10 p.m. onward. On the next morning, a blood sample was taken in the overnight-fasted condition, after which subjects received another dose of 15 mg/kg body weight CDCA or placebo. Thereafter, subjects were moved to the department of nuclear medicine and placed on a bed at room temperature (23°C). To guarantee a comfortable position during the measurements, subjects were placed in a semi-supine position. Indirect calorimetry was used to measure energy expenditure during the two hours of the measurements. After the first hour of measurements, 50 MBq of [^{18}F]FDG was injected through a venous catheter. After injection, subjects were instructed to lie still to prevent uptake of FDG in the muscles. Measurements were continued for another hour, after which the subject was moved to the PET/CT scanner (Gemini TF PET-CT, Philips) for quantification of metabolically active BAT. Three hours after CDCA ingestion, another blood sample was drawn. All measurements were repeated after one week, and the order of the placebo and CDCA measurements was randomly assigned in a crossover design (Anderson, 1980), so that each subject received CDCA on one occasion and placebo on the other occasion. A minimal washout period of 7 days was taken between the experiments. The investigator and subjects were blinded to the treatment allocation during the experiment.

On a separate day, subjects came to the laboratory after an overnight fast for the determination of body compensation using dual X-ray absorptiometry (DXA, Hologic, type Discovery A), and to determine maximal BAT activity under cold-exposed conditions, as we have described previously (van der Lans et al., 2013).

PET Scanning Protocol and Analysis

One hour before the PET-CT scan, subjects were intravenously injected with 50 MBq (1.35 mCi) of [^{18}F]FDG. Imaging started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a PET scan. A total of 6–7 bed positions (5 min per bed position) were necessary to cover the area where BAT is usually found (i.e., abdominal, thoracic, and neck region). The PET image was used to determine the [^{18}F]FDG uptake, and the CT image was used for PET attenuation correction and localization of the [^{18}F]FDG uptake sites.

The scans were analyzed using PMOD software (version 3.0; PMOD Technologies). Two researchers (EPMB, BB) interpreted the PET/CT images.

We used the fixed-volume method (van der Lans et al., 2014) to determine the average standard uptake value (SUV mean) and maximum standard uptake value (SUV max) in pairs of right- and left-sided volumes of interest (VOIs) of 8×8×8 mm size. Analyses were carried out on scans after cold exposure, placebo, and CDCA intervention. VOIs were placed and analyzed in supraclavicular BAT, subcutaneous WAT, skeletal muscle, liver, and brain. The same anatomic locations were used in the analysis between all three experiments, as described earlier by our group (van der Lans et al., 2014). The SUV of BAT was measured and averaged over the right and left supraclavicular fat depots by placing the VOIs right and left in the area of maximum SUV activity within the depot, with verification of fat tissue by appropriate negative Hounsfield units (HU) for fat tissue. To determine the average [^{18}F]FDG uptake in the deltoid muscle and the triceps and biceps muscle, we analyzed the middle (thickest) part of these muscles in both arms. The uptake in the erector spinae muscle was measured in the cervical (C-7), thoracic (Th-8), and lumbar (L-3) part of the muscle. The subcutaneous WAT was measured in the dorsolumbar region near vertebra L-3. The middle part of the liver was selected for analysis of liver uptake, and we avoided uptake in the large blood vessels by carefully distinguishing between the liver segments. Adipose tissue was distinguished from muscle tissue by means of HU on CT scan (adipose tissue −10 to −180 HU, skeletal muscle 15 to 100) (van der Lans et al., 2013; Vosselman et al., 2012).

Blood Analysis

Blood was collected from the antecubital vein for analyses of several blood variables. Plasma concentrations of free fatty acids (NEFA-HR set; Wako Chemicals), free glycerol (Glycerol kit; R-Biopharm), total glycerol (ABX Triglyceriden CP; Horiba ABX), and glucose (ABX Glucose HK CP; Horiba ABX) were measured on a COBAS PENTRA centrifugal spectrophotometer (Horiba ABX). Plasma triglyceride concentrations were calculated by subtracting free glycerol from the total glycerol concentrations, and serum insulin was analyzed on a Gamma Counter (2470 Automatic Gamma Counter Wizard2 Wallac; Perkin-Elmer) with a Human Insulin specific RIA kit (Millipore). Serum TSH was measured by an Electrochemiluminescence Immunoassay Kit on a COBAS 6000 system (Roche Diagnostica), and free thyroxine (FT4) was analyzed by a solid-phase time-resolved fluoroimmunoassay kit on an AutoDELFIA system (PerkinElmer). Plasma inflammatory marker C- reactive protein (CRP) was measured with a particle-enhanced immunoturbidimetric assay on a COBAS PENTRA system (Horiba ABX). BA concentrations were determined in plasma by phase liquid chromatography associated to tandem mass spectrometry (LC-MS/MS, in Multiple Reaction Monitoring [MRM] mode) after extraction by protein precipitation. The use of internal deutered standards allowed the quantification.

Human Primary Adipocyte Cultures

The collection of stromal vascular fraction was obtained from BAT and subcutaneous white adipose tissue from the same individual during thyroid surgery in patients with a normal thyroid function using a collagenase digestion and was reviewed and approved by the ethics committee of Maastricht University Medical Center (METC 10-3-012, NL31367.068.10).

Written informed consent (n = 4) was obtained before surgery. Collected cells were grown to confluence before differentiation was initiated. The stromal vascular fraction derived from BAT was incubated with bFGF (4 ng/ml) during 2 days before confluence was achieved. Differentiation was initiated for 7 days via differentiation medium made up out of biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethasone (100 nM), IBMX (250 μM), rosiglitazone (5 μM), T3 (2 nM), and transferrin (10 μg/ml). Specifically for the differentiation of the stromal vascular fraction derived from BAT, BMP4 (4 ng/ml) was included in the differentiation medium. Cells were transferred to maintenance medium consisting of biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethasone (10 nM), T3 (2 nM), and transferrin (10 μg/ml) until mature adipocytes had formed.

In Vitro Oxygen Consumption in Differentiated Adipocytes

The stromal vascular fraction derived from adipose tissue biopsies was plated in XF96-well cell culture plate. After cells were differentiated into mature adipocytes as described in the previous section, oxygen consumption and mitochondrial function were measured using the XF96 extracellular flux analyzer from Seahorse Biosciences. Cells were incubated for 1 hr at 37°C in unbuffered DMEM (2 mM GlutaMAX, 1 mM sodium pyruvate, 1.85 gl^{-1} NaCl, and 25 mM glucose). Basal oxygen consumption was measured followed by injection of 2 μM oligomycin and subsequently followed by injection of the compounds of interest. Oligomycin and CDCA, GW4064, and LCA were purchased from Sigma-Aldrich. The TGR5 agonist CpdA 3-(2,6-difluorobenzylthio)-5-(3,4-dimethoxybenzyl)-4-(4-fluorophenyl)-4H-1,2,4-triazole was

synthetized according to patent WO2010/093845. Data are plotted as a percentage compared to uncoupled respiration following oligomycin.

RNA Isolation and Gene Expression Analysis

Total RNA was extracted from mature adipocytes using the miRNEasy kit from QIAGEN according to the manufacturer. cDNA was created using the high-capacity RNA-to-cDNA-kit from Applied Biosystems. Gene expression data were normalized to TATA box-binding protein (*TBP*) and further analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers for *D2* (Hs00988260_m1) and *UCP1* (Hs00222453_m1) were from Applied Biosystems. SYBR-green qPCR primers were as follows: *PGC1α*: forward primer 5′-TGCTGAAGAGGGAAAGTGA GCGATTAGTTGA-3′, reverse primer 5′-AGGTGAAAGTGTAATACTGTTGGTT GA-3′; *AP2* (*FABP4*): forward primer 5′-CCTTTAAAAATACTGAGATTTCCT TCA-3′, reverse primer 5′-GGACACCCCCATCTAAGGT-3′; *HOXC9*: forward primer 5′-GGACTCGCTCATCTCTCACG-3′, reverse primer 5′-AAAATCGCTA CAGTCCGGCA-3′; *TGR5*: forward primer 5′-CAGTGTCGACCTGGACTTGA-3′, reverse primer 5′-TAACGGCCAGAGGAGCTTTA-3′. Primers for *TBP* and *TBX1* have been described previously (Wu et al., 2012).

Western Blot Analysis

Brown adipocytes were harvested in Laemmli buffer. Proteins were separated using SDS-PAGE followed by transfer to nitrocellulose membranes. Proteins were detected and quantified using the Odyssey from LI-COR Biosciences. The antibody against *UCP1* (ab10983) was purchased from Abcam. Beta-actin (A5316) was detected using an antibody from Sigma-Aldrich.

Statistical Analysis

Statistical analysis was performed by PASW Statistics version 20.0 for MacBook Pro. Reported data are expressed as means ± SD where indicated. The primary endpoints in this study were BAT activity and energy expenditure. Total BAT activity was expressed in kBq and standard uptake values (SUV; as calculated by uptake [kBq/ml] per injected dose [kBq] per patient weight [g]). BAT activity of each region was determined by the average SUV (SUV mean) times the volume of the region (cubic centimeter), expressed as SUV total. Repeated measurements were compared using Student's t test. p values of < 0.05 were considered statistically significant. Group size estimations were based upon a power calculation to minimally yield an 80% chance to detect a significant difference in BAT activity and EE of $p < 0.05$ between the treatments. For qPCR analysis, differences between two means were analyzed using a Student's t test. For respiration experiments, differences were analyzed using a two-way ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.07.002.

AUTHOR CONTRIBUTIONS

E.P.M.B. and E.B.M.N. designed and performed the experiments, analyzed data, and wrote the manuscript. KH.M.R. and G.S. assisted during the experiments. B.H., B.B., N.D.B, F.M., B.S., W.D.v.M.L, and P.S. contributed to the design of the study, analyzed and interpreted the data, and reviewed and edited the manuscript. A.T. and M.K. performed the analyses of total bile acids and bile acid profiles. B.D. and J.C. synthetized the CpdA. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors report no conflicts of interest relevant to this article.

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