Physiological concentrations of β-hydroxybutyrate do not promote adipocyte browning☆

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ABSTRACT

Aims: Previous work has demonstrated that ketogenic diets promote white fat browning; however, the exact mechanisms underlying this phenomenom have yet to be elucidated. Recently, an *in vitro* study showed that supraphysiological concentrations of β-hydroxybutyrate (βHB) had a strong influence on the induction of adipocyte browning. On the other hand, concentrations in the physiological range, achieved through ketogenic diets and prolonged fasting produce values of 1–3 mM and 4–7 mM, respectively. Herein, we investigated the impact of physiological concentrations of βHB on metabolism, and the expression of uncoupling protein 1 (UCP1) and other browning markers in adipose tissues.
Main methods: The effects of βHB on adipocyte browning were investigated *in vitro*, using primary cultures of isolated visceral and subcutaneous fat cells and cultured 3T3-L1 adipocytes, and *in vivo*.
Key findings: It was determined that βHB failed to induce changes in the oxidative capacity, citrate synthase activity or browning gene expression patterns in isolated adipocytes, and did not exert a permissive effect on β-adrenergic agonist-induced browning. In addition, 3T3-L1 adipocytes differentiated following βHB treatment exhibited downregulated *Ucp1* expression levels, a result that was recapitulated in the subcutaneous adipose tissue of Wistar rats after βHB salt treatment. Rats administered βHB salts also presented reduced brown adipose tissue UCP1 protein expression.
Significance: The mechanisms underlying ketogenic diet-induced browning of adipocytes are not known. The results from the present study indicate that physiological concentrations of βHB are not responsible for this phenomenon, despite the observed βHB-mediated downregulation of UCP1 expression.

1. Introduction

The phenomenon of adipocytes expressing uncoupling protein 1 (UCP1) within white fat depots is known as "browning", and can be triggered by β-adrenergic stimulation. When appropriately stimulated, these adipocytes are called beige, and classically referred to as brown adipose tissue (BAT). In response to the elevated UCP1 expression levels and augmented cellular respiration, these cells develop thermogenic characteristics [1].

It is known that the activation of BAT thermogenesis produces beneficial metabolic effects, such as reduced body adiposity and increased insulin sensitivity [2]. Despite not being accepted as the most relevant adipose cells for the activation of body thermogenesis [3], the emergence of thermogenically competent beige adipocytes within white adipose depots also appears to induce the same beneficial metabolic effects as BAT activation [4]. Indeed, inhibiting white adipose tissue (WAT) browning increases the susceptibility of the cells to the development of metabolic dysfunctions, such as obesity, glucose intolerance, and inflammation, and decreases energy expenditure and the uptake of glucose into the tissue. It has also been reported that the transplantation of fats depots, known to be predisposed to browning (*i.e.* inguinal subcutaneous fat), promotes systemic metabolic benefits

in diet-induced obese mice [5].

Previous studies demonstrated that ketogenic diets can effectively stimulate BAT thermogenesis activation and white fat browning [6,7]. However, results from β-adrenergic receptor knockout mice fed a ketogenic diet indicated that BAT thermogenic activation is induced by the sympathetic nervous system, and demonstrated that white fat browning is not impaired [8]. Due to the fact that the lack of β-adrenergic receptors abolishes the tissue response to the sympathetic nervous system and β-adrenergic activation, these results strongly suggest that circulating blood factors produced in response to a ketogenic diet are directly involved in the induction of white fat browning. Indeed, ketone bodies could potentially modulate fat browning, since this serum metabolite is produced during ketogenic feeding.

Exogenous ketone supplementation models suggest that ketones, and not the ketogenic diet, are responsible for the observed UCP1 up-regulation in WAT [9]. Ketone bodies have been classified as browning activator metabolites, and in cultured adipocytes, β-hydroxybutyrate (βHB) has been shown to directly induce the expression of genes related to the browning phenomenon, such as *Ucp1* and *Cidea* [10]. However, these results were observed with supraphysiological concentrations of βHB (15–50 mM), which are only observed during diabetic ketoacidosis, and the effects of physiological concentrations of this ketone body have yet to be investigated.

Caloric restriction, ketogenic diets, and fasting are examples of situations where ketogenesis is activated and ketosis can be detected without the development of ketoacidosis [11,12]. For example, diet manipulations can increase ketonemia levels from below 0.1 mM up to 1–3 mM, and prolonged fasting/starvation and exogenous ketone intake can result in even higher levels (4–7 mM) [12]. Since ketosis can be provoked *in vivo* by dietary interventions, the impact of physiological concentrations of βHB on the metabolism and browning of WAT has extremely important consequences and is worthy of investigation.

2. Methods

2.1. Experiments with isolated adipocytes

2.1.1. Adipocyte isolation

Male specific pathogen free (SPF) Wistar rats (11–12 weeks) from the Animal Resource Center of the Institute of Biomedical Sciencesat the University of São Paulo, in Brazil, were anesthetized with sodium thiopental (4 mg/100 g body weight) and decapitated. Approximately 1 g of the subcutaneous (inguinal) and visceral (epididimal) fat pads were collected, minced into small pieces with scissors and digested in collagenase buffer, as previously described [13]. The concentration of the isolated adipocyte suspensions were determined according to Di Girolamo et al. [14].

2.1.2. Isolated adipocytes treatment

Visceral and subcutaneous adipocytes (3.5×10^6 cells) were incubated in horizontal culture bottles (25 cm^2) containing DMEM media, containing 5 mM glucose, 5% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Incubations with non-toxic concentrations of (±)-Sodium 3-hydroxybutyrate (βHB) (Sigma Chemical, St. Louis, MO, USA), as determined by XTT cytotoxicity assays (data not shown), were performed for 24 or 48 h. Each animal represented one experimental unit ($n = 1$), and the number of experiments performed is provided in the figure legends.

2.1.3. Metabolic capacity of isolated adipocytes

After the βHB treatments, isolated adipocytes were washed and suspended in EHB buffer [Earle's salts, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 4% bovine serum albumin (BSA)]. We then monitored the cells ability to oxidize [2-^{14}C]-Acetate or incorporate it into lipids, as previously described [13]. The results are reported in nmol.(10^6 cells.h)$^{-1}$.

Gene	Sense	Antisense
Ucp1	GGCAAAAACAGAAGGATTGC	TAAGCCGGCTGAGATCTTGT
Ppargc1a	TTGCCCAGATCTTCCTGAAC	TGAGGACCGGCTAGCAAGTTT
aP2	AATCCCCATTTACGCTGATG	TCACCTGGAAGACAGCTCCT
Pparg	CGAGTCTGTGGGGATAAAGC	CAAACCTGATGGCATTGTGA
CycloA	GCCGATGACGAGCCCTTG	TGCCGCCAGTGCCATTATG

2.2. Culture and treatment of 3T3-L1cells

3T3-L1 cells were plated in culture dishes (60 mm diameter), with 1.2×10^6 cells/dish (~57% of dish confluence). The DMEM culture medium contained 25 mM glucose, 1 mM of sodium pyruvate, 3.7 g/L of sodium bicarbonate, 100 U/mL penicillin and 100 mg/mL streptomycin, pH of 7.4 (Gibco). Culturing the cells until confluence and subsequent differentiation was performed according to Zebisch et al. [15]. Day '0' was considered the moment when the differentiation cocktail was added to the culture and also marked the beginning of βHB treatment. After 8 days of the differentiation protocol, adipocytes were easily distinguished by the identification of clear lipid accumulation and expression of mature adipocyte genes (*i.e. aP2* and *Pparg*). All experiments were performed in triplicate.

2.3. In vivo studies

Wistar Rats (10 weeks old) consuming a standard diet, received DL-βHB mineral salts (KetoForce by Prototype Nutrition, Savind Inc. - IL, USA) in their drinking water for 4 weeks, as described in Caminhotto et al. [16]. Ketone body concentrations were measured during lights on/off periods with Freestyle Optium XCEED (Abbott Diabetes Care, Alameda, CA, USA) and Freestyle Optium β-Ketone strips (see Caminhotto et al. [16] for more details). The treatment resulted in ketonemia, with an overall increase of 36% (0.65 to 0.89 mM). Animals were then anesthetized and euthanized by decapitation. In addition to interscapular BAT, perirenal (prWAT), subcutaneous inguinal (scWAT) and epididymal (epWAT) white fat pads were collected, frozen in liquid nitrogen and stored at −80 °C.

2.4. Maximal citrate synthase activity

Following βHB treatment, extraction buffer, containing 50 mM Tris-base, 1 mM EDTA, pH 7.4, was added, and the cells were homogenized using a vortex. Reactions were initiated by adding 10 μL of the sample and oxaloacetate to the reaction buffer (100 mMTris-base, 0.2 mM DTNB, 0.1 mM Acetyl-CoA and 1% Triton) This methodology was adapted from ALP et al. [17]. Enzymatic activity was calculated from the slope of absorbance curve (change in absorbance at 412 nm over time). Protein concentration was quantified using the Bradford assay [18], and used to normalize the enzymatic activities.

2.5. Gene expression

Gene expression was assessed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). For isolated adipocytes, cells were homogenized with TRIzol® Reagent immediately after βHB treatment (24 or 48 h) and stored at −80 °C. For white and brown adipose tissue, 100 mg of tissue was used. The PureLink® RNA Mini Kit (Ambion by Life Technologies, Cat. No. 12183-018A, USA) was used for RNA extraction. RNA was quantified using an Epoch (Biotek®) or NanoDrop (Thermo Scientific) spectrophotometer. Briefly, 2 μg of RNA was treated with 0.32 μL of DNAse (0.4 U/μL in DNAse buffer) and 4.2 mM MgCl$_2$ in a final volume of 20 μL for 30 min at 37 °C. The DNase was then inactivated by incubating the samples at 75 °C for 10 min. These DNA-free RNA samples were then added to a solution containing
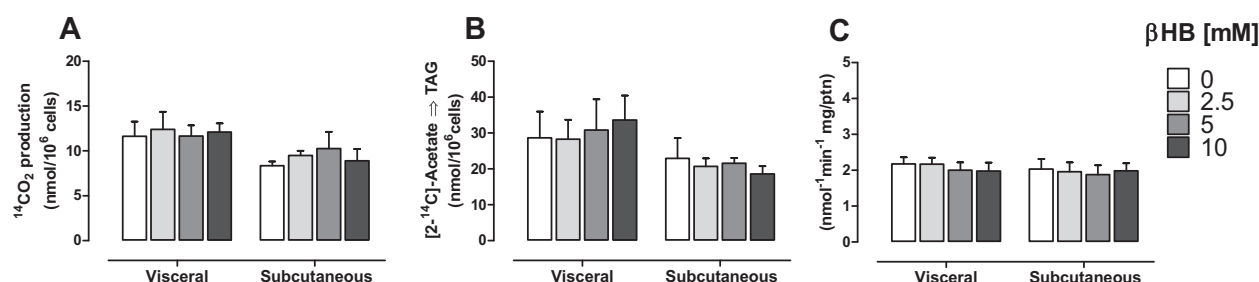
Fig. 1. Metabolic markers of isolated fat cells. Visceral and subcutaneous adipocytes were incubated with physiological concentrations [0, 2.5, 5 and 10 mM] of the ketone body βHB for 24 h, and the (A) oxidative, (B) lipogenic capacities, as well as (C) maximal citrate synthase activity were evaluated. No significant differences were detected. When evaluating oxidative and lipogenic capacities $n = 4$ for visceral and $n = 3$ for subcutaneous, from 4 independent experiments, and for experiments measuring enzymatic activity $n = 5$ from 3 independent experiments. One-way ANOVA was used to compare the treatment concentrations *vs.* respective point 0. Data are presented as the mean $\pm$ SEM.
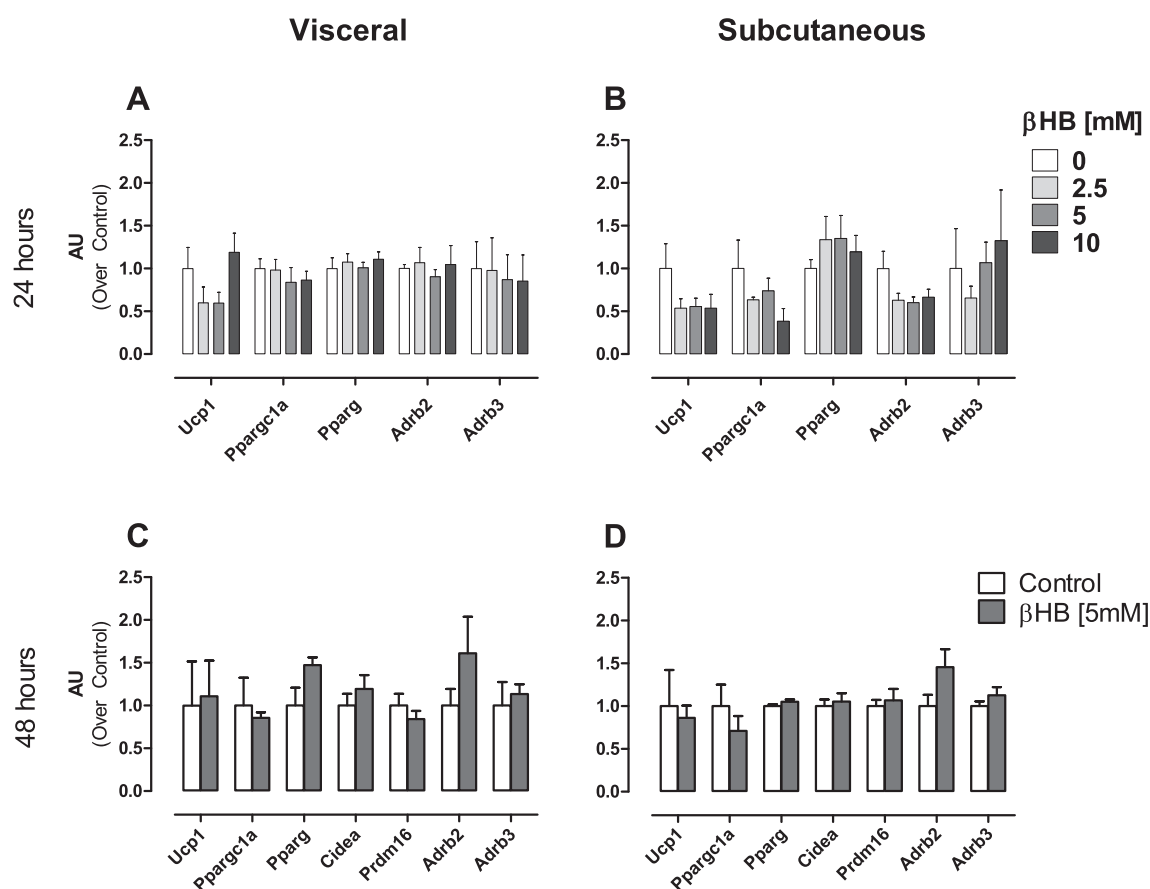

Fig. 2. Browning gene expression pattern in isolated fat cells following βHB treatment. Visceral and subcutaneous adipocytes were incubated with physiological concentrations of the ketone body βHB for 24 (A, B) or 48 (C, D) hours. Typical gene expression found during browning stimulation did not occur. For the experiments that lasted 24 h n = 5 from 3 independent experiments, and for experiments that lasted 48 h n = 4 from 1 experiment. One-way ANOVA was used to compare treatments *vs.* respective point 0 in panels A and B, and Student's *t*-test was used to evaluate the data presented in panels C and D. Data are presented represent as the mean $\pm$ (SEM).

0.5 μL of Superscript® III enzyme (Invitrogen) in 10 μL of 5× buffer (1 enzyme: 20 buffer), 5 μL of 100 mM DTT, 10 μL of a dNTP mix (containing 2.5 mM of each base) and 1.3 μL of Random Primer (3 μg/μL). The final reaction volume for each sample was 50 μL. Complementary DNA (cDNA) was synthesized by reverse transcription (RT) in a thermocycler (Eppendorf®, Germany) under the following cycling conditions: 10 min at 25 °C, 50 min at 50 °C, and 15 min at 70 °C. RT-PCR was performed using 40 ng of cDNA and pre-designed TaqMan Gene Expression Assays (Applied Biosystems, UK) for Uncoupling protein 1 (*Ucp1*, Rn00562126_m1), Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Ppargc1a*, Rn01430718_m1), Peroxisome proliferator-activated receptor gamma (*Pparg*, Rn00440945_m1), Cell death-inducing DFFA-like effector a (*Cidea*, Rn04181355_m1), PR domain containing 16 (*Prdm16*, Rn01516224_m1), Cytochrome c oxidase subunit VIIIb (*Cox8b*, Rn00562884_m1), Adrenoceptor beta 2 (*Adrb2*, Rn00560650_s1), Adrenoceptor beta 3 (*Adrb3*, Rn00565393_m1) and Beta-2 Microglobulin (*B2m*, Rn00560865_m1) or Actin Beta (*Actb*, Rn00667869_m1). *B2m* or *Actb* were used as the internal control genes. BAT was employed as a positive control for genes related to browning. RT-PCR amplifications were carried out in a StepOnePlus® Real Time PCR System (AppliedBiosystems) for 40–60 cycles. Relative quantification of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method.
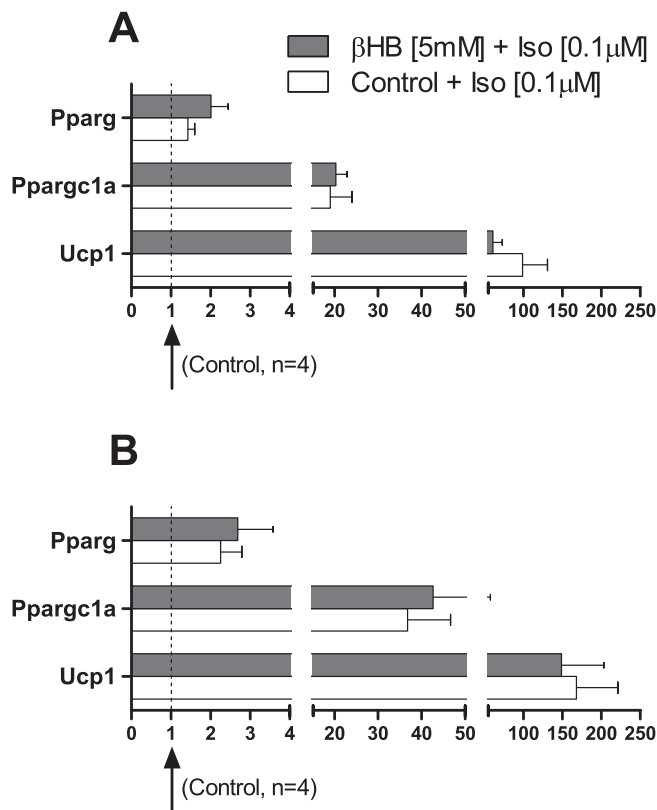
Fig. 3. Effect of βHB on β-adrenergic-induced browning. (A) Visceral and (B) subcutaneous adipocytes were treated with 5 mM βHB for 24 h, washed and incubated with 0.1 μM of the β-adrenergic agonist isoproterenol for 6 h. Typical gene expression found during browning stimulation occurred; however, no permissive effect of previous incubation with the ketone was found. n = 4 from 1 experiment. Student's *t*-test. Data are presented as the mean ± SEM.
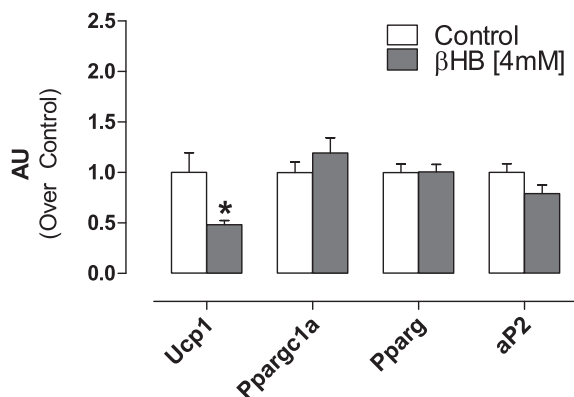

Fig. 4. Effect of βHB on *Ucp1* gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were differentiated in the absence or presence of 4 mM βHB. *Ucp1* mRNA levels decreased by 52%. No differences were found in *Ppargc1*, *Pparg* and *aP2* gene. n = 6 from 6 independent experiments. * *P* < 0.05. Student's *t*-test. Data are presented as the mean ± SEM.

For 3T3-L1 cells, homogenization was achieved with QIAzol® Lysis Reagent. RNA was extracted according to the recommendations of the manufacturer. RNA quantification was done with an Epoch (Biotek®) spectrophotometer. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) with 2 μg of RNA. The RT mix contained 1 μL of MultiScribe® Reverse Transcriptase (50 U/μL) in 2 μL of 10× RT buffer, 0.8 μL of 25× dNTP (100 mM) and 1 μL of OligodT (50 μM). PCR was performed using QuantiFast SYBR Green PCR kit (Qiagen GmbH, Dusseldorf, Germany) in a Rotor Gene-Q

device (Qiagen) for 40 cycles. Primers sequences are provided in Table 1. CycloA was used as internal control gene.

2.6. Western blotting

White and Brown fat pads, stored at −80 °C, were homogenized in a buffer composed of 135 mM NaCl, KCl 2.7 mM, 1.02 mM MgCl$_2$, 10 mM EDTA, 5 mM Na$_4$P$_2$O$_7$, 0.01 mM NaF, 5% Triton X-100 (from 20% stock solution), 13% glycerol (from 75% stock solution), 20 mM Tris 20 mM, 0.002 mM phenylmethylsulfonyl fluoride, 0.003 mM Na$_3$VO$_4$, 0.1 mM Okadaic acid and 0.002 mM protease inhibitors cocktails.

The homogenate was centrifuged (12,000 rpm, 40 min, 0 °C), the supernatant was collected and total protein (excluding the fat cake) was quantified using the Bradford assay [18]. Equal amounts of total protein (6 μg for BAT and 30 μg for WAT) were diluted in Laemmli buffer and separated on 8% polyacrylamide gels (100 V). The proteins were then transfered from the gels to nitrocellulose membrane (2 h, 80 V). The membranes were stained with ponceau-S to confirm transfer, and to determine the efficiency of protein transfer. After blocking the membranes, an Anti-UCP1 (ab23841) primary antibody (1:1000) was added and incubated overnight at 4 °C. The following day the blots were washed and then incubated with a secondary antibody conjugated to peroxidase (1:5000). Immunoreactivity was then visualized using the enhanced chemiluminescence (ECL) in a Syngene G:BOX® imaging system. The intensity of the bands on the blots was measured using the Syngene G:BOX® software and the results were expressed in arbitrary units (AU). All results were normalized to the constitutive expression of β-Actin or Vinculin.

3. Results

3.1. Effect of physiological concentrations of βHB on isolated adipocyte browning

It was hypothesized that adipocytes submitted to a browning stimulus would exhibit an increase in cellular respiration and mitochondrial biogenesis markers, as well as a specific gene expression pattern. In order to test this hypothesis, we first evaluated the effects of physiological concentrations of the ketone body βHB [0, 2.5, 5, 10 mM] on the oxidative and lipogenic capacities of the adipocytes. Acetate is a well-utilized metabolic substrate of adipose tissue [19]. Herein, we employed [2-^{14}C]-Acetate to assess the mitochondrial capacity, since it is a precursor of acetyl-CoA and its utilization circumvents the initial reactions required for the oxidation of glucose and fatty acids, up to citrate acid cycle. Moreover, maximal citrate synthase activity has been used as a marker of mitochondrial function because it is well correlated with mitochondrial mass and oxidative capacity [20,21] and is considered a proxy of mitochondrial density [22].

As shown in Fig. 1, neither visceral nor subcutaneous adipocytes exhibited significant differences in the oxidative (A) and lipogenic (B) capacities, and failed to display changes in maximal citrate synthase activity (C) after 24 h of incubation with physiological concentrations of βHB (2.5–10 μM).

Previous studies have shown that following a browning stimulus there is an upregulation in the expression of *Ucp1, Cidea, Ppargc1 and Prdm16*. However, after 24 h no significant changes in the expression of *Ucp1, Ppargc1a, Pparg, Adrb2* or *Adrb3* were detected in visceral (Fig. 2A) or subcutaneous (Fig. 2B) samples, at any of the βHB concentrations tested (2.5–10 mM), when compared to controls (0 mM). Furthermore, when exposing similar samples to 5 mM βHB for 48 h, similar results were observed (Fig. 2C and D). Thus, the results indicated that physioloigical concentrations of βHB were not capable of altering the expression of genes related to the browning phenotype or mitochondrial biogenesis.

Next we investigated the effect of the β-adrenergic agonist isoproterenol on *Pparg, Ppargc1a* and *Ucp1* expression in isolated
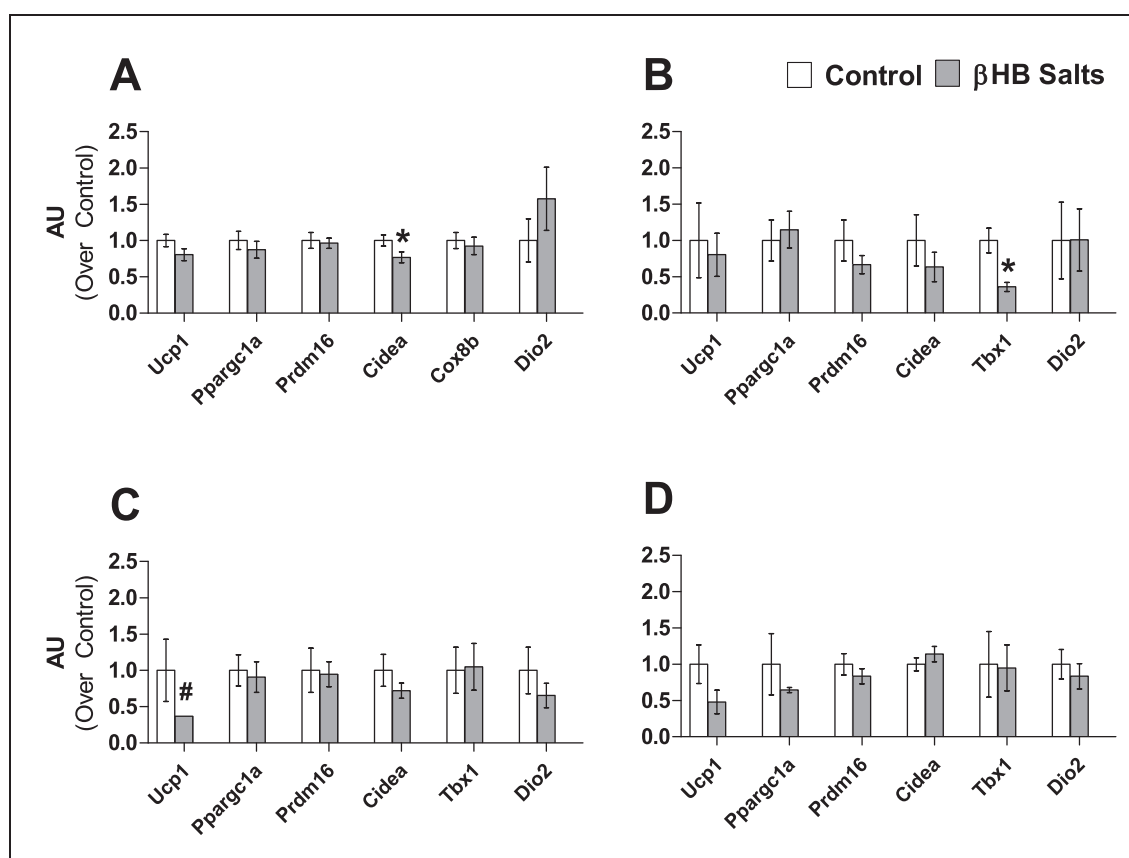
Fig. 5. Effect of βHB ingestion on browning marker genes in different types of adipose tissues. Wistar rats were administered DL- βHB mineral salts for 4 weeks and the gene expression analyses were performed with different fat pads: (A) BAT, (B) prWAT, (C) scWAT and (D) eWAT. *Cidea* was downregulated by 23% in BAT, *Tbx1* was downregulated by 64% in prWAT, *Ucp1* was downregulated by 63% in scWAT (8 out of 9 samples where *Ucp1 +*) and no significant differences in gene expression were observed in eWAT. $n = 9$ from 2 independent experiments. Student's *t*-test. *$p > 0,05$; **$p > 0,01$; # only one sample was *Ucp1* positive in treated group. Data are presented as the mean ± SEM.

adipocytes from visceral (Fig. 3A) and subcutaneous fat pads (Fig. 3B) and the effect of βHB on β-adrenergic-induced browning. As expected, subcutaneous fat cells responded with higher expression levels of *Ucp1* and *Ppargc1a*, when compared to control cells. No permissive effect of previous incubation with the ketone was found.

3.2. Ucp1 expression in 3T3-L1 adipocytes following βHB exposure

In order to define whether long-term treatment with a physiological concentration of βHB could induce a browning gene expression pattern, 3T3-L1 adipocytes were differentiated in the presence of 4 mM βHB. As shown in Fig. 4, *Ucp1* gene expression was attenuated by 52% in 3T3-L1 adipocytes ($p > 0.05$) treated with the ketone body. On the other hand, the expression levels of *Ppargc1a*, which encodes for the PGC1α protein and is a critical regulator of mitochondrial biogenesis, was not altered. Additionally, *Pparg* and *Ap2*, both markers of adipogenesis, were not significantly different following ketone body treatment. Taken together the results suggest that the control and treated adipocyte cells exhibit similar rates of differentiation.

3.3. Effects of in vivo βHB salt ingestion on white fat depot browning and UCP1 expression

It was previously reported that the oral administration of βHB salts significantly increased ketonemia in the absence of any other dietary intervention [16]. Thus, we used this *in vivo* animal model to evaluate the isolated effect of ketones on browning markers in white and brown fat pads.

Analyzing the expression of browning genes (*Ucp1, Ppargc1a, Prdm16, Cidea, Dio2*) and a specific beige adipocyte gene (*Tbx1*) revealed the expression of some *browning* related genes was found to be fat pad specific (Fig. 5). For example, *Cidea* was downregulated by 23% in BAT (Fig. 5A), *Tbx1* was downregulated by 64% in prWAT (Fig. 5B), *Ucp1* was downregulated by 63% in scWAT (only 8 of 9 samples where *Ucp1 +*) (Fig. 5C) and no significant differences in gene expression were observed in eWAT (Fig. 5D).

Lastly, we evaluated UCP1 protein content, which is indicative of thermogenic programming, by western blot analyses (Fig. 6A). It was found that scWAT and epWAT, both in the absence and presence of βHB, were UCP1 negative. In BAT, UCP1 expression was significantly lower (-34%), while there was no significant differences detected in prWAT (Fig. 6B). There were also no differences in maximal citrate synthase activity when comparing control to βHB treated animals (Fig. 6C). Notably, different types of fat pads exhibited different maximal citrate synthase activities, with the highest activity being detected in BAT (Fig. 6C).

4. Discussion

Previous experiments performed with high concentrations of ketone bodies, showed that βHB is a strong inducer of browning [10]. This result could, at least partially, explain the thermogenic program triggered in the WAT of animals fed a ketogenic diet [6–8]. However, in the present study, our results failed to support these conclusions.

Under adequate stimulation, adipocytes respond with the development of a beige phenotype in diverse experimental models. Herein, the
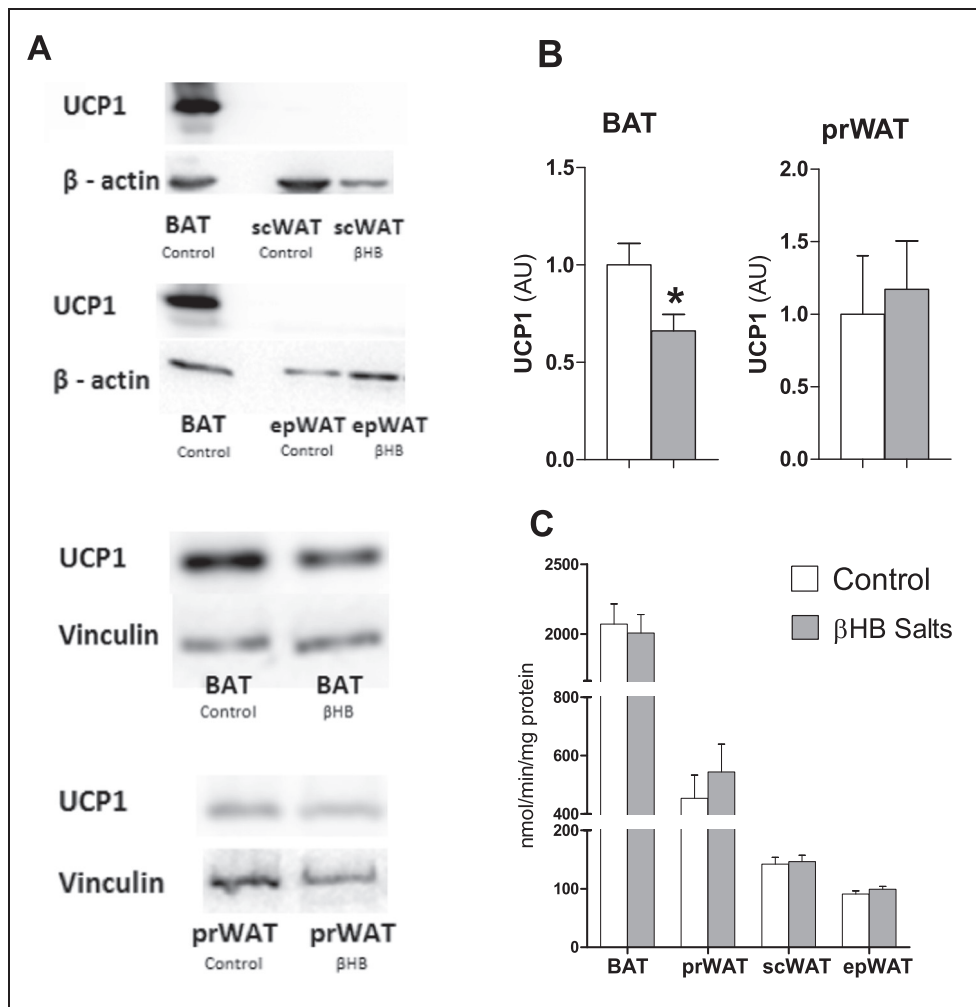
Fig. 6. Effect of βHB ingestion on browning protein markers in different types of adipose tissue. Wistar rats were administered DL- βHB mineral salts for 4 weeks, and UCP1 protein expression was evaluated in different fat pads. (A) Representative western blots of UCP1 expression in scWAT, epWAT, BAT and prWAT. scWAT and eWAT were found to be UCP1 negative; (B) UCP1 expression in BAT and prWAT. UCP1 expression was downregulated in BAT and unchanged in prWAT. n = 6–7 from 2 experiments. (C) Maximal citrate synthase activity in BAT, prWAT, scWAT and epWAT. There were no significant alterations were detected in any of the analyzed samples. n = 9 from 2 experiments. Student's t-test. *p > 0,05. Data are presented as the mean ± SEM.

β-adrenergic agonist increased the expression of genes related to the thermogenic program and mitochondrial biogenesis of white adipocytes (Fig. 3). It is plausible that the same effects could also occur in 3T3-L1 cells and in vivo, since different compounds and experimental conditions were already evaluated in similar models [23–25]. However, at physiological concentrations, βHB was ineffective at inducing the browning of adipocytes. The ketone body treatments also did not permit β-adrenergic agonist-induced browning. Furthermore, it was observed that 3T3-L1 adipocytes, differentiated under the βHB treatment displayed downregulated Ucp1 gene expression, while certain adipocyte differentiation markers were left unchanged (Fig. 4), thus indicating normal adipogenesis with fewer signs of a beige phenotype. Furthermore, in inguinal scWAT, our in vivo model of ketone supplementation also presented downregulated Ucp1 gene expression (Fig. 5C). Additionally, we detected reduced Tbx1 gene expression in prWAT (Fig. 5B), which was previously shown to be a specific marker of beige adipocytes [1]. Taken together these results suggest that βHB inhibits the browning of adipocytes. Interestingly, prWAT was the only white fat depot that was positive for UCP1 expression, as evidenced by the western blot analysis (Fig. 6A) and displayed the highest citrate synthase activity (Fig. 6C), when compared to other WATs. These results are consistent with previous studies reporting elevated browning markers in prWAT, when compared with other fat pads in both mice and rats [24,25].

While our results are not in accordance with Carriere. et al., [10], another study showed that an acute infusion of βHB, to achieve physiological ketosis (~1.2 mM), decreased UCP1 mRNA levels in the

kidney tissue of mice [26]. It is conceivable that this ketone body could impair browning markers (i.e. UCP1 expression), since βHB is a ligand of a Gi-protein-coupled receptor. This receptor family decreases cAMP formation by inhibiting adenylate cyclase, and antagonizing β-adrenergic receptor signaling. It should be pointed out that besides representing a significant pathway of browning induction, β-adrenergic activation also promotes the activation of lipolysis. For that reason, ketones are recognized as lipolytic inhibitors in vitro [27]. It was previously reported that rats supplemented with βHB salts exhibited a negative correlation between serum lipolysis products and ketonemia [16], indicating a possible inhibition of lipolysis through the activation of a Gi-protein-coupled receptor by ketones, which may be in turn responsible for the observed downregulation of UCP1 expression in the present study.

Our results are also in agreement with the idea that ketone bodies are a signaling metabolite of energy restriction, which through anti-catabolic effects influence the physiological axes to conserve energy. For example, reduced energy mobilization by lipolysis inhibition, spare glucose and amino acid utilization, and decrease energy expenditure by lowering the sympathetic tone [28]. Indeed, such βHB-related anti-catabolic effects could account for the observed effects on browning markers and the attenuated UCP1 expression (−34%) in the BAT of βHB supplemented animals.

As shown by Srivastava et al. [9], activiation of the BAT thermogenic program, in rats supplemented with exogenous ketones, may be dependent on other factors present in that model. For example, in that study, ketonemia exceeds the physiological concentrations found in the

ketogenic diet (0.5–3 mM) and reached a peak of 7 mM. We do not know whether there is an *in vivo* threshold for ketonemia that must be exceeded for the induction of the browning phenomenon, as observed *in vitro* by Carriere et al., [10]. Therefore, it is necessary to search for and identify other agents, besides ketones, that could account for the high thermogenic program associated with the ketogenic diet in animal models. It is conceivable that the ketogenic diet promotes hormonal interactions that result in the browning phenomenon. For example, high concentrations of fibroblast growth factor 21 (FGF21) have been detected in animals fed a ketogenic diet, and this hormone was previously shown to be a mediator of several metabolic adaptations associated with ketosis [8,29]. Additionally, it was reported that FGF21 enhances the thermogenic program in WAT, both *in vitro* and *in vivo* [24], and could the responsible for ketogenic diet-induced browning. It is also worth mentioning that the ketogenic diet, as well as the aforementioned ketone ester diet [9], provoke an intense reduction in serum insulin levels [7], and that insulin can effectively reduce mitochondrial respiration and uncoupling in BAT and subcutaneous inguinal adipose tissue [30]. Thus, the involvement of other endocrine/metabolic events cannot be completely ruled out.

5. Conclusion

In conclusion, while the causes of ketogenic diet-mediated browning remain inconclusive, our study demonstrated that physiological concentrations of βHB are not responsible for this phenomenon. In fact, under certain conditions, this ketone body can downregulate UCP1 gene and protein expression, as well as other browning gene markers in BAT and WAT.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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