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**Effects of exogen ketosis induced by 1,3-butanediol on
adipose organ and hippocampus**

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INTRODUCTION

The Ketone bodies

Ketone bodies (KBs) represent a group of three small, hydrophilic molecules of low molecular weight: acetone (Ac), β -hydroxybutyrate (β -OHB), and acetoacetate (AcAc) (Figure 1). KBs are produced endogenously from free fatty acids by the liver during periods of limited glucose availability, such as starvation or carbohydrate restriction, and serve as an alternative fuel source for the brain and peripheral tissues [1]. Their biosynthesis occurs primarily in the liver [2], where fatty acid-derived acetyl-CoA serves as the main substrate for ketogenesis [3]. However, increasing evidence now indicates that extrahepatic tissues also possess a limited ketogenic capacity, including intestinal crypts [4], kidneys [4], T cells [5], astrocytes [6,7], and adipocytes [8]. The capacity of these tissues or cell populations to produce KBs depends on the expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), the key rate-limiting enzyme that governs ketogenesis [3].

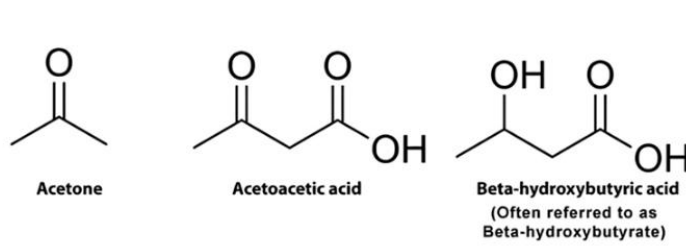


Figure 1. **Ketone bodies structure**

Under physiological conditions, circulating KBs range from 0.05 to 0.3 mM, but their concentrations markedly increase during fasting, prolonged exercise, or ketogenic diet adherence, reaching up to 5–7 mM without causing ketoacidosis [1,9]. During extended starvation or uncontrolled type 1 diabetes, β -OHB levels may even exceed 10 mM, indicating the onset of ketoacidosis [1].

Syntheses, transport, utilization of Ketone bodies

When lipolysis predominates over lipogenesis, elevated free fatty acids enhance acetyl-CoA (Ac-CoA) availability, which under carbohydrate restriction is diverted from the Krebs cycle toward ketogenesis [3]. Within the mitochondrial matrix, thiolase, HMG-CoA synthase (HMGCS2), and HMG-CoA lyase sequentially convert Ac-CoA into acetoacetate (AcAc), which can be reduced to β -OHB or spontaneously decarboxylated to acetone [10]. β -OHB is the most abundant ketone in the circulation

[11] and the AcAc/ β -OHB ratio in sera reflects the hepatic redox state, serving as a marker of metabolic balance [11]. Ketogenesis is closely coordinated with fatty acid β -oxidation and regulated by hormonal and transcriptional cues, including glucagon and PPAR α signaling, which upregulate HMGCS2 expression during fasting [11] and by insulin that suppress lipolysis and HMGCS2 activity. Collectively, these mechanisms ensure that ketogenesis operates as a rapid and flexible metabolic adaptation to nutrient fluctuations, maintaining energy homeostasis under both physiological and stress conditions [12].

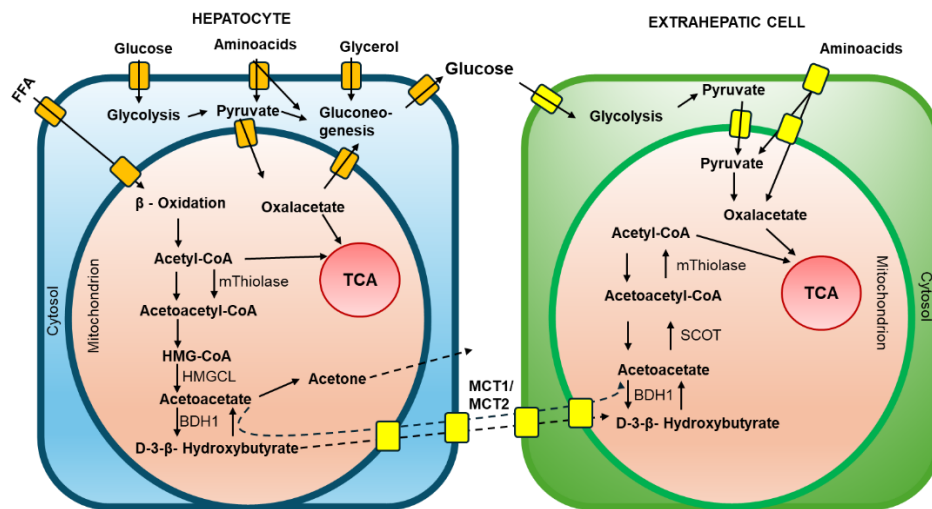


Figure 2. Overview of the ketogenesis and ketolysis pathways described in the text FFA, free fatty acids; mThiolase, mitochondrial thiolase; HMGCS2, hydroxy methylglutaryl-CoA synthase; HMGCL, HMG-CoA lyase; BDH1, mitochondrial β OHB dehydrogenase; MCT1/2, monocarboxylate transporter 1 and 2; SCOT, succinyl-CoA:3-oxoacid-CoA transferase; CS, citrate synthase

KBs synthesized in the liver are exported via monocarboxylate transporters (MCT1, MCT2), delivered to the bloodstream and directed to extrahepatic organs. The expression of MCT1 and MCT2 increases during fasting or energy stress, facilitating the redistribution of hepatic ketones to peripheral tissues [13]. Upon arrival to the mitochondria of distant organs, ketone bodies become utilized for energy, where they are converted back to acetyl-CoA and oxidized in the Krebs cycle [11,14].

The first step of ketolysis involves succinyl CoA-oxoacid transferase (SCOT), which converts acetoacetate to acetoacetyl CoA and represents the rate-limiting step for the utilization of ketones for energy scope. High concentrations of acetoacetate feedback negatively on SCOT to decrease ketone conversion. Finally, acetoacetyl CoA is converted to acetyl CoA by methylacetoacetyl CoA thiolase. Acetyl CoA may be turned

into citrate and churned through the citric acid cycle to produce FADH₂ and NADH, or it can be converted to oxaloacetate and used in gluconeogenesis.

While β -OHB and AcAc serve as efficient energy substrates for the brain, heart, skeletal muscle, and kidney, the liver cannot oxidize them, due to the absence of succinyl-CoA:3-oxoacid CoA transferase (SCOT). Even the erythrocytes are unable to use KBs for energy purpose as they lack mitochondria.

As an energetically efficient pathway operating inversely to lipogenesis, ketone oxidation yields more ATP per unit of oxygen than fatty acids, providing a metabolic advantage under hypoxic or high-demand conditions, particularly in the heart and brain [15,16].

Beyond their energetic role, KBs function as signaling metabolites that modulate cellular redox status and metabolic homeostasis in distant organs [1,17]. This function is described in the following paragraphs.

Nutritional ketosis

Ketogenesis and the utilization of KBs as alternative energy substrates represent adaptive metabolic responses that become particularly relevant during fasting, prolonged exercise, or carbohydrate restriction [11,16].

Nutrient intake during meals triggers significant changes in ketone metabolism, through actions that influence pancreatic hormones and catecholamines, as well as molecular changes at the organ level. Following a carbohydrate-rich meal, increased circulating glucose levels promotes insulin release from pancreatic β cells, which not only stimulates glucose utilization in peripheral tissues [18] but also suppresses lipolysis in adipose tissue and the subsequent delivery of fatty acids to the liver, thus reducing ketogenesis. Conversely, diets high in fatty acids and low in carbohydrates, often referred to as ketogenic diets, promote ketogenesis rather than attenuate it, since the low carbohydrate content limits the insulin response of pancreatic islet β cells and the resulting antilipolytic effects.

Although nutritional ketosis can be achieved through adherence to a ketogenic diet, or fasting this approach often suffers from low compliance rates due to its restrictive nature. Moreover, long-term adherence may predispose individuals to nutritional imbalances, including deficiencies in essential micronutrients such as vitamins, minerals, and dietary fiber, unless carefully managed and supplemented. For these

reasons, exogenous ketone formulations, such as ketone salts, ketone esters, and some β -OHB precursors, such as 1,3-butanediol, have emerged as valuable tools to induce ketosis and ketosis related signalling effect, without dietary restriction, thus providing an alternative means to manipulate metabolism for potential therapeutic purposes across a range of chronic diseases [19,20]. Exogenous induced ketosis does not reduce insulin nor augment fatty acid oxidation [21].

In recent years, the global interest for such supplements have expanded rapidly, driven by claims of enhanced mental clarity, improved physical performance, and appetite regulation [22]. Although β -OHB can be delivered in salt form, its clinical use is limited by the associated high mineral load, which may increase blood pressure and cause adverse metabolic effects [23,24].

Among the precursors of β -OHB is 1,3-butanediol (BD), a Food and Drug Administration approved organic diol widely used as a food flavoring solvent and considered a potential synthetic food for long-duration space missions [25]. Extensive toxicological studies have confirmed that BD is safe and well tolerated [26]. BD represents an alternative strategy to induce ketosis without carbohydrate restriction [27,28], since, once ingested, BD undergoes hepatic oxidation to β -OHB, leading to a dose-dependent elevation in circulating ketone levels with an approximate 6:1 ratio of β -OHB to AcAc [16].

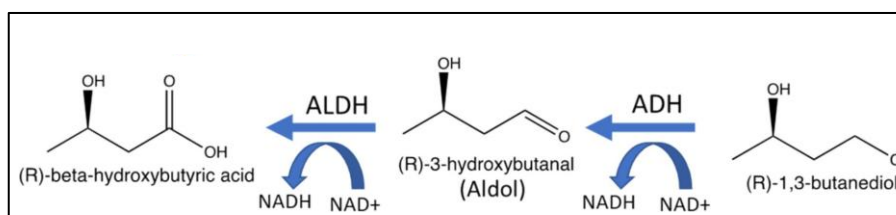


Figure 3. Pathway of β -OHB formation from 1,3-butanediol. In liver the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are involved.

Thus, BD administration allows for a controlled rise in circulating KBs, mainly β -OHB, without the concurrent hormonal alterations typically induced by fasting or very low carbohydrate intake [29]. Beyond its ketogenic role, BD displays several physiological effects, including endothelium-dependent vasodilation [30] anti-inflammatory and neuroprotective actions [31] and improved hypothalamic leptin sensitivity in obese mice [32].

Effects induced by ketone bodies

Traditionally, ketogenic diets have been applied in clinical settings for pharmaco-resistant epilepsy [33] and GLUT1 deficiency syndromes [34]. However, the physiological relevance of KBs extends well beyond their energetic role, with accumulating evidence for pleiotropic effects on redox balance, cellular signaling, and epigenetic regulation [1]. In this context, recent studies have demonstrated that nutritional ketosis, induced by ketogenic diet, fasting, as well as administration of ketone bodies precursor may exert therapeutic benefits across metabolic, cardiovascular, and neurodegenerative disorders [35–37]. These benefits are often attributed to the intrinsic anti-inflammatory actions of KBs and their capacity to drive histone β -hydroxybutyrylation (Kbhb) (see also below) [38]. Consistently, chronic β -OHB elevation has been linked to enhanced mitochondrial biogenesis, improved insulin sensitivity, and reduced oxidative stress, supporting β -OHB as a broader metabolic modulator [39].

Nutritional ketosis has gained attention in sports and performance medicine as a state in which circulating β -OHB levels exceed 0.5 mM, achieved through fasting, ketogenic diets, or exogenous ketone supplementation [40,41]. While studies on exercise performance have yielded mixed results [42–44], evidence from randomized trials and meta-analyses indicates that acute exogenous ketones do not consistently enhance high-intensity or time-trial performance; the effects depend on the compounds administered (ketone ester vs. ketone salt), dose, timing, and intensity, and may be constrained by gastrointestinal tolerance and acid–base shifts [44,45].

At the level of skeletal muscle, AcAc and β -OHB exert systemic metabolic actions, most notably on substrate selection and anti-catabolic pathways [46]. Specifically, ketone infusion suppresses glycolysis while stimulating protein synthesis and attenuating proteolysis [16]. In agreement *vitro* findings show that ketone exposure blunts atrophy signaling, enhances mitochondrial function, and lowers selected ceramide species [47,48]. Importantly, in rodent muscle, R-(but not S) β -OHB can impair insulin-stimulated glucose uptake and fiber-type–specific manner (oxidative > glycolytic), underscoring the relevance of enantiomer-specific effects [49]. During exercise, the escalating ATP demand drives whole-body substrate turnover [50]; inducing ketosis pre-exercise with BD β -OHB monoester can lower carbohydrate

utilization and increase reliance on intramuscular lipids, likely via inhibition of adipose lipolysis and the consequent reduction in circulating FFAs [43].

In the adult brain, glucose is the dominant fuel under normal conditions; nonetheless, classic work showed that, during starvation, ketone bodies can supplant glucose as the main substrate, providing up to two-thirds of cerebral energy [16]. Experimentally elevating circulating ketones, via β -OHB infusion or MCT ingestion, yields a dose-dependent increase in brain ketone uptake and oxidation [51,52], with a reciprocal reduction in cerebral glucose utilization [51]. Mechanistically, KBs cross the blood–brain barrier through MCT1/MCT2, whose expression and transport activity are up-regulated by fasting and energy stress, thereby augmenting cerebral ketone delivery [53]. Beyond serving as oxidative substrates, β -OHB engages neuroprotective signaling consistent with antioxidant and anti-inflammatory phenotypes [54]. Clinically, ketogenic MCT supplementation improved cognition over 6 months in mild cognitive impairment, and ketone-ester protocols in older adults induce robust, safe ketosis with favourable effects on brain metabolic biomarkers, supporting translational plausibility [55,56]. Overall, NK appears to enhance or preserve cognition across diverse preclinical and human settings [57]. In traumatic brain injury (TBI), blunt-trauma models demonstrate reduced tissue damage/edema and improved cognitive recovery with ketogenic interventions, and early clinical data support feasibility and safety; definitive efficacy in sport-related TBI, however, will require larger, multi-setting trials [58].

Ketone metabolism has become increasingly relevant in cardiovascular research. In heart failure (HF), a condition characterized by impaired substrate use and mitochondrial dysfunction, KBs function as efficient oxidative fuels and signaling molecules, enhancing mitochondrial function, attenuating oxidative stress, and dampening inflammation [59–61]. Enhanced ketone oxidation in failing hearts appears to represent adaptive metabolic reprogramming that supports ATP production and preserves contractility [62,63]. Translational and clinical studies further indicate that acute elevation of circulating β -OHB can augment cardiac performance in HF: controlled infusion studies report increased cardiac output (up to ~40%) without compromising myocardial energetic efficiency, consistent with a fuel-supportive, hemodynamically favourable profile [61,64]. Mechanistically, failing myocardium upregulates ketone uptake and oxidation, in line with the relatively high phosphate-to-oxygen (P/O) ratio of ketones, more ATP per unit O_2 consumed, an index of oxidative efficiency, and potential attenuation of inflammation and vascular dysfunction

[15,65,66]. While phosphocreatine/ATP responses may vary by phenotype, these data support a context-dependent therapeutic window for ketone delivery in HF [67].

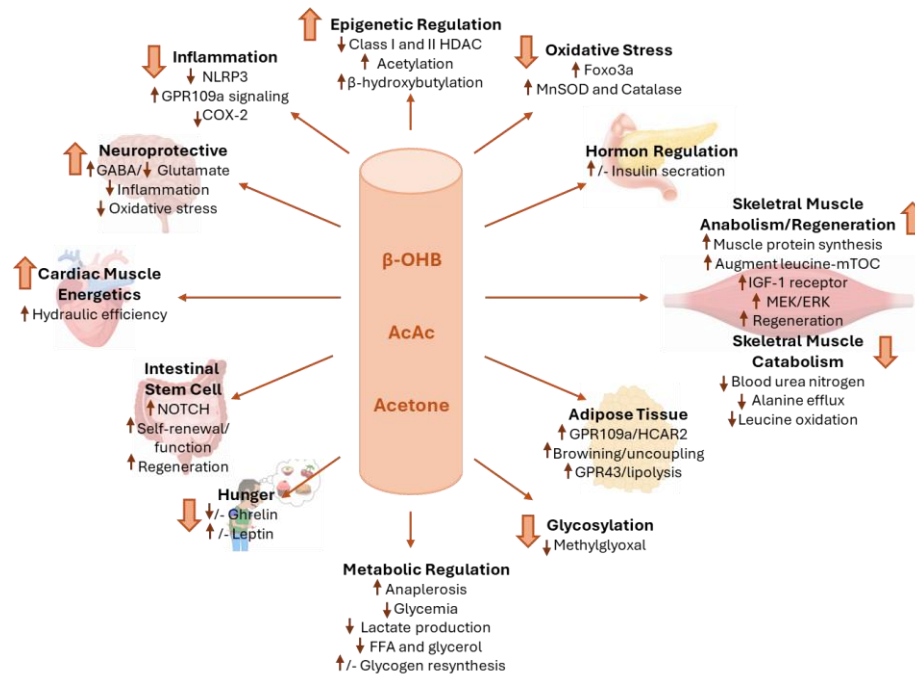


Figure 4 Systemic actions of ketone bodies. β -hydroxybutyrate (β -OHB), acetoacetate (AcAc), and acetone exert pleiotropic effects on multiple tissues, including neuroprotection, improved cardiac energetics, modulation of intestinal stem cell function, regulation of appetite hormones, enhanced insulin secretion, support of skeletal muscle growth/regeneration, and metabolic remodeling in adipose tissue.

Signalling pathway of ketone bodies

As previously stated, ketones are more than just substrates supporting ATP production, as ketones (primarily β -OHB) are also important signalling molecules. KBs are recognized ligands for multiple GPCRs capable of modulating gene expression and inflammatory tone. One of the key GPCRs associated with ketone signalling is GPR109A, also known as hydroxy-carboxylic acid receptor 2. Other GPCRs involved in ketone signalling include GPR41 and GPR43, which have been shown to interact not only with short-chain fatty acids but also with ketones [68]. Notably, β -OHB (but not AcAc) antagonizes GPR41, whereas AcAc has been described as an endogenous ligand for GPR43, decreasing intracellular cAMP. β -OHB can also act independently of GPCRs by blocking NLRP3 [69].

In parallel, KBs directly and rapidly tune adipose lipid metabolism. In adipocytes, R- β -OHB engages HCAR2/GPR109A, dampens NF- κ B signaling, and acutely suppresses lipolysis [70] (Figure 5). Consistent with this, exogenous ketone (EK) delivery, whether via R-BD R- β -HB monoester ingestion or infusion of R- β -HB or racemic β -OHB,

reduces plasma FFAs and glycerol, with or without changes in insulin, in an apparent dose-dependent fashion [43,52,71–73]. By contrast, AcAc can activate GPR43 and promote lipolysis by increasing adipose lipoprotein lipase activity [74]. Consequently, the direction of the lipolytic response depends on both the means of inducing ketosis and the prevailing ketone species: prolonged fasting/KDs generally favor lipolysis and FFA mobilization, whereas EKs that predominantly raise R- β -HB tend to inhibit lipolysis via HCAR2/GPR109A and acutely lower circulating FFAs at rest and during exercise [43,70] (Figure 5). Notably, chronic EK regimens that markedly elevate AcAc have been linked to adipose atrophy not fully explained by reduced caloric intake [75], underscoring the need to delineate how distinct NK paradigms regulate adipose lipid turnover.

Several studies also support the ability of ketones to influence gene expression, which may be mediated via epigenetic regulation secondary to modulating histone deacetylases (HDACs), essential enzymes controlling the acetylation status of histones [16,76]. β -OHB appears to selectively inhibit class I HDACs, and it can remodel chromatin via histone β -hydroxybutyrylation. This modification, mediated by p300 and reversed by HDACs and sirtuins, regulates genes involved in oxidative metabolism, vascular repair, and mitochondrial function [38,77–80]. Kbhb has thus emerged as an adaptive epigenetic mechanism that integrates metabolic state with transcriptional reprogramming [81,82]. In addition to epigenetic actions, anti-inflammatory effects of KBs have been linked to SIRT3 activation and suppression of mitochondrial ROS generation [5]. Through these converging pathways, β -OHB acts as a unifying metabolic signal that coordinates energy metabolism, oxidative balance, and immune regulation [83,84].

Collectively, these findings highlight the multifaceted nature of ketone bodies as not only efficient fuels but also potent regulators of mitochondrial function, gene expression, and immune homeostasis [1,11].

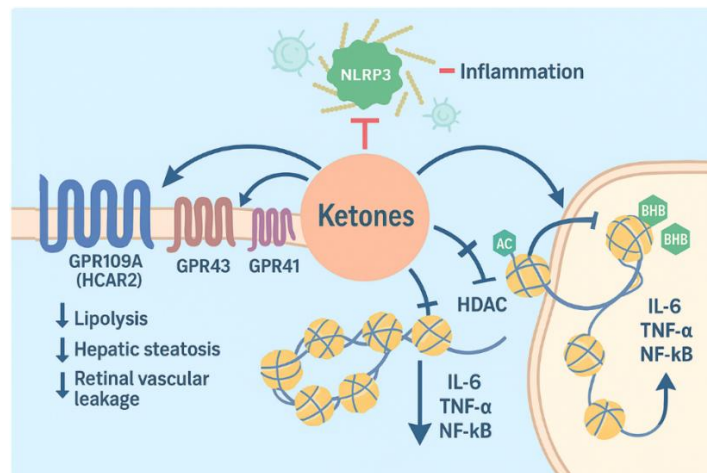


Figure 5 Ketone-regulated cell signalling. Ketones modulate multiple cellular signalling pathways, exerting diverse physiological effects. By activating GPCRs, specifically GPR109A, GPR43 and GPR41, ketones suppress lipolysis and hepatic steatosis. Additionally, they promote gene expression either by inhibiting HDAC or through β -hydroxybutyrylation. Ketones also mitigate inflammation by inhibiting the NLRP3 inflammasome. AC, acetylation; β HB, β -hydroxybutyrylation; GPCRs, G protein-coupled receptors; HDAC, histone deacetylases; NLRP3, NLR family pyrin domain containing 3.

AIM OF THE STUDY

The systemic effects of KBs are increasingly studied, however their target tissues and actions remain only partially known.

The adipose organ, composed principally of white (WAT) and brown (BAT) adipose tissues, plays critical role in metabolic health. WAT, other than storing excess of energy as triglycerides, secretes hormones that regulate metabolism, hunger, and satiety [85,86]. Healthy WAT can expand to accommodate extra energy, preventing fat from accumulating in other organs. However, when WAT function is impaired, particularly in obesity, it can lead to inflammation, ectopic fat deposition in lean tissue, increasing the risk for metabolic diseases such as insulin resistance and heart disease.

BAT plays a thermogenic role, converting energy deriving from substrate oxidation into heat, thanks to the abundance of mitochondria and the peculiar presence within them of the Uncoupling 1 protein (UCP1), which uncouples substrate oxidation from ATP synthesis [87]. In some conditions (eg, cold exposure), in addition to using stored fats as substrates, BAT requires additional energy supplies such as serum glucose and

lipids, thus improving the glucose metabolism and lipid profiles in certain conditions [88]. In adult humans, the amount of detectable BAT tissue inversely correlates with body mass index and fat mass, and positively correlates with the resting metabolic rate [89]. Consequently, BAT is emerging as an effective new target to counteract some metabolic disorders such as, among others, the metabolic syndrome [90].

Some studies indicate that both WAT and BAT act as peripheral targets of ketone signaling [8,70,91]. Concerning WAT very high levels of β -OHB has been reported to inhibit lipolysis, thus indicating that ketone bodies can exert a negative feedback on their own production by reducing fatty acid release by the tissue. In addition, β -OHB in vitro added to 3T3-L1 adipocyte reduce ROS via augmentation of antioxidative stress factors [8].

Conversely, in BAT, some studies suggest that increase in KBs, obtained by ketogenic diet or ketone ester administration to animal model under high fat diet, stimulates mitochondrial biogenesis [92,93]; however, the effects of KBs on BAT mitochondrial bioenergetics/ thermogenic capacity, which play a central role in regulating the overall BAT physiology, remain poorly characterized.

In light of the above, the importance of identifying novel KBs-induced effects and the molecular pathways they influence on adipose organ lies in the central role it plays in energy homeostasis, metabolic rate, endocrine signalling, and disease progression and for developing new therapeutic strategies targeting metabolic disorders. Based on this consideration we evaluated the effects of exogenous ketosis induced by BD administration on WAT and BAT functionality. BD was chosen in view of its ability to induce a rapid increase in β -OHB concentrations, essential for distinguishing between the acute effects induced by β -OHB signalling and the long-term adaptive mechanisms. Indeed, understanding the rapid effects of KBs not only provides insight into the ketones' primary signaling pathways and target tissues but also allows for a clearer interpretation of their role in acute metabolic regulation, stress responses, and cellular homeostasis [1,37].

Literature and data reported in the present thesis indicate that BD reduce energy intake [37] by approximately 15%, thus we considered it interesting to extend the study to the effects of a mild energy restriction of the same entity and duration of that induced by BD treatment on BAT physiology. This would contribute to obtain a clearer framework

for understanding the effect of BD on BAT, by helping to disentangle the direct effects induced by β -OHB formation from those mediated by changes in energy availability.

The central nervous system also represents a major site of KBs action. However, the effects of β -OHB on brain physiology under normal conditions are still poorly characterized, as most evidence derives from pathological models linking β -OHB to neuroprotection in disease contexts [22,96,97]. Investigating how elevated β -OHB levels modulate neural and metabolic homeostasis in healthy animals could therefore provide novel insights into its physiological roles. Of particular interest is the hippocampus, a brain region essential for learning and memory, thus alterations of its functional or structural integrity can prelude the onset of neurodegenerative events [98]. Considering the central role of the hippocampus and the deleterious consequences of perturbations of its homeostasis, we find it interesting to evaluate the effects induced by enhanced levels of β -OHB, achieved by BD administration, on mechanisms involved in the ability of this brain area to respond to stress, namely inflammation, redox balance, autophagy, and endoplasmic reticulum stress. In this case, the effect of BD treatment was compared to that observed in rats undergoing a restricted dietary regimen, isoenergetic with the BD group, to further unveil the effects of this specific molecule.

The outcomes of this research are documented and discussed in the following research articles. In particular, research article 1 concerns the effect of BD on WAT; research article 2 concerns the effect of BD on BAT; research article 3 concerns the effect of mild CR on BAT physiology; research article 4 concerns the effect of BD on hippocampus.

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Article

1,3-Butanediol Administration Increases β -Hydroxybutyrate Plasma Levels and Affects Redox Homeostasis, Endoplasmic Reticulum Stress, and Adipokine Production in Rat Gonadal Adipose Tissue

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Abstract: Ketone bodies (KBs) are an alternative energy source under starvation and play multiple roles as signaling molecules regulating energy and metabolic homeostasis. The mechanism by which KBs influence visceral white adipose tissue physiology is only partially known, and our study aimed to shed light on the effects they exert on such tissue. To this aim, we administered 1,3-butanediol (BD) to rats since it rapidly enhances β -hydroxybutyrate serum levels, and we evaluated the effect it induces within 3 h or after 14 days of treatment. After 14 days of treatment, rats showed a decrease in body weight gain, energy intake, gonadal-WAT (gWAT) weight, and adipocyte size compared to the control. BD exerted a pronounced antioxidant effect and directed redox homeostasis toward reductive stress, already evident within 3 h after its administration. BD lowered tissue ROS levels and oxidative damage to lipids and proteins and enhanced tissue soluble and enzymatic antioxidant capacity as well as nuclear erythroid factor-2 protein levels. BD also reduced specific mitochondrial maximal oxidative capacity and induced endoplasmic reticulum stress as well as interrelated processes, leading to changes in the level of adipokines/cytokines involved in inflammation, macrophage infiltration into gWAT, adipocyte differentiation, and lipolysis.

Keywords: ketone bodies; ROS; antioxidant enzymes; Nrf2; endoplasmic reticulum stress; adipokines; mitochondrial respiratory complexes

1. Introduction

Ketogenesis is a metabolic process leading to the production of ketone bodies (KBs), namely beta-hydroxybutyrate (β -OHB), acetoacetate (AcAc), and acetone. KBs are produced in the liver hepatocytes when excessive fatty acid oxidation and acetyl coenzyme A (acetyl-CoA) production occur [1]. Hepatocytes do not express enzymes that allow the use of KBs as metabolic substrates; KBs are released into the bloodstream and are sent to extrahepatic tissues, where, through ketolysis, they are converted to acetyl-CoA. The latter enters the Krebs cycle to produce energy [1,2].

In addition to being energy molecules, KBs play “non-canonical” roles in the control of energy homeostasis by acting as signal molecules [1,3,4] and by exerting different cellular

effects through changes transduced by epigenetic, post-translational, and cell surface signaling mechanisms. In particular, KBs promote proteins post-translational modifications in histone proteins and the inhibition of class I histone deacetylases (HDACI) [5], thus modulating gene transcription. Both β -OHB and AcAc modulate signaling through G protein-coupled receptors (GPRs), with GPR41 and GPR109 specific for β -OHB [6,7] and GPR43 specific for AcAc [8].

Numerous studies have highlighted the clinical benefits of ketosis in various diseases and neurological and metabolic disorders, due to KBs' cytoprotective actions, actuated by stimulating mitochondrial biogenesis, mitochondrial antioxidant enzymatic defense systems, and preventing lipid and protein oxidative damages [9]. In this context, ketosis, induced by ketogenic diets or by administration of ketone esters, has been used for the treatment of some forms of epilepsy [10], and nowadays, it appears to play a neuroprotective role in neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases [11]. Furthermore, KBs can counteract inflammation and non-alcohol-dependent liver disorders and appear to have cardioprotective effects as well [9]. More studies indicate that white adipose tissue (WAT) is a target of KBs signaling [7,12,13], but the effects they induce on WAT physiology are only partially known.

WAT accumulates lipids to redistribute during fasting to other tissues and organs [14] and actively regulates lipid and carbohydrate metabolism in various organs and tissues by secreting multiple biologically active molecules called adipokines. Some adipokines exert local autocrine and paracrine actions, modulating the migration of the immune cell into the tissue, adipogenesis, and adipocyte metabolism [15]. Other adipokines are secreted in the blood, thus allowing the adipocytes to play significant roles in feedback mechanisms at the systemic level, such as the control of energy balance, the regulation of eating behavior, caloric intake, glucose availability, and energy expenditure. Furthermore, other adipokines are released by WAT in response to stressful conditions or obesity, mainly visceral, and are involved in the onset of insulin resistance, metabolic syndrome, and its complications [15].

The function of WAT is intricately linked with redox changes, and lipogenesis and adipogenesis are controlled by the redox shift [16]. In addition, adipocyte functionality is profoundly regulated by the endoplasmic reticulum (ER), which is crucial for lipid synthesis and droplet formation, protein folding and secretion, and Ca^{2+} homeostasis. Indeed, alterations in the ER functionality have been reported to affect WAT lipolysis and inflammation [17–19]. The protein folding process within ER has a high energy cost; it is profoundly dependent on redox homeostasis and is strictly linked to mitochondrial functionality. The ER lumen has a slightly oxidizing environment that is functional for the formation of disulfide bonds in structural, membrane, and secretory proteins. Variations of redox conditions in ER lumen, towards either oxidizing or reducing direction, can disrupt the protein folding mechanism, thus enhancing the production of misfolded proteins and activating the unfolded protein response (UPR^{ER}) and ER stress [20,21]. When under stress, ER relays calcium signals to mitochondria, which activate substrate oxidation and ROS production, thus leading to mitochondrial dysfunction and establishing a vicious cycle [22].

Given the ability of KBs to influence the redox state and the need for an adequate redox state for WAT physiology, here we studied the effects caused by the enhancement of KBs plasma levels on WAT functionality. Throughout the study, we focused our attention on visceral WAT, in particular on the large gonadal WAT depot (gWAT), as it is of particular concern because it is a key player in metabolic derangements much more than subcutaneous WAT [23–25]. Our goal was to shed light on the direct effects induced by KBs, regardless of the multiple pleiotropic effects that occur under physiological conditions in which their levels increase, such as fasting, physical exercise, or following the administration of a ketogenic diet.

Furthermore, 1,3-butanediol (BD) is a di-alcohol that is readily oxidized in liver hepatocytes yielding a rapid enhancement in β -OHB concentration; in particular, in rodents, BD in vivo administration has been used as a strategy to provoke "exogenous" ketosis since it was demonstrated to produce a rapid dose-dependent elevation of β -HOB serum

levels, thus providing an alternative method to elevate β -OHB in place of nutrient deprivation [26,27].

In the present work, we administered *in vivo* BD to rats for 14 days to provoke “exogenous” ketosis. Since β -HOB has been reported to reduce food assumption [28,29], we also evaluated the rapid effect induced by BD within 3 h from its *in vivo* administration to avoid the confounding effect of reduction of energy intake.

We focused on gWAT antioxidant capacity, ER stress, mitochondrial functionality, and lipolysis. In addition, we evaluated changes in tissue morphology and adipokines production, following 14 days of adaptation of the tissue to elevated β -OHB levels.

2. Materials and Methods

2.1. Animals

Male Wistar rats of about 270 g were housed one per cage in a temperature-controlled room maintained at 22 ± 2 °C under a 12 h dark/light cycle.

Three groups of animals were used: the first group consisted of control animals receiving a single *i.p.* administration of physiological solution at the beginning of the treatment (named C), the second group consisted of animals receiving a single *i.p.* administration of BD (2.5 mol/100 g b.w.) and euthanized 3 h from the *i.p.* administration (named BD 3 h), and the third group received a single *i.p.* administration of BD (2.5 mol/100 g b.w.) followed by the oral administration in drinking water (10%) for 14 days (named BD 14 d). All animals were fed with a standard diet *ad libitum* (4RF21, Mucedola, Italy) and had free access to water or BD water solution.

Drinking BD solution was prepared and replaced daily. Food and BD solution intake was monitored daily, while animal body weight was monitored three times a week. BD treatment did not affect fluid intakes, being about 24 mL/day for all the groups. The energy of BD was taken into account for the evaluation of the energy intake of the rats.

B-OHB levels were monitored both in control and in KBs treated rats, after 3 h from either the *in vivo* administration of BD and vehicle or after 14 days of treatment, to assess the efficacy of the treatments. Glucose levels were detected after 12 days of treatments after a brief period of food removal (5 h).

Venus blood samples were obtained from a small cut on the tail, and D β -OHB and glucose were detected using a ketone body meter (Glucomen Areo β -ketone sensor).

At the end of the treatment, the rats were anesthetized using an *i.p.* injection of sodium Tiopental (40 mg/kg b.w) and euthanized by decapitation. gWAT was excised, weighed, and immediately processed or frozen in liquid nitrogen and stored at -80 °C for later analysis.

This study was carried out under the recommendations in the EU Directive 2010/63 for the Care and Use of Laboratory Animals. All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health (Authorization n° 776/2021-PR.). Every effort was made to minimize animal pain and suffering.

2.2. Histological Analysis

Samples of gWAT pads were fixed through immersion in 4% (*v/v*) formaldehyde in 0.1 M phosphate buffer (overnight, 4 °C). The samples were dehydrated in ethanol, cleared, and embedded in paraffin blocks. The tissues were cut into serial 6 μ m thick sections and hematoxylin-eosin staining was used for morphological examination. Sections were viewed with a Nikon Eclipse 80i light microscope (Nikon Instruments, Milan, Italy) at 20 $\times$ magnification. Images were obtained with a Sony DS-5M camera connected to an ACT-2U image analyzer.

2.3. Lipolysis

Lipolysis was evaluated by detecting the diffusion of glycerol from gWAT to the medium [30]. First, 100 mg of gWAT was incubated for 1 h at 37 °C in 500 μ L of Krebs

Ringer buffer (12 mM HEPES, 121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂) supplemented with 2% FA-free bovine serum albumin (BSA) and 0.1% glucose. Parallel incubations, performed in the absence and in the presence of 10 µM isoproterenol, were executed in order to detect basal and isoproterenol-stimulated lipolysis, respectively. During incubations, samples were under continuous slight shaking and were gassed with 95% O₂–5% CO₂. At the end of the incubation period, an aliquot of the medium was used for the analysis of glycerol content. A commercially available glycerol reagent kit (Merck Life Science S.r.l., Milano, Italy) was used. The absorbance-based enzyme assay kit was converted to fluorescence-based detection through the inclusion of the H₂O₂-sensitive dye Amplex UltraRed, following Clark et al. [31].

2.4. Western Blot

First, 100 mg of gWAT was homogenized in 300 µL RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with a broad-range antiprotease cocktail (Merck, Life Science S.r.l., Milano, Italy). Homogenates were left on ice for 1 h and then centrifuged at 17,000× *g* for 30 min at 4 °C; the resulting supernatants were collected.

Thereafter, 25 µg protein from gWAT lysates was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat dry milk and were subsequently incubated overnight with the appropriate primary antibodies of interest. The second day, membranes were washed three times in TBS-Tween and then were incubated with secondary horseradish peroxidase coupled antibody and processed for enhanced chemiluminescence detection using Excellent Chemiluminescent Substrate Kit (Elabscience, Houston, TX, USA).

The following primary antibodies have been used throughout the study: anti-superoxide dismutase 2 (SOD-2) (ab 13533; abcam, Cambridge, UK), anti-catalase (CAT) (ab 16731 abcam, Cambridge, UK), cocktail of antibody used to detect CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1 and CV-ATP VA subunits (oxophos ab110413, abcam), anti eIF2-α (L57A5, Cell Signaling, Beverly, MA, USA), anti (ser 51) eIF2α phosphorylated form (D9G8, Cell Signaling), anti-Calnexin (ab22595 abcam, Cambridge, UK), anti peroxiredoxin-3 (PRDX-3) (ab 73349 abcam, Cambridge, UK), anti 78-kDa glucose-regulated protein (GRP78-BiP) (ab 108613 abcam, Cambridge, UK), anti glyceraldehyde phosphate dehydrogenase (GAPDH) (3E8AD9 Invitrogen, Monza, Italy), nuclear factor erythroid 2-related factor 2 (Nrf-2) (E-AB-32280 ElabScience, Houston, TX, USA), anti-Tumor Necrosis Factor Alpha (TNF α) (ab1793, abcam, Cambridge, UK). Primary antibodies were diluted 1:1000, with the exception of anti (ser 51) eIF2α phosphorylated form and cocktail of antibody oxophos that were diluted 1:250.

Protein bands were quantified through densitometric analysis (Image J software 1.52t) and normalized based on loading controls GAPDH.

2.5. Determination of gWAT ROS Levels, Lipid and Protein Oxidative Damages, and Total Antioxidant Capacities

gWAT ROS levels were measured following the ROS-induced conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the fluorescent dichlorofluorescein (DCF) [32]. Briefly, aliquots of homogenate (25 µg proteins) were incubated in 200 µL of monobasic phosphate buffer 0.1 M, pH 7.4 supplemented with 10 µM DCFH-DA. Then, FeCl₃ was added (final concentration 100 µM), and the mixture was incubated for 30 min. The conversion of DCFH-DA to the fluorescent product DCF was measured using a microplate reader (Tecan Infinite 200 pro plate reader) with excitation and emission wavelengths of 485 and 530 nm, respectively. The conversion of DCFH to DCF in the absence of homogenate was detected to evaluate the background. A standard curve of DCF has been used in order to calculate the pmol DCF formed.

Lipid and protein oxidative damages were determined in gWAT by evaluating the lipid hydroperoxides levels and protein-bound carbonyls (CO) as reported elsewhere [33,34]

The total antioxidant capacity of gWAT was determined by following the decolorization, at 734 nm, of the mono-cation radical of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{•+}), produced by oxidation by 245 mM potassium persulfate of 7 mM ABTS, due to 0.01 mg of gWAT proteins [35]. The calibration curve was obtained using a stock solution of 3,5-Di-tert-4-butylhydroxytoluene (BHT). Total antioxidant capacity was expressed as BHT Equivalents · mg⁻¹ protein.

2.6. Adipokines Content Analysis

A Proteome Profiler rat Adipokine Array kit (R&D Systems, Minneapolis, MN, USA) has been used for the parallel determination of the relative levels of selected Adipokines following the manufacturer's indication.

2.7. Statistical Analysis

Data were analyzed with the GraphPad Prism 6.02 (San Diego, CA, USA) software system. One-way analysis of variance method (ANOVA), followed by the "Tukey correction for Multiple Comparison" or Student's *t*-test were used to establish the statistical significance of the differences between experimental groups. Differences were considered statistically significant at *p* < 0.05.

3. Results

3.1. BD Administration Reduces Epididymal gWAT Mass and Adipocyte Size and Promotes Lipolysis

The administration of BD was effective in inducing ketosis since it induces a significant increase in serum (D) β-OHB levels. Three hours after BD i.p. administration (BD 3 h), β-OHB serum levels reached values 5.5-fold higher than the control, and after 14 days of treatment with BD added to drinking water (BD 14 d), the values were about 2.6-fold higher than the control (Figure 1). At the end of the 14 days of treatment, BD 14 d rats gained less weight than C (Table 1) and showed a concomitant lower energy assumption and gWAT weight. After 5 h of food removal, no difference in glucose plasma levels was found.

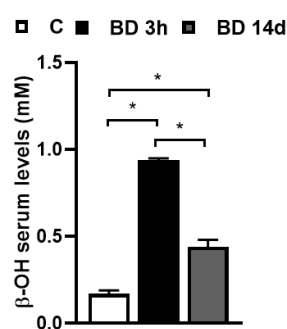


Figure 1. Effect of BD treatment on (D) β-OHB serum levels, detected after 3 h from BD i.p. administration (BD 3 h) and after 14 days of oral administration (BD 14 d). Values represent the mean ± SE of 10 different animals. * *p* < 0.0001.

Morphological analysis indicates that 14 days of BD administration induces a reduction in adipocyte size (Figure 2); in addition, in gWAT, parenchyma macrophage infiltration and an enhancement in vascularization can be observed (Figure 2A). A reduction in tissue lipid accumulation has also been observed in the liver of BD 14 d group (Supplementary Figure S1).

BD induces a significant increase in basal lipolysis that was already evident three hours after the administration of their precursor. The observed increases were +23% and +31% in BD 3 h and BD 14 d vs. C, respectively. BD also stimulated isoproterenol-stimulated lipolysis, and the effect reached statistical significance only in the BD 3 h group vs. C (Figure 2B).

Table 1. Effect of 14 days administration of BD to rat on body weight gain, energy intake, and gWAT weight.

	C	BD 14 d
Body weight at the beginning of the treatment (g)	273 ± 7	271 ± 4
Body weight at the end of the treatment (g)	329 ± 6	300 ± 7 **
Body weight gain (g)	55 ± 3	29 ± 4 ***
Energy intake (kJ)	3840 ± 363	2828 ± 348 ***
gWAT weight (g)	4.5 ± 0.4	3.2 ± 0.1 **
Glucose serum levels (mg/dL)	112 ± 6	120 ± 2

Values represent mean ± SE of 10 animals. ** $p < 0.01$, *** $p < 0.001$ vs. C.

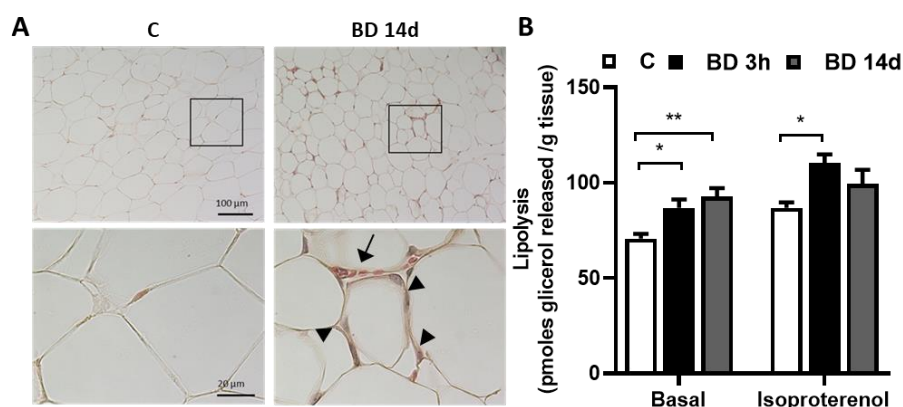


Figure 2. Effect of 14 days BD administration on gWAT morphology and lipolysis. (A) shows a representative histological analysis of gWAT from control (C) and BD-treated rats (BD 14 d). Below is the enlargement (100×) of the framed areas. Note the presence of capillaries (arrow) and infiltrating (inflammatory) cells/macrophages (arrowhead) around the adipocytes in BD 14 d-compared to C-gWAT. Hematoxylin and Eosin staining. (B) represents Basal and isoproterenol-stimulated lipolysis. Values represent the mean ± ES of 8 different animals * $p < 0.05$, ** $p < 0.01$.

3.2. Effect of BD on Redox Homeostasis

To test if BD could affect gWAT redox homeostasis, we evaluated the tissue's ROS levels, the oxidative damage to lipids and proteins, and the tissue's antioxidant capacity (Figure 3).

BD was efficacious in reducing tissue ROS levels. The BD effect was observed already 3 h after its administration and persisted after 14 days of treatment. ROS levels were reduced by 52% and 46% in BD 3 h and BD 14 d vs. C, respectively. BD also protected proteins and lipids from oxidative damage. Indeed, when compared to C, gWAT lipid hydroperoxide levels were significantly reduced by 47% and 33% in BD 3 h and BD 14 d vs. C, respectively. Furthermore, carbonylated protein levels were strongly reduced in both BD 3 h (−80%) and BD 14 d (−60%) vs. C.

Concerning gWAT antioxidant capacity, we detected both soluble and enzymatic components by performing the ABTS assay and evaluating the protein levels of antioxidant enzymes. BD rapidly improved tissue soluble antioxidant capacity, which increased by 102% in the BD 3 h vs. C group, and this effect persisted after 14 days of treatment. It also enhanced SOD-2, PRDX-3, and CAT protein levels. The increases observed were +116% and +75% in BD 3 h and BD 14 d vs. C, respectively, in the case of SOD-2; +159% and +147% in BD 3 h and BD 14 d vs. C, respectively, in the case of CAT; and +619% and +666% in BD 3 h and BD 14 d vs. C, respectively, in the case of PRDX-3. The levels of Nrf-2, known as the main orchestrator of the cellular antioxidant response, were significantly and rapidly enhanced by BD, and the increases observed were +205% and 147% in BD 3 h and BD 14 d vs. C, respectively.

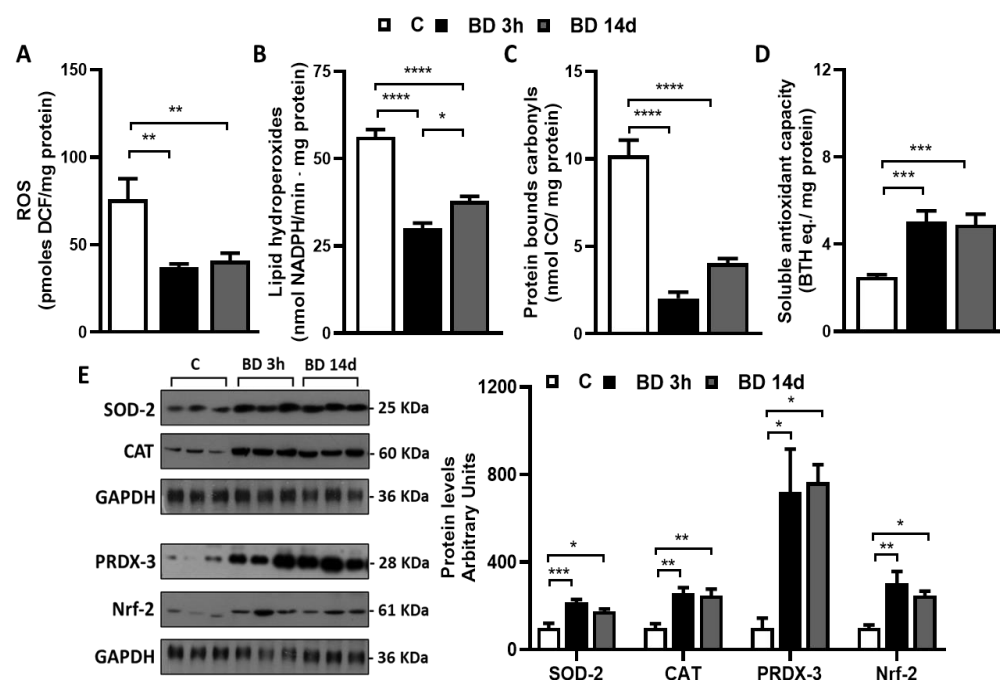


Figure 3. Effect of BD on gWAT redox homeostasis. Total ROS (A), lipid hydroperoxides (B), protein oxidative damage (C), and tissue soluble antioxidant capacity (D). Values are means $\pm$ SEM of 8 different rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Enzymatic antioxidant capacity (E). Representative Western Blot of Superoxide dismutase-2 (SOD-2), catalase (CAT), peroxiredoxin-3 (PRDX-3), and Nuclear factor erythroid-related factor 2 (Nrf2) (E). GAPDH was used as loading control (25 μ g of protein/rat/lane). Histograms represent the quantification of data. Data were normalized to the value obtained for control animals, set as 100. Values represent the mean $\pm$ SEM of 6 different rats or 3 rats in the case of PRDX-3.

3.3. Effect of BD on gWAT ER Stress and Inflammation

To evaluate the ability of BD to influence ER stress, we detected the protein levels of its specific markers, namely GRP78/BiP and calnexin (Figure 4A). Within 3 h after its administration, BD increased their protein levels, and the effect observed persisted after 14 days of treatment. GRP78/BiP levels were enhanced by +77% and +160% in BD 3 h and BD 14 d vs. C, respectively. The increases in calnexin levels were +96% and +123% in BD 3 h and BD 14 d vs. C, respectively.

BD enhanced both total and phosphorylated levels of eIF-2 α (ser 51); the effect was evident within 3 h from its administration (Figure 4B). No significant changes in the ratio phosphorylated eIF-2 α /total eIF2- α were observed between groups.

We also evaluated if BD could also lead to inflammation by evaluating TNF- α protein levels that were significantly higher in BD 3 h (+217%) and BD 14 d (+304%) vs. C.

3.4. Effect of BD on gWAT Oxidative Capacity and Mitochondrial Respiration Complexes Abundance

To test if BD could affect gWAT mitochondrial oxidative capacity, we detected tissue levels of mitochondrial respiratory complexes' specific subunits, as an index of tissues complexes' abundance, and Complex IV/cytochrome oxidase (COX) activity, as an index of the maximal mitochondria oxidative capacity.

BD increased all the respiratory complexes, the effect being rapidly manifested (Figure 5A). Concerning complex IV/cytochrome oxidase, the protein levels of its specific subunits MTCO1 were increased by about 300% in both BD 3 h and BD 14 d groups vs. C. Despite this, cytochrome oxidase activity, expressed as nmoles O/min mg proteins, was unaffected by BD at both treatment durations (Figure 5B). In addition, specific COX activity, obtained by normalizing tissue COX activity for the tissue amounts of complex IV,

was significantly reduced in both BD 3 h and BD 14 d compared to C (−79% and −76%) (Figure 5C).

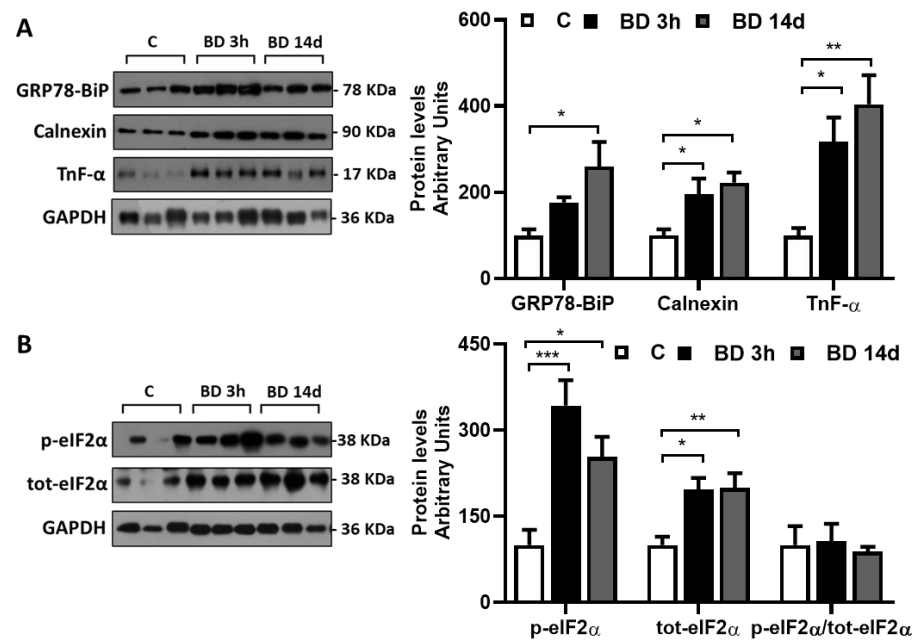


Figure 4. Effect of BD on gWAT levels of proteins linked to ER and cellular stress involved in ER stress and UPR^{ER} response detected. Representative Western blots of GRP78/BiP, Calnexin and TnF-α (A), and total and phosphorylated (ser 78) levels of eIF2α (B) were performed in gWAT lysate. GAPDH was used as loading control (25 µg of protein/rat/lane). Histograms represent the quantification of data. Data were normalized to the value obtained for control animals, set as 100. Values represent the mean ± SEM of 6 different rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

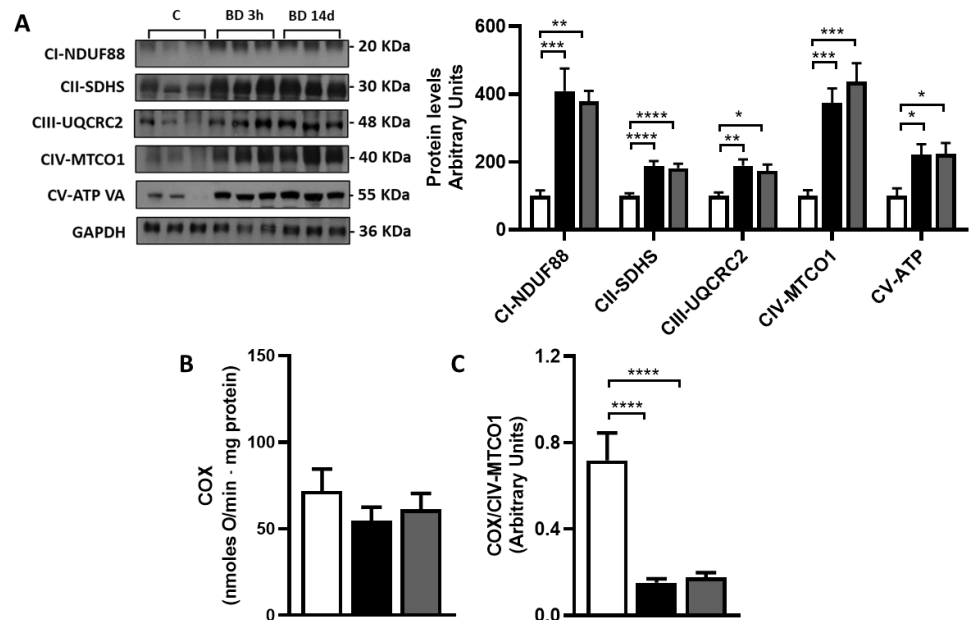


Figure 5. Effect of BD on gWAT mitochondrial respiratory complexes abundance and cytochrome oxidase activity. (A) shows representative Western Blots of specific subunits of the five mitochondrial respiratory complexes, namely CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP VA. GAPDH was used as loading control (25 µg of protein/rat/lane). Histograms represent the

quantification of data. Data were normalized to the value obtained for control animals, set as 100. Values represent the mean $\pm$ SEM of 6 different rats. (B,C) show Cytochrome oxidase/complex IV activity reported as nmoles O/min mg tissue proteins (B) or normalized for Complex IV levels and expressed in arbitrary units (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Values represent the mean $\pm$ SEM of 8 different rats.

3.5. Administration of BD for 14 Days Affects Specific gWAT Adipokines/Cytokines Level

Since adipokines/cytokines production could be affected by changes in gWAT mass and adipocytes size, we tested if the administration of BDs for 14 days could affect the adipokines levels in gWAT by performing an adipokine array. Representative blots were reported in Figure 6. When compared to C, the BD 14 d group showed an increase in chemotactic protein-1 (MCP1, +88%); Preadipocyte factor 1 (Pref-1, +79%); Advanced glycation endproduct receptor (RAGE, +62%); Regulated on activation, normal T cell expressed and secreted (RANTES, +120%); Plasminogen activator inhibitor-1 (PAI-1, +77%); TIMP metalloproteinase inhibitor 1 (TIMP-1, +64%); Interleukin-10 (IL10, +60%). In addition, downregulation of the adipokines Interleukin-1 (ICAM-1) and Leptin was observed (−26% and −34%, respectively, vs. C).

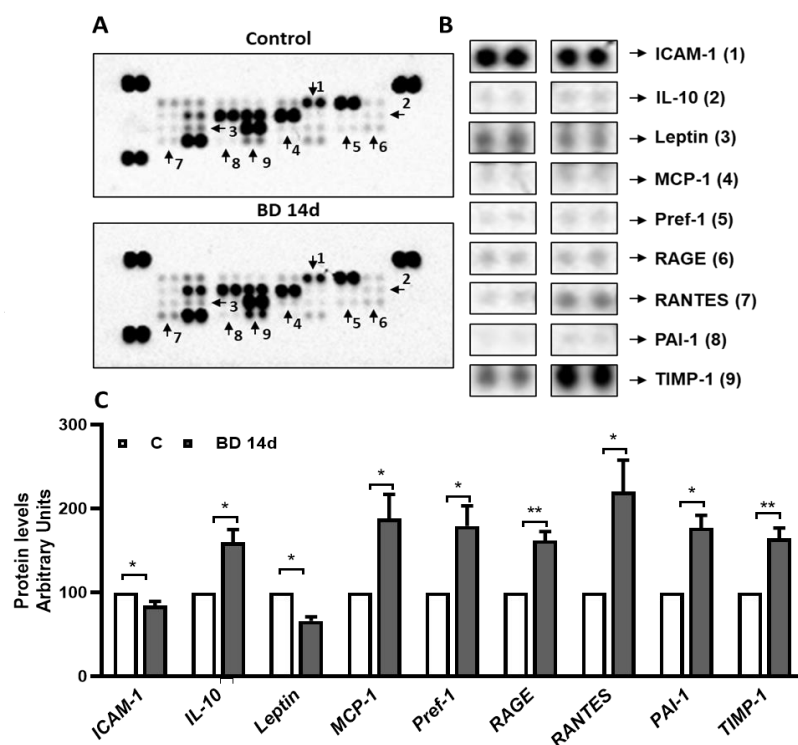


Figure 6. Effect of administration of BD for 14 days on gWAT adipokines profiles. Representative adipokine profile detected in gWAT from C and BD 14 d rats, by an adipokine protein array (A). The arrowheads indicate signals with significant changes. Magnifications of the spot of proteins whose intensity resulted statistically different are reported in (B). Histograms represent the quantification of relative levels of adipokines with observable changes (C). The values represent the mean $\pm$ SEM of 3 different samples, each one obtained by a pool of two different animals. Data were normalized to the value obtained for control animals, set as 100. * $p < 0.05$, ** $p < 0.01$.

4. Discussion

WAT is known to have a profound effect on overall energy metabolism, and a growing number of studies shed light on its paracrine and endocrine signaling function. Our study reports that BD greatly affects gWAT physiology and highlights the role played by its main product β -OHB on the tissue, whose effects are already evident within 3 h after the BD administration. The rapid effects induced by BD highlight that this molecule can act

independently of its ability to reduce energy intake and determines long-term complex metabolic adaptations.

β -OHB plays a role in suppressing oxidative stress induced by oxidant treatment through increasing endogenous antioxidant capacities [5], and we report that BD enhances the whole tissue antioxidant soluble capacity, promotes the overexpression of proteins involved in enzymatic antioxidant capacity (SOD-2, PRDX-3, and CAT), and reduces tissue ROS content and the oxidative damages to lipids and proteins. Our data, showing the ability of BD to enhance SOD-2 and catalase levels in gWAT, agree with that reported in the literature indicating the effectiveness of β -OHB, *in vitro* added to 3T3-L1 adipocyte, to reduce ROS via augmentation of antioxidative stress factors [12]. Data also agree with the evidence that knockout mice for 3-Hydroxy-methyl-glytaryl-CoA synthetase 2, the first limiting enzyme in ketone bodies production, show a reduction in gWAT antioxidant enzymes [12]. The discovery that BD effects on the antioxidant system are already evident 3 h after BD administration extends the concept to the short-term action of β -OHB.

Multiple metabolic processes might be involved in the effect of BD to increase tissue soluble antioxidant capacity (for review, see Ref. [36]). Among these is the ability of β -OHB (i) to increase the mitochondrial Q/QH₂ ratio and to decrease the NAD⁺/NADH ratio [37]; (ii) to decrease the glycolytic flux and force glucose-6-phosphate along the pentose phosphate pathway, thus leading to the production of two equivalents of NADPH; and (iii) to increase mitochondrial acetyl-CoA approximatively 15-fold, thereby increasing the flux of the citrate–pyruvate and citrate–isocitrate cycles, each of which includes a NADP⁺ to NADPH reduction step, catalyzed by malic enzyme and isocitrate dehydrogenase, respectively [38,39]. The β -OHB mediated increase in NADPH results in enhanced levels of reduced glutathione, thioredoxin, vitamins C and E, and other essential antioxidants [40]. Further detailed studies are needed to investigate if and how these pathways are enhanced in gWAT from BD-treated rats.

Among the transcriptional factors involved in the upregulation of antioxidant genes by the β -OHB in several tissues is Nrf2 [41]. We report that BD administration rapidly enhances Nrf2 protein levels in gWAT associated with increased levels of antioxidant enzymes content. However, the ability of β -OHB to activate gene transcription through inhibiting class I histone deacetylases (HDACs) could also be involved in the antioxidant effect of BD [5].

It is important to consider that a huge improvement of whole-tissue soluble antioxidant capacity, together with the overexpression of antioxidant enzymatic systems, leads to an excess of reducing equivalents that deplete ROS and drive the cells to reductive stress [42]. The change in redox homeostasis toward reductive stress profoundly affects ER functionality. In the ER, protein folding requires an oxidative environment; thus, the occurrence of reductive stress leads to the loss of protein disulfide bond formation and induces alteration in ER homeostasis (i.e., ER stress) and the activation of the UPR^{ER} [21]. These processes appear to manifest in the gWAT from BD-treated animals, as revealed by the potent antioxidant effect of BD described above, and by the rapid increase in levels of proteins involved in UPR^{ER}, such as calnexin and BIP, that would counteract protein unfolding to limit ER stress.

One of the pathways of UPR^{ER} involves the phosphorylation of eIF-2 α on ser 51. It is known that eIF2- α , together with GTP molecule and a charged initiator methionine tRNA, forms the active ternary complex that is fundamental for the translation initiation and ensures the correct identification of the start codon. P-eIF2 α leads to a reprogramming of protein synthesis: the global protein synthesis is attenuated (thus reducing the influx of proteins into the ER), and the translation of a specific subset of mRNAs is enhanced through alternative non-AUG translation [43]. Here, we reported the ability of BD to enhance both P-eIF2 α and total eIF2- α protein levels, with an unchanged ratio between them. Plausibly, the enhancement of total eIF2- α levels prevents the decrease in global protein synthesis that is generally associated with UPR^{ER}, and it is possible to speculate its involvement in the rapid increase in specific protein levels induced by BD.

ER stress is strictly interrelated with mitochondrial dysfunction, and dysfunction on either side can compromise cell homeostasis and lead to metabolic derangements [22]. In addition, the occurrence of reductive stress has been reported to lead to mitochondrial functionality failure in adipose cells and myoblast [44,45]. In the present paper, we detected the activity of cytochrome oxidase/complex IV in gWAT homogenate, as an index of tissue mitochondrial maximal oxidative capacity, and we observed that BD administration did not affect it, despite being effective in increasing the tissue mitochondrial complex subunit IV levels, thus leading to a reduction in specific cytochrome oxidase activity (obtained by the ratio COX activity/Complex IV MCO1). These data could be indicative of the occurrence of mitochondrial dysfunction. However, further experiments concerning mitochondrial bioenergetic processes and mitochondrial ROS production are needed to clarify (i) whether changes in mitochondrial functionality occur in BD-treated rat samples and (ii) the relationship between the alteration of redox balance towards reductive stress and mitochondrial functionality in gWAT.

The occurrence of ER stress and mitochondrial dysfunction affects the ability of WAT to store lipids and tissue inflammation [17,46–48]. It has been reported that the induction of ER stress, both in vivo and in vitro, efficiently activated lipolysis in adipocytes [17,49]. In addition, in adipose tissue, ER stress leads to a pro-inflammatory state through the activation of pathways involved in the regulation of inflammatory cytokines release [23] (see also above). Furthermore, the proinflammatory cytokine TNF- α activates signaling pathways leading to adipocyte triglycerides breakdown [49,50]. Our data shed light on the ability of BD to stimulate lipolysis rapidly, being the effect plausibly mediated by the occurrence of ER stress, as well as by TNF- α . In the literature, the ability of β -OHB to inhibit lipolysis by binding to GPR109A receptors is also reported [7]. GPR109A shows EC₅₀ for β -OHB of about 0.8 mM, which is similar to the concentration we found after 3 h from the administration of BD (0.94 mM) but higher than that observed following 14 days oral administration of BD in drinking water (0.46 mM). Thus, it is possible to speculate that in our experimental condition, the activation of lipolysis induced by ER stress and TNF- α prevails on its inhibition mediated by GPR109A.

Following 14 days of treatment with BD, we found a reduced accumulation of lipids in gWAT, as revealed by the lower tissue weight and the reduced adipocyte size. The above-interrelated processes leading to lipolysis (i.e., change in cell redox homeostasis, the occurrence of ER stress, reduction in mitochondrial oxidative capacity, inflammation), that manifest rapidly and persist after 14 days of BD treatment, could have contributed to the reduction of lipid accumulation in gWAT. However, data obtained in the BD 14 d group indicate that other processes could also have been involved; among these are changes in the level of adipokines involved in inflammation and adipocyte differentiation and the reduction in energy intake. It should be mentioned that BD taste may have contributed to influencing the appetite of the rats even if our data indicate that BD-treated rats drank an amount of solution similar to the water consumed by control rats, thus indicating that the treatment with BD was well tolerated by rats. Plausibly, the reduction of food intake could be due to the increase in β -OHB serum levels induced by BD. In fact, Langhans et al. [28,29] reported that a single subcutaneous administration of β -HOB decreased food intake. Furthermore, Isoda et al. [51] reported the ability of BD to reduce food intake when administered by gavage to mice fed a high-fat diet, by sensitizing the action of leptin at the hypothalamic level.

By running an adipokines array, we reported that BD administration for 14 days induced changes in the levels of multiple adipokines/cytokines involved in inflammation, macrophages recruitment, and activation; among them were MCP1, RANTES, RAGE, and PAI-1, other than the TNF α cited above.

MCP1 affects adipocyte function, directs monocyte recruitment, and activates proinflammatory macrophages [23]. In line with this, in rats chronically treated with BD, associated with enhanced MCP1 levels, we observed macrophage infiltration into the gWAT (shown by histological analysis) and TNF- α enhanced levels. This observation is sup-

ported by the increased levels of RANTES, which participates in the recruitment of blood monocytes by triggering their adhesion and transmigration to/through adipose tissue endothelial cells [52], and of PAI-1, which play a role in the migration of macrophages and whose deletion or inhibition causes a significant reduction in M1 macrophages in the WAT of mice fed a high-fat diet [53].

Regarding RAGE, it is expressed at high levels in macrophages/monocytes, and the activation of this receptor by its ligands induces macrophage activation and mediates monocyte/macrophage chemotaxis and upregulation of inflammatory factors [54]. Thus, the upregulation of RAGE by BD treatment agrees with macrophage recruitment and inflammation. The evidence that BD also up-regulates the anti-inflammatory cytokine IL-10 suggests a limitation of the overall pro-inflammatory response. Plausibly, TNF- α itself could modulate IL10 upregulation by KBs, as suggested by studies reporting that IL-10 is up-regulated by TNF- α in vitro as well as in obesity both in humans and rodents [55].

Histological analysis indicates that 14 days of BD treatment induces an increase in capillaries surrounding adipocytes. The angiogenesis process is based on the fine regulation of protease/antiprotease balance; indeed, despite increased proteolysis being considered fundamental for endothelial cell migration and invasion, the inhibition of proteases plays an important permissive role during angiogenesis by preserving matrix integrity [56,57]. In line with this, after 14 days of treatment with BD, the vascularization of the tissue is enhanced, together with the levels of the serine protease inhibitor PAI-1 and the metalloprotease inhibitor TIMP-1. Some adipokines up-regulated by BD could contribute to the reduction of gWAT mass by inhibiting adipogenesis that, in adults, is essential for adipocyte turnover and maintenance of adipose tissue; among these are the already cited TNF- α [58], TIMP-1 [59], PAI-1 [60], as well as Pref-1 [61].

The data here reported also indicate that BD regulates WAT endocrine signaling. Leptin plays a crucial role in energy homeostasis by regulating the food intake and the oxidation rate of substrates [62]. Leptin production increases with increasing fat mass and adipocyte size. The reduction in leptin tissue production observed in BD-treated rats compared to control ones is consistent with their reduced fat mass and adipocyte diameter. On the other hand, these data contrast with the reduced energy intake observed in BD-treated animals, since notoriously leptin has an inhibitory effect on food intake, by activating hypothalamus POMC neurons and inhibiting the NPY one (recently reviewed in Obradovic et al., 2021 [63]). However, recent data have shown that the treatment with BD and the consequent increase in β -OHB levels increase the sensitivity of hypothalamic neurons involved in hunger circuitry and satiety to leptin [51], in line with our observations.

In this context, it should be underlined that the reduction in food intake observed in rats receiving BD administration for 14 days could have contributed to some of the effects here described. The evidence that most of the effects of BD-induced ketosis are observed within 3 h indicates that they are independent of the reduction in energy intake. However, further detailed studies will be necessary to define the real contribution of β -OHB per se and that due to the reduction of energy intake in such animals. Since the reduction in energy intake itself leads to an increase in KBs serum levels [62,64], the use of an animal model in which endogenous ketone body production is inhibited (i.e., 3-hydroxy-methylbutyryl-CoA synthetase 2 knockout [12]) would be helpful to discriminate between the roles played by ketosis and energy intake reduction in the effects induced by long-term BD administration, and this is a limitation of our study.

5. Conclusions

Based on our data, we can suggest that BD treatment, by strongly enhancing the antioxidant capacity of the gWAT, shifts redox homeostasis toward reductive stress and, thus, to those interrelated consequences occurring at the level of ER and mitochondria that lead to lipolysis, tissue inflammation, and changes in gWAT adipokine/cytokines production.

In conclusion, by taking advantage of BD-induced increase in β -OHB levels, advances have been made in the understanding of the role played by KBs in influencing gWAT physiology. One of the most interesting and novel aspects reported in the present paper concerns the evidence that the effects induced by BD rapidly manifest and persist after 14 days of treatment. Studies based on a longer-term treatment with BD will allow to establish if these effects persist or if the occurrence of tissue adaptations leads to resolving ER stress and inflammation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox12071471/s1>, Figure S1: Effect of 14 days BD administration on liver morphology.

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RESEARCH ARTICLE

Research Article 2

Rapid modulation of interscapular brown adipose tissue mitochondrial activity by ketosis induced by 1,3-butanediol administration to rats

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Abstract

Some studies indicate that brown adipose tissue (BAT) represents a promising target in the fight against dysmetabolic diseases, with indications suggesting it as a potential target for the effects of ketone bodies. We investigate whether the elevation of plasma levels of the ketone body β -hydroxybutyrate, achieved through the in vivo administration of its precursor 1,3-butanediol (BD) to rats, could impact interscapular BAT (iBAT) mitochondrial biochemistry and functionality. We examined the effects induced by BD within 3 h and after 2 weeks of treatment. A large-scale quantitative proteomics approach, coupling liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis, and Western blot associated with functional studies by respirometry allowed us to evaluate the changes in iBAT mitochondrial protein expression and bioenergetics induced by BD. BD administration increased β -hydroxybutyrate plasma levels,

Abbreviations: AcAc, acetoacetate; ACN, acetonitrile; ANOVA, one-way analysis of variance; BAT, Brown adipose tissue; BD, 1,3-butanediol; BSA, Bovine serum albumin; CID, collision-induced dissociation; DDA, Data Dependent Acquisition mode; FDR, false discovery rate; G3P, glycerol-3-phosphate; GDP, guanosine di-phosphate; HCOOH, formic acid; HDACI, class I histone deacetylases; HSP-60, heat shock protein 60; iBAT, interscapular BAT; Kbh, β -hydroxybutyrylation; KBs, Ketone bodies; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; LFQ, label-free quantification; mG3PDH, mitochondrial glycerol-3-phosphate dehydrogenase; OXPHOS, oxidative phosphorylation complexes; PEP, posterior error probability; PPARs, peroxisome proliferator-activated receptors; PTM, post-translational modification; UCP1, Uncoupling protein-1; VDAC, voltage-dependent anion channel; β -OHB, β -hydroxybutyrate.

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which correlated with an enhancement in iBAT mitochondrial respiration rate, likely due to the activation of the respiratory chain and uncoupling protein-1. The proteomic analysis demonstrated that BD influenced the mitochondrial levels of specific subunits belonging to the five respiratory complexes, uncoupling protein-1, and proteins involved in propanoate metabolism. BD administration also induced lysine β -hydroxybutyrylation of mitochondrial proteins, including specific subunits of the respiratory chain complexes and uncoupling protein-1. Most of the BD-induced effects were observed within 3 h of its administration and persisted/increased after 2 weeks of treatment. In conclusion, by using BD to increase β -hydroxybutyrate levels, we provide evidence supporting the role of β -hydroxybutyrate as a signaling molecule capable of rapidly modulating BAT physiology by acting at the mitochondrial level.

KEYWORDS

brown adipocyte, ketone bodies, metabolism, mitochondria, thermogenesis

1 | INTRODUCTION

Brown adipose tissue (BAT) plays a thermogenic role due to its ability to dissipate the energy obtained by substrate oxidation into heat. BAT expresses high levels of uncoupling protein-1 (UCP1), a protein localized into the mitochondrial inner membrane that mediates the diffusion of protons from the intermembrane space toward the matrix, a process that is not coupled with the synthesis of ATP.^{1,2} Studies in rodents indicate that to support this energy-dissipating process, BAT increases the oxidation of glucose, amino acids, and lipids; thus, the activation of BAT thermogenesis can improve metabolic dysregulation, particularly in the context of diet-induced obesity, characterized by an impaired thermogenic BAT function.² In humans, the presence of ¹⁸Fluorodeoxyglucose positive BAT appears to be inversely associated with factors like obesity, visceral fat, hyperglycemia, and type-2 diabetes and seems to be associated with a healthier metabolic phenotype in obesity³ and cardiometabolic health,⁴ suggesting that BAT could be a promising target for treating these conditions.³⁻⁵ However, in humans, the limited amount of BAT and the methods used to assess its presence, based on ¹⁸FDG uptake, cast doubts on its true potential.⁶⁻⁸ Despite this, significant efforts are being made to identify factors that could influence BAT mitochondrial physiology.

Ketone bodies (KBs) [acetoacetate (AcAc), acetone, and β -hydroxybutyrate (β -OHB)] are produced in the liver mitochondria under physiological conditions of enhanced fatty acids oxidation, such as prolonged fasting, calorie restriction, intense physical activity, or even in disease states, such as diabetic ketoacidosis. Hepatocytes do

not use KBs as energy substrates, but rather release them into the bloodstream; thus, KBs reach the extrahepatic tissues, where, through ketolysis, they are converted to acetyl-CoA, which enter the Krebs cycle to produce energy.^{9,10} The metabolic importance of KBs is not restricted to their role as an energy source, since they also play a role as signaling molecules.¹¹⁻¹⁶ Signaling activated by β -OHB seems to occur through interaction with membrane receptors coupled to G proteins.^{10,11,15,16} Moreover, KBs can induce post-translational modification (PTM) of proteins and, through lysine β -hydroxybutyrylation (Kbhb), β -OHB is covalently attached to lysine ϵ -amino groups. The amount of protein adduct depends on the β -OHB concentration and involves proteins localized in various cellular compartments, including the nucleus and mitochondria.^{14,17-19} In this context, it has been reported that β -OHB promotes the PTM of histone proteins and the inhibition of class I histone deacetylases (HDACI), thereby modulating gene transcription.^{14,17,18} In addition, several proteins undergo Kbhb modifications in response to experimental conditions that increase β -OHB in the liver.¹⁹

KBs have multiple beneficial effects on the whole organism since they counteract inflammation and non-alcoholic liver disorders, exert cardioprotective effects, and induce a reduction in body weight by increasing energy expenditure and reducing appetite and hunger.⁹⁻¹¹ KBs may improve athletic performance, although conflicting evidence exists regarding this aspect.^{9,20} KBs stimulate mitochondrial biogenesis and enhance antioxidant enzymatic defense systems.^{10,14,21,22} Noteworthy, several physiological conditions characterized by increased KBs serum levels are strictly associated with changes in BAT physiology. In calorically restricted animals, BAT may

change its function from an energy-consuming system to an energy reservoir system,²³ with reduced thermogenesis.^{24,25} On the other hand, some studies indicate that exercise may activate BAT, with myocyte-secreted factors (known as “exerkines”) playing a role in such activation.²⁶ Ketogenic diet administration to mice for 4 weeks has been reported to enhance UCP1 and mitochondrial proteins.^{27,28} This diet increases plasma-free fatty acids levels, likely leading to metabolic inefficiency, by the activation of UCP1, mediated by the peroxisome proliferator-activated receptors (PPARs).^{27,28} Thus, physiological ketosis can induce pleiotropic effects that could mask or confound the roles played by KBs in BAT activation. Studies in which the enhancement in KB_s serum levels was achieved by exogenous administration of KBs or their precursor, by pointing to a direct effect of KBs on the tissue, may give different outcomes depending on β -OHB serum levels reached. Indeed, the administration of Ketone ester D- β -hydroxybutyrate-(R)-1,3 butanediol monoester to high fat diet-fed obese rats, leading to about a 6-fold increase in β -OHB serum level, enhanced tissue UCP1 protein levels and specific markers of BAT activation.²⁹ On the other hand, a modest enhancement of β -OHB (about 1.36 fold), obtained by the administration of β -OHB in drinking water for 4 weeks to rats, reduced BAT UCP1 levels.³⁰ In addition, although studies in the literature suggest that KBs could affect BAT mitochondrial biogenesis,^{27,29} there is still a lack of research on the ability of KBs to influence BAT mitochondrial bioenergetics. Moreover, no studies are available on the possible rapid/short-term effect elicited by KBs on BAT mitochondria.

Given the role played by both KBs and BAT in regulating energy homeostasis and that of mitochondria in BAT physiology, it is crucial to give further insight into their interaction. To this end, we chose to induce “exogenous ketosis” by *in vivo* administering 1,3-butanediol (BD) to rats since the liver rapidly metabolizes it to β -OHB, and produces dose-dependent enhancement in β -OHB levels.^{31,32} The use of BD allows us to shed light on the putative short-term effect of β -OHB on BAT mitochondria and, at the same time, to evaluate the mitochondrial adaptation to prolonged exposure to high levels of β -OHB.

We focused our attention on the ability of BD to affect interscapular BAT (iBAT) mitochondrial biochemistry and functionality. Thus, we conducted a large-scale quantitative proteomics analysis by coupling liquid chromatography with tandem mass spectrometry (LC-MS/MS) and Western blot analysis to compare the expression of mitochondrial proteins in response to stimulation with BD, and to associate them with changes in mitochondrial functionality, both detected in the isolated mitochondrial enriched fraction. Furthermore, considering that Kbhb is

crucial for β -OHB signaling, we evaluated whether it occurs in BAT mitochondria from BD-treated rats.

2 | MATERIALS AND METHODS

2.1 | Animal treatments

Male Wistar rats weighing 275–300 g (about 10 weeks old) were obtained from Envigo RMS Srl (Udine Italy). Rats were housed in a temperature-controlled room at $22 \pm 1^\circ\text{C}$ under a 12:12-h light–dark cycle.

In the present study, animals were treated as previously reported.²² Three animal groups were used. Group “C” comprised control rats that received a single *i.p.* administration of a physiological solution at the beginning of the treatment. Group “BD-2w” comprised rats receiving a single *i.p.* administration of BD at the start of the treatment (2.5 mmol/100 g b.w.), followed by the oral administration of BD dissolved in drinking water (10% w/w) for 2-weeks. Group “BD-3h” consisted of rats receiving a single *i.p.* administration of BD (2.5 mmol/100 g b.w.) and sacrificed 3 h after the injection.

Rats were fed with a standard diet *ad libitum* (4RF21, Mucedola, Italy) and had free access to water or BD drinking solution that was replaced daily; food, water, and BD drinking solution intake were monitored daily. The amount of BD consumed by rats through drinking was considered to evaluate the animal's total energy intake, and the energy of BD consumed by rats was added to that of food intake. A values of 6 Kcal/gr BD was considered as reported by others.^{33–35}

BD treatment did not affect fluid intakes (about 2–0 mL/day for all the groups).

β -OHB plasma levels were monitored both in control and in BD-treated rats, after 3 h from either the *in vivo* administration of BD and vehicle or after 2 weeks of treatment, to assess the efficacy of the treatments. Venus blood samples were obtained from a small cut on the tail, and (D) β -OHB was detected using a ketone body meter (Glucomen Areo β -ketone sensor).

At the end of the treatments, rats were anesthetized by *i.p.* administration of a single dose of sodium Tiopental (40 mg/Kg b.w.) and euthanized by decapitation. Tissues were excised, weighed and iBAT was immediately processed or frozen in liquid nitrogen and stored at -80°C for later analysis.

This study was carried out in agreement with recommendations of the EU Directive 2010/63 for the Care and Use of Laboratory Animals. All efforts were made to minimize the rats' suffering and pain. All animal protocols used throughout this study were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health (Authorization n° 776/2021-PR).

2.2 | Histological analysis

Samples of iBAT were fixed by immersion in 4% formaldehyde in 0.1 M phosphate buffer (overnight at 4°C). The samples were dehydrated in ethanol, cleared, and embedded in paraffin blocks. The tissues were cut into serial 6- μ m-thick sections; hematoxylin–eosin staining allowed morphological examination. The sections were viewed with a Nikon Eclipse 80i light microscope (Nikon Instruments, Milan, Italy). Images were obtained with a Sony DS-5M camera connected to an ACT-2U image analyzer. The average area of adipocytes and the frequency distribution were calculated from three sections per rat and at least four rats for each group (>250 adipocytes counted per group). Adipocyte size distribution is presented as percentage of the total amount of cells. Computed values were imported into Prism 5 (GraphPad Software, San Diego, CA, USA) for data analysis.

2.3 | Mitochondrial isolation

Immediately after excision, iBAT was deprived of all visible white adipose tissue contaminations and immersed in isolation buffer [220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, 5 mM EGTA, and 0.5% fatty acid-free bovine serum albumin (BSA), pH 7.4]. Little pieces were homogenized in a Potter-Elvehjem homogenizer, and the homogenate obtained was centrifuged at 10000g for 10 min, at 4°C. Floating fat was removed, while the pellet was then re-suspended in the original volume and centrifuged at 500g for 10 min; the resulting supernatant was centrifuged at 8000g to obtain a mitochondrial pellet. Mitochondrial pellets were washed twice, re-suspended in a minimal volume of isolation medium, and kept on ice.

The mitochondrial respiration rate was evaluated polarographically using a Clark-type electrode (Hansatech Instruments Ltd., UK) at 37°C. Isolated mitochondria (0.2 mg/mL) were incubated in a respiration medium made of 80 mM KCl, 50 mM HEPES (pH 7.0), 1 mM EGTA, 5 mM K_2HPO_4 , and 0.5% fatty acid-free BSA (w/v). Respiration was started by adding glycerol 3-phosphate (G3P) (6 mM) or succinate (10 mM). Successively, guanosine diphosphate (GDP) (2 mM) was added to the respiration medium.

2.4 | Mitochondrial membrane potential

Mitochondria (0.3 mg/mL) were incubated in the respiration medium supplemented with oligomycin (1 μ g/mL) and nigericin (80 ng/mL). Respiration rate was

determined as described above in mitochondria energized with G3P (6 mM) or succinate (10 mM) as substrate, in the absence and presence of 2 mM GDP. Membrane potential was assayed in 96-well plates using a fluorescence microplate reader (Tecan Infinity 200-Pro) using the positively charged dye safranin O, which changes fluorescence intensity linearly proportional to the mitochondrial membrane potential. The safranin O signal for each condition was measured at excitation and emission wavelengths of 540 ± 25 nm and 590 ± 25 nm, respectively, either in the absence of any substrates, in the presence of either G3P (6 mM) or succinate (10 mM) as substrate, or in the presence one of the two substrates and GDP (2 mM). The relative decrease in fluorescent signal upon energization of the mitochondria was proportional to the membrane potential; the results were reported as the absolute magnitude of this change in fluorescence, with larger changes in relative fluorescence units indicating higher membrane potentials.

The respiration rate was plotted as a function of the membrane potential to obtain the kinetic response of the overall reactions involved in the oxidation of substrates and the synthesis of ATP.

2.5 | Mitochondrial lysate preparation and western blot

Mitochondria were isolated as described above, with isolation buffer supplemented with antiprotease cocktails (Sigma Aldrich). Mitochondria were diluted in RIPA buffer, left on ice for 1 h, and then, centrifuged at 17000g for 30 min at 4°C. The resulting supernatants were collected, and the protein concentration was determined using Bio-Rad's DC method (Bio-Rad Laboratories, RRID:SCR_008426 Hercules, CA).

Primary antibodies used throughout the study were the following: anti-UCP1 (AB1426; Merk Sigma Aldrich), anti-mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH) [(GDP2, EPR14259) ab188585 Abcam], a cocktail of antibody used to detect CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP VA subunits of oxidative phosphorylation complexes (Total OXPHOS Rodent WB Antibody Cocktail, ab110413, Abcam). Anti-voltage dependent anion channel (VDAC, ab GTX114187 GeneTex), anti-heat shock protein 60 (HSP-60, GTX110089 geneTex), anti-vinculin (PA5-8026 Invitrogen, Thermofisher), and anti- β hydroxybutyryl-lysine (Kbhb, PTM-1202RM, PTM BIO). The primary antibodies were diluted 1:1000 except for anti- β hydroxybutyryl-lysine that was diluted 1:500 and anti-Total OXPHOS that was diluted 1:250.

2.6 | In solution digestion

For the proteomics analysis, a pool (5 rats) of mitochondrial lysates for each condition (C, BD-3h, and BD-2w) was used. For the preparation of the pools, mitochondria were isolated from individual tissues, and some aliquots were frozen in liquid nitrogen. Mitochondrial aliquots from the different tissues used ($n=5$ for each treatment) were processed simultaneously to obtain individual mitochondrial lysates. After evaluating the protein content of each mitochondrial lysate, the samples (20 μ g of protein for each sample) were pooled together. The mitochondrial lysate pool (55 μ g of protein) was subjected to a protein extraction protocol with four volumes of cold acetone. The samples were centrifuged for 10 min at 10000rpm. The pellets were dried and resuspended in denaturing buffer (6M Urea, 25mM ammonium bicarbonate). The cysteines were reduced by adding 100mM DTT (dissolved in denaturing buffer) to the protein suspension and incubated at 60°C for 1h. After cooling the samples, the reduced cysteines were alkylated with 120mM iodoacetamide and incubated in the dark for 1h at room temperature. DTT (100mM) was added for 1h at room temperature to quench the alkylation process. For enzymatic digestion, trypsin 1 μ g/ μ L was added to each sample in a ratio enzyme:substrate 1:50. The enzymatic hydrolysis was performed overnight at 37°C in a thermostatic bath. Each sample was treated with the same desalting procedure using stage tips containing 3 layers of 3M Empore C18 membrane. Stage tips were washed with 100 μ L 0.1% formic acid (HCOOH) and peptides eluted with 50 μ L of 50% acetonitrile (ACN) and, subsequently, with 80% ACN, both acidified with 0.2% HCOOH. The peptide mixture was dried in a vacuum Speed-Vac, and re-suspended in 50 μ L 5% ACN, 0.2% HCOOH.

2.7 | LC-MS/MS analysis

The mixture was analyzed by LC-MS/MS on an LTQ Orbitrap XL system (Thermo Fisher) equipped with a nano-LC Proxeon nanoEasy-II system. Peptides were fractionated onto a C18 reverse-phase capillary column (5 μ m biosphere, 75 μ m ID, 200 mm length) at flow rate of 250 nL/min. A linear gradient from 10% to 60% of eluent B (0.2% formic acid, 95% acetonitrile LC-MS Grade) was used over 200 min. Mass spectrometric analyses were carried out in Data Dependent Acquisition mode (DDA): from each MS scan, spanning from 300 to 1800 m/z, the five most abundant ions were selected and fragmented in the collision-induced dissociation (CID) modality. Mass tolerance was set at 10 ppm and Orbitrap resolution at 15000.

2.8 | Bioinformatics MaxQuant, proteomics data analysis

Raw data files were processed by using MaxQuant software (1.6.8.0 version).³⁶ The following parameters were used for raw data processing: trypsin enzyme specificity, 3 missed tryptic cleavages, oxidation of methionine, formation of pyroGlu from N-terminal glutamine (Q) or glutamic acid (E), β -hydroxybutyryl-lysine as variable modifications and cysteine (C) carbamidomethylation as a fixed modification. Identification parameters included minimum peptide length of 6 amino acids, minimum of 1 peptide (both razor and unique peptide). Peptide tolerance of 10 ppm and fragment mass tolerance of ± 0.2 Da. All proteins were filtered according to a false discovery rate (FDR) of 0.01% applied both at peptide and protein levels and a maximum peptide posterior error probability (PEP) of 1. The derived peak list generated by Quant. exe (the first part of MaxQuant) was searched using the Andromeda search engine integrated into the MaxQuant against the specific fasta file of *Rattus Norvegicus* obtained from the UNIPROT web site.

2.9 | Data processing

MaxQuant output files were subsequently processed using Perseus (RRID:SCR_015753) (version 1.6.8.0)³⁷ software platforms. An experimental design template was used to get merged replicate experiments (each data set contained two technical replicates) into a single column containing all the proteins into every sample. Contaminants, reverse, and only identified by site hits were filtered out. Expression values of label-free quantification (LFQ) intensity were log2 transformed and only the protein rows containing a minimum of 2 valid values were maintained within Perseus matrix. Missing values were replaced by random numbers drawn from a normal distribution with a width of 0.3 and a down shift of 1.8. The complete list of proteins (332-dataset) included UniProt id, gene name, protein name, score, and sequence coverage % (Supplementary dataset, File S2). Finally, the proteins were grouped for BD/C LFQ intensity ratio and ordered for upregulated (>1.2 fold change) and downregulated (<0.83 fold change).

2.10 | String and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways software

The STRING software (RRID:SCR_005223) was used to integrate all known and predicted associations between

regulated proteins, including both physical interactions as well as functional associations.³⁸ A full STRING network (the edges indicate both functional and physical protein associations), a medium confidence of 0.4, and k-means clustering (network is clustered to a specified number of clusters) were set.

KEGG enrichments were obtained by String and by the KEGG database (RRID:SCR_012773)³⁹ by converting Uniprot id into KEGG annotation (genome annotation database).

2.11 | Statistical analysis

Functional data and Western blots were analyzed with the GraphPad Prism RRID:SCR_002798 software system version 6. Student's *t*-test or one-way analysis of variance (ANOVA), followed by the "Tukey correction for Multiple Comparisons" post-hoc test was used to establish the statistical significance of the differences between experimental groups. Differences were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Administration of 1,3-butanediol to rats enhances plasma β -OHB levels and affects rats' body weight gain, adipose tissue weight, and iBAT morphology

The *in vivo* administration of BD was effective in increasing β -OHB plasma levels. Within 3 h from the *i.p.* administration (BD-3h group), β -OHB serum levels were about 6 fold than C. When the *i.p.* administration was followed by 2 weeks of BD administration in drinking water (BD-2w group), β -OHB levels were ~2.5 fold that of C (Figure 1A). These data confirm the effectiveness of the treatments and our previous data.²²

Due to the fast increase in β -OHB plasma levels observed following *i.p.* BD administration, group BD-3h allows us to evaluate the rapid response of iBAT mitochondria to a β -OHB enhancement. Maintaining high levels of β -OHB for 2 weeks in the BD-2w group enables us to assess the long-term adaptation of iBAT mitochondria to high β -OHB exposure.

At the beginning of the treatment, all the rats showed similar body weights (average of 286 gr). At the end of 2 weeks of treatment, the BD-2w group gained less weight than the control (−50%) (Figure 1B). During the 2 weeks of treatment, BD-2w had a lower energy intake (−15%) compared to C (Figure 1C); additionally, the ratio between energy intake and body weight

gain (i.e. an index of the rough energy efficiency) was significantly enhanced in BD-2w versus C (Figure 1D), thus confirming what already reported in our previous paper.²² Whole animal VO₂, detected on a subset of animals, was enhanced by BD treatment for two weeks (Figure S1). BD influences the contribution of adipose tissues to the total rat weight. The contribution of iBAT was slightly but significantly enhanced by 13%, and that of gonadal white adipose tissue was reduced by about 18% in BD-2w compared to C. The contributions of liver, skeletal muscle, and heart weight were not affected by BD treatment (Table 1).

Isolated iBAT tissues from BD-2w rats appear darker in color. Histological analysis of iBAT parenchyma showed typical multilocular cells that in the BD-2w group presented a reduced average size. Indeed, after 2 weeks of BD treatment, a decrease in the percentage of large adipocytes was observed associated with a concomitant increase in the percentage of small adipocytes (Figure 1E–H).

3.2 | Proteomics analysis of iBAT mitochondrial lysates

Proteins obtained by a pool of iBAT mitochondrial lysates were subjected to a classical *in-solution* digestion for a large-scale shotgun bottom-up approach performed by LC–MS/MS analysis. Data obtained from high-resolution mass spectrometry requires a powerful platform with quantitative proteomics software to avoid erroneous protein identification and to obtain statistically reliable quantitative information. In our experiment, MS/MS raw data were processed by using MaxQuant, a software useful for protein identification and LFQ toward the comparison of precursor ion peaks across runs, allowing reproducible protein measurements. The protein group file was loaded on Perseus software to visualize and filter proteins according to the "Material and methods" steps. A total of 1028 proteins was identified by using MaxQuant stringent criteria of threshold based on a target decoy database approach and by setting a value of 0.01 FDR at the peptide spectrum match level, corresponding to a 99% confidence score. Approximately 33% of proteins were selected by the steps of Perseus filtering according to the criteria reported in the material and method section, allowing a dataset of 332 proteins (Figure 2).

Within the 332-dataset, further filtering was performed to chase those proteins with marked dysregulation along the samples by comparing LFQ intensity of BD-2w versus the C sample. More than 50% of proteins displayed a dysregulation due to the fold change (ratio of LFQ intensity of BD vs. C) higher than 1.2 times or less than 0.83 times. The

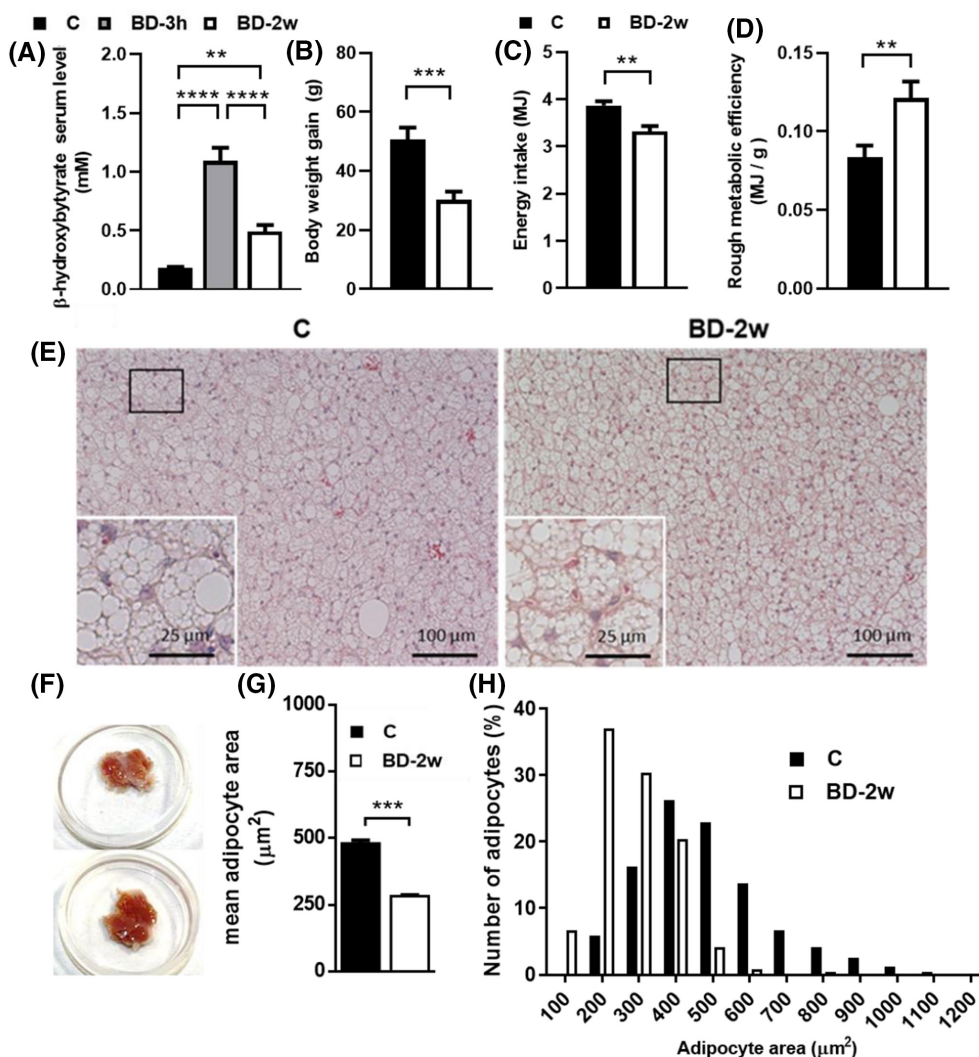


FIGURE 1 Effect of BD administration on β -OHB plasma levels, rats' parameters, and iBAT morphology. (A) β -OHB plasma levels, (B) body weight gain, (C) energy intake, (D) rough metabolic efficiency. Values represent mean $\pm$ SEM of 13–14 animals. (E) iBAT hematoxylin & eosin staining; inset represents high magnification of the frame areas, (F) tissues explant, (G) mean cell size, and (H) cell size distribution analysis of iBAT from C and BD-2w rats. ** $p < .01$; *** $p < .001$; **** $p < .0001$.

TABLE 1 Effect of BD treatment for 2 weeks on the contribution of tissues to the rat weight.

	C	BD-2w
Liver weight/body weight $\times 100$	3.38 ± 0.09	3.15 ± 0.08
Heart weight/body weight $\times 100$	0.27 ± 0.01	0.28 ± 0.01
Skeletal Muscle-Tibialis weight/body weight $\times 100$	0.25 ± 0.03	0.29 ± 0.04
Skeletal Muscle-Soleus weight/body weight $\times 100$	0.054 ± 0.002	0.054 ± 0.003
Interscapular Brown Adipose Tissue weight/body weight $\times 100$	0.087 ± 0.006	$0.114 \pm 0.008^*$
Gonadal White Adipose Tissue weight/body weight $\times 100$	1.37 ± 0.08	$1.13 \pm 0.06^*$

Note: Results represent the mean $\pm$ SEM of 14–13 different animals.

* $p < .05$ versus C.

hierarchical clustering heatmap performed by using Perseus showed a visual representation of the relative expression levels of the 177 proteins, suggesting a strong clustering

between C and BD-3h because of an intermediate behavior of protein expression in BD-3h. Interestingly, the heatmap of exclusive up-regulated proteins displayed a change

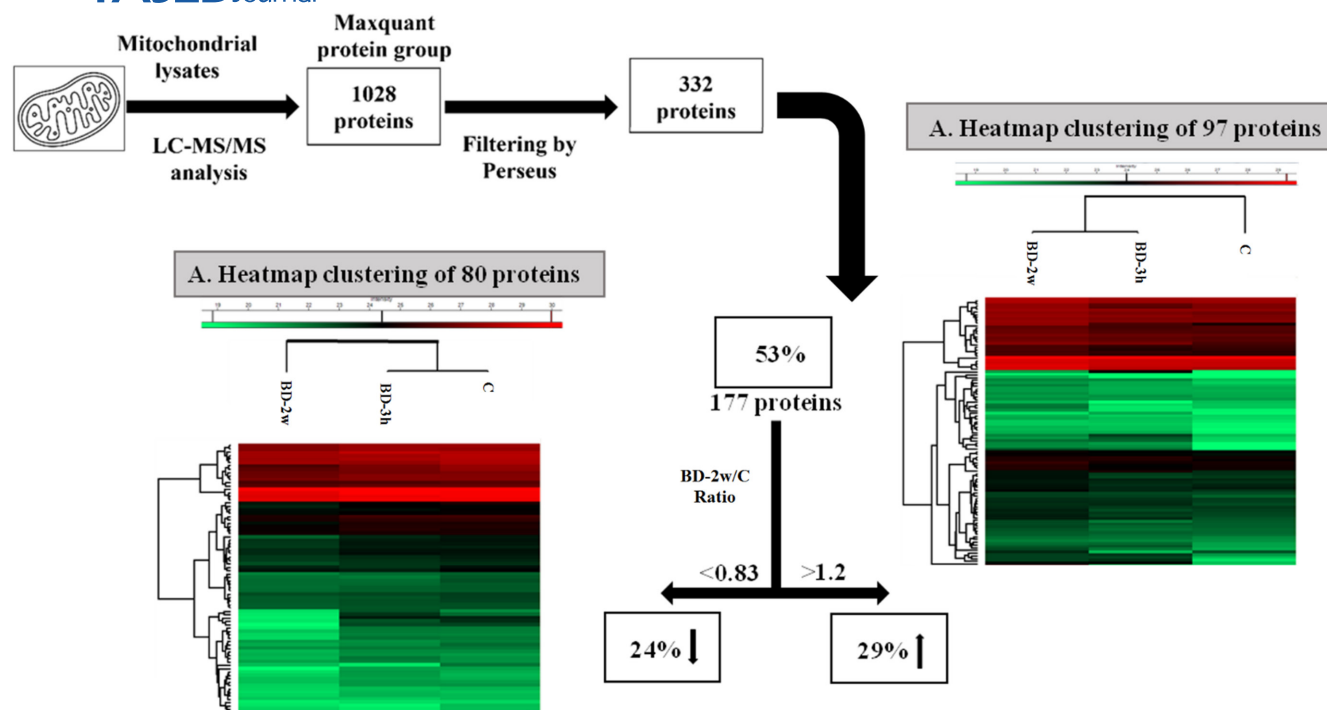


FIGURE 2 Filtering steps for the selection of a dataset of significant proteins. Heat map hierarchical clustering of 80 down-regulated proteins (A) and 97 up-regulated proteins (B) detected along the C, BD-3h, and BD-2w. Each value is the result of two technical replicates obtained by a pool of 5 rats.

of clustering by reducing the distance between BD-2w and BD-3h, thus suggesting that the changes in the abundances of up-regulated proteins of BD-2w and BD-3h resulted to be more similar when compared to C samples (Figure 2, panel A). On the contrary, BD-2w resulted to be more distinct from the other two samples in the heatmap of down-regulated proteins highlighting different changes in protein abundance following the 2w stimulation (Figure 2, panel B).

Protein name, UniProt Id, gene name, sequence coverage [%], score, and the ratios of BD-2w/C and BD-3h/C LFQ intensity are reported in Supplementary data (File S2).

3.3 | BD treatment affects the levels of proteins involved in oxidative phosphorylation: Results from proteomic analysis and western blot

Among the up-regulated proteins, 35% were mitochondrial proteins involved in oxidative phosphorylation. All mitochondrial proteins involved in the oxidative phosphorylation were picked out from the String analysis where functional and physical protein associations were visualized from a big network of lines and edges, obtained from a value of 0.4 interaction score (medium confidence) (Figure S2A). After converting UniProt id codes into the relative KEGG annotations, the results displayed that the

proteins belong to the five mitochondrial respiratory complexes as reported in colored boxes highlighted within the KEGG oxidative phosphorylation (Figure S2B). Figure 3 illustrates the change in protein expressions of subunits belonging to the five respiratory complexes, as well as of UCP1 due to BD treatments. A significant fold change greater than 2 was observed for some proteins when analyzing BD-2w versus C or BD-3h versus C.

In Complex I, seventeen subunits were upregulated in the BD-2w group, while none of the detected subunits were downregulated. Notably, ND2, Ndufb7, and Ndufb8 showed the most changes. In the BD-3h group, four subunits were upregulated (with Ndufb7 and ND2 being the most affected), whereas thirteen subunits were downregulated. In Complex II, peptides from three different subunits were detected by MS. Among them, two subunits (Shdha and Shddb) were upregulated in the BD-2w group versus C, and one subunit (Shdhc) was upregulated in the BD-3h group versus C. Other than enhancing complex II subunits, treatment with BD for 2 weeks induced a 3.8-fold enhancement of Succinate dehydrogenase assembly factor 1 (Sdhaf1), a protein involved in the assembly of the complex, compared to C.

For what Complex III was concerned, upregulation of three subunits was observed in the BD-2w group versus C, whereas no changes were observed in the BD-3h group. In Complex IV, seven subunits were upregulated in the BD-2w group. Notably, CoI, Cox6a1, and Cox8b showed a fold change of BD-2w/C greater than 2. In the BD-3h

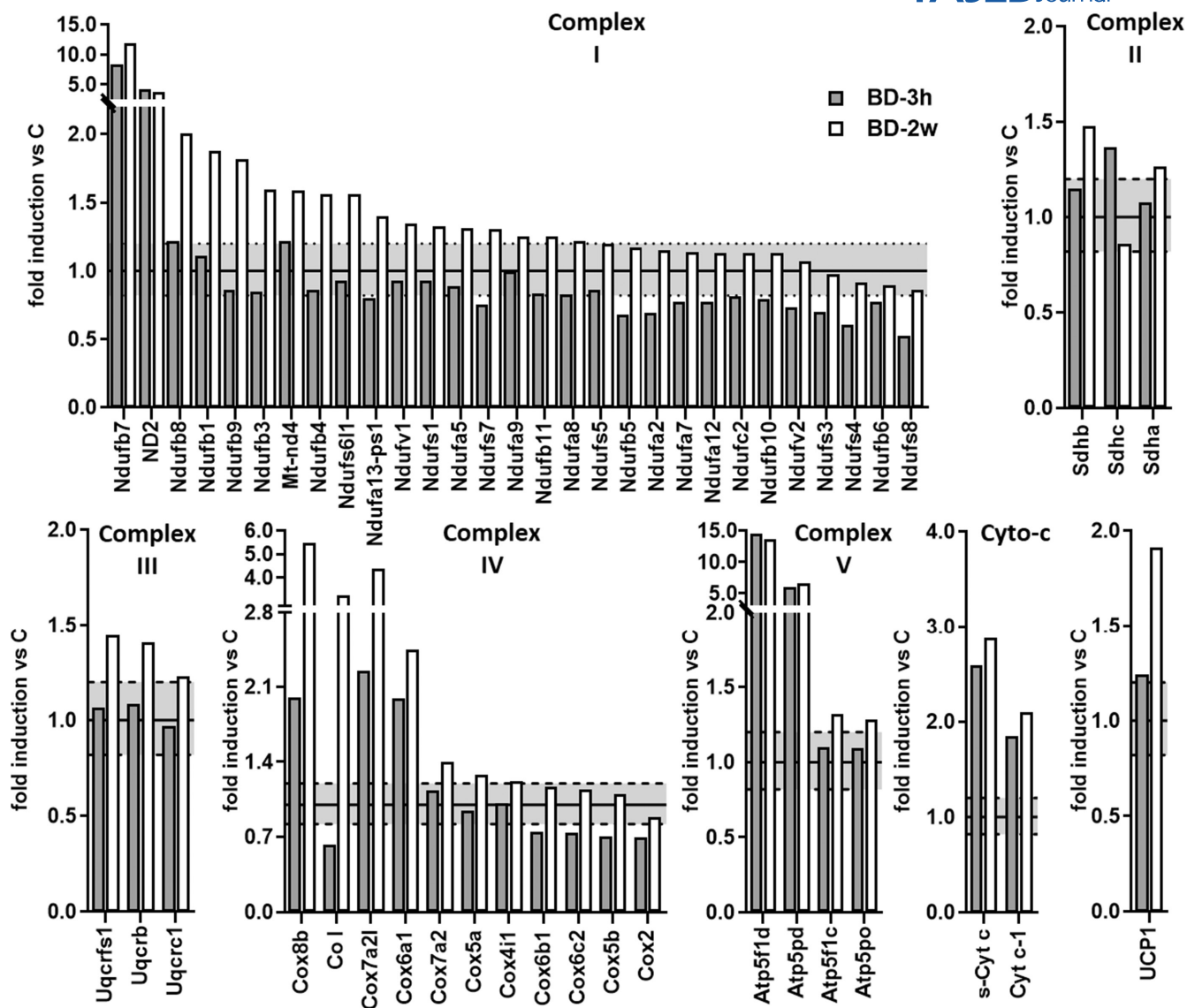


FIGURE 3 Effect of BD on mitochondrial respiratory chain complexes subunits and UCP1 protein levels. Relative abundance of iBAT mitochondrial proteins determined by the label-free proteomics approach. Data are summarized for proteins that can be assigned to respiratory chain complexes. Gene names for subunits that have changed have been reported. The values with a fold >1.2 are an expression of upregulation, whereas those with a fold <0.82 are indicative of a downregulation. The upper dotted line represents the value 1.2, and the lower dotted line represents the value 0.82. Each value is a result of two technical replicates obtained by a pool of 5 rats.

group, upregulation of Cox8b, Cox7a2l, Cox6a1 was also observed, with a fold change BD3-h/C of about 2. A downregulation of CoI, Cox6b1, Cox6c2, Cox5b, Cox2 subunits was observed in the BD-3h group compared to C.

In Complex V, four subunits were upregulated in the BD-2w group versus C, with Atp51fd and Atp5pd showing a fold change greater than 2. Similar stimulation was observed in the BD-3h group compared to C. In both BD-2w and BD-3h groups, increases in Cytochrome c subunits (s-Cyt c and Cyt-c1) were detected compared to C.

Moreover, the MS analysis also revealed a progressive increase in UCP1 protein levels in the BD-3h and BD-2w groups compared to C (Figure 3). These data were also obtained by Western blot analysis (Figure 4).

Additionally, Western blot analysis showed that BD treatment significantly enhanced the levels of specific subunits of mitochondrial respiratory complexes, whereas it did not affect mG3PDH protein levels (Figure 4). These data are in line with proteomic ones.

3.4 | BD affects levels of proteins involved in Propionyl CoA-metabolism and Succinyl-CoA formation and ketone bodies metabolism

According to KEGG analyses some proteins regulated by BD belong to the network of propanoate metabolism

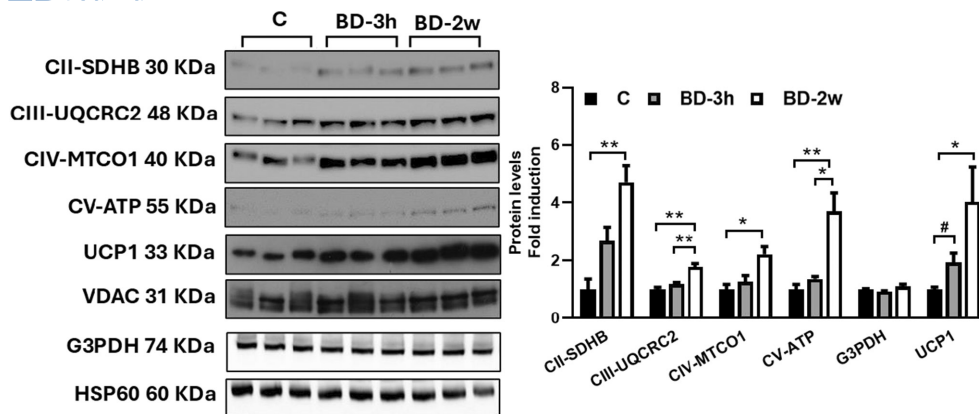


FIGURE 4 Representative Western blots of respiratory complexes subunits (CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP VA), mG3PDH, and UCP1 detected in isolated mitochondria from iBAT of C, BD-2w, and BD-3h rats (15 μ g of protein/mouse/lane). VDAC or HSP60 was used as the loading control. Histograms represent the quantification of data. Data were normalized to the value obtained for C animals, set as 1. Values represent the mean $\pm$ SEM of 3 or 6 different rats. * $p < .05$, ** $p < .01$; #ns by ANOVA, $p = .018$ by t -test.

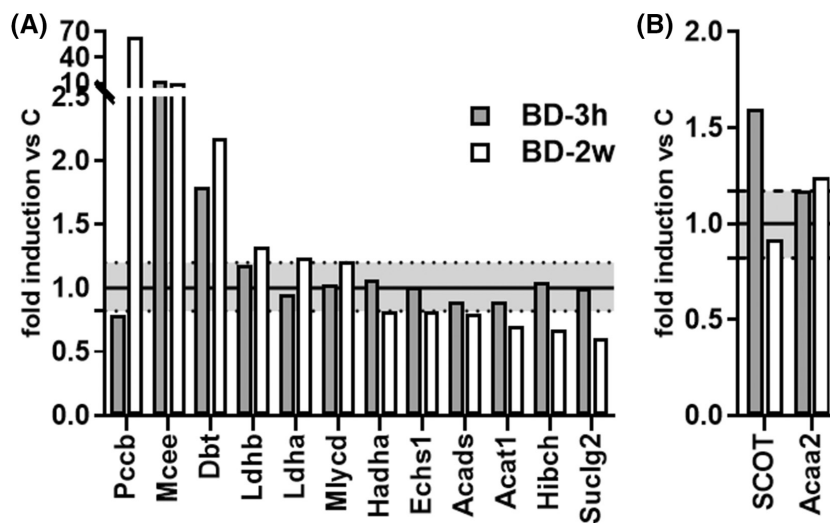


FIGURE 5 Effect of BD on the levels of proteins involved in propanoate metabolism (A) and ketone bodies metabolism (B). Relative abundance of iBAT mitochondrial proteins involved in propanoate metabolism affected by BD treatments. Gene names of changed proteins have been reported. The values with a fold >1.2 are an expression of upregulation versus C, whereas those with a fold <0.82 are indicative of a downregulation versus C. The upper dotted line represents the value 1.2, and the lower dotted line represents the value 0.82.

(see File S1 and Figure S3). BD treatment for 2 weeks increased the levels of enzymes involved in Propionyl-CoA metabolism, leading to Succinyl-CoA formation. Specifically, Propionyl-CoA carboxylase beta chain (Pccb) and Methylmalonyl CoA epimerase (Mcee) exhibited significant fold changes in BD-2w compared to C, with a remarkable 64-fold and 13-fold increase, respectively (Figure 5A). The effect of BD on Methylmalonyl CoA epimerase protein expression was already observed within 3h from its administration (Figure 5A). On the other hand, levels of enzymes involved in the pathways leading to Acetyl-CoA from propionyl CoA are reduced by 2 weeks BD treatment, among these are: Short-chain specific acyl-CoA dehydrogenase (Acads), Enoyl-CoA hydratase (Echs1), Trifunctional enzyme subunit alpha

(Hadha), 3-hydroxyisobutyryl-CoA hydrolase mitochondrial (Hibch) (Figure 5A).

BD treatment enhances levels of enzymes involved in ketone bodies metabolism such as Succinyl-CoA:3-Ketoacid CoA transferase (SCOT) within 3h and 3-ketoacyl-CoA thiolase (Acaa2), that resulted slightly enhanced in BD-2w compared to C (Figure 5B).

3.5 | BD rapidly enhances mitochondrial respiration rate by affecting the activity of the respiratory chain and UCP1

To assess the impact of BD on iBAT mitochondrial functionality, we measured oxygen consumption by using

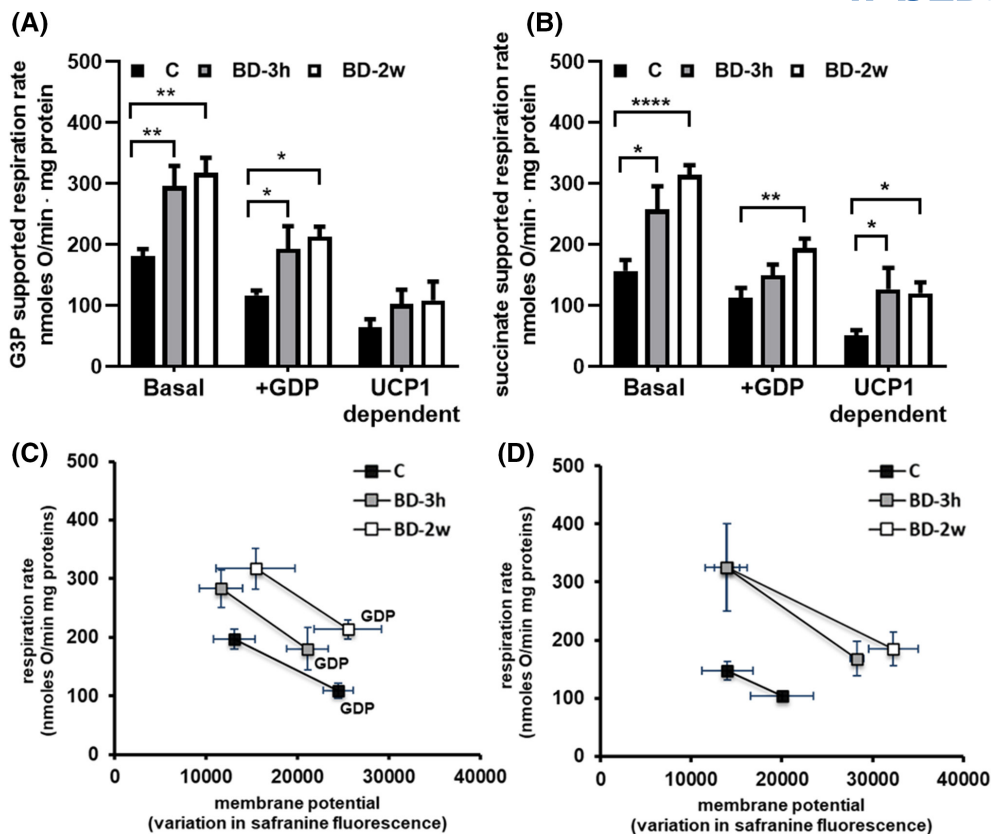


FIGURE 6 Effect of BD on mitochondrial respiration and the overall activity of reactions involved in the oxidation of substrates. To detect mitochondrial respiration, mitochondria were energized with either G3P (A) or succinate (B). Respiration was evaluated in the absence or the presence of GDP (2 mM). UCP1-dependent respiration was obtained by subtracting GDP-inhibited respiration from basal respiration. Values represent the mean $\pm$ SEM of 7–8 different rats. Measurements were performed in duplicate. * $p < .05$, ** $p < .01$, **** $p < .0001$. To detect the overall activity of reactions involved in the oxidation substrates, membrane potential and mitochondrial respiration were detected in parallel experiments in mitochondria energized with G3P (C) or succinate (D), in the absence (basal) and the presence of GDP. Values represent the mean $\pm$ SEM of 5 different samples.

two different respiratory substrates: glycerol-3-phosphate (G3P) (Figure 6A) and succinate (Figure 6B). BD treatment leads to an increase in mitochondrial respiration regardless of the respiratory substrate used. This effect was observed within 3 h from BD administration and persisted after 2 weeks of treatment. Specifically, compared to the C group, mitochondria from the BD-3h group exhibited higher respiration rates, with a similar increase (+64%) in the presence of G3P (Figure 6A) or succinate (Figure 6B) as substrates. The administration of BD for 2 weeks led to increases in respiration rate of +74% and +100% versus C, in the presence of G3P or succinate, respectively.

As expected, the addition of GDP to inhibit the UCP1 activity resulted in a reduction in mitochondrial respiration rate. However, mitochondria from the BD-treated rats still showed a higher respiration rate compared to the C group, even in the presence of GDP. When looking at UCP1-linked respiration (obtained by subtracting GDP-inhibited respiration from basal respiration), BD treatment enhanced it, and the stimulatory effect reached

statistical significance in the presence of succinate as a respiratory substrate.

The ability of BD to stimulate respiration, even when UCP1 activity is inhibited by the presence of GDP, suggests that BD may affect the reactions involved in substrate oxidation. To explore this possibility, we evaluated the impact of BD treatment on the overall kinetic of the reactions involved in the oxidation of substrates and in the genesis of proton motive force, including the respiratory chain (Figure 6C,D). When comparing the plots obtained from the three groups, we observed distinct patterns between the BD-3h and BD-2w groups compared to C: the plots for BD-3h and BD-2w do not overlap with those for C and indicate a change in the overall substrate oxidation kinetics in the former two groups relative to C. In other words, at any given mitochondrial-membrane potential, mitochondria from the BD-3h and BD-2w groups showed a higher respiration rate. This indicates an increase in respiratory chain activity induced by BD administration. Noteworthy, this effect was observed after 3 h and

persisted after 2 weeks of treatment. Data from Figure 6D also indicate that a process of proton leak is stimulated by BD treatment, in fact in basal condition, when UCP1-mediated proton leak is active, mitochondria from BD-2w and BD-3h groups respire more to maintain the same membrane potential.

3.6 | Ketone bodies induce Kbbh of mitochondrial proteins

We then investigated the Kbbh on mitochondrial proteins by combining Western blot and MS data. Western blot analysis was performed by using an antibody against β -hydroxybutyryl-lysine residues, and we used the liver homogenate as a positive control. In liver lysate, we revealed an increase in Kbbh proteins in BD-3h and BD-2w versus C (+127% and +134%, respectively), thus confirming data already reported in the literature,¹⁹ and extending the effect observed to a short-term (within 3 h). Interestingly, we also observed an increase in protein Kbbh in iBAT mitochondria from BD-3h and BD-2w groups versus C

(Figure 7). These results led us to evaluate if some subunits of respiratory chain complexes and UCP1 could undergo Kbbh, by proteomics approach based on mass spectrometry (see methods). Indeed, as reported in Table 2, some specific subunits of respiratory chain complexes I (Nufa9, Nufa10, Nufs4), II (Sdhh), III (Uqcrrs1, Uqcrrc2), and V (Atp5f1c) displayed lysine residues modified with Kbbh in the BD-3h group, while UCP1 presents two lysine residues that undergo to Kbbh.

4 | DISCUSSION

The manipulation of BAT's energy-burning capacity has the potential to yield metabolic benefits, making it an attractive target for interventions against dysmetabolic diseases and obesity,^{2,3,5} although the current contribution of BAT to whole-body energy expenditure in humans is still a matter of dispute.^{7,8}

The findings presented in this study demonstrate that the administration of BD and the resultant "exogen ketosis" significantly impact iBAT functionality, with

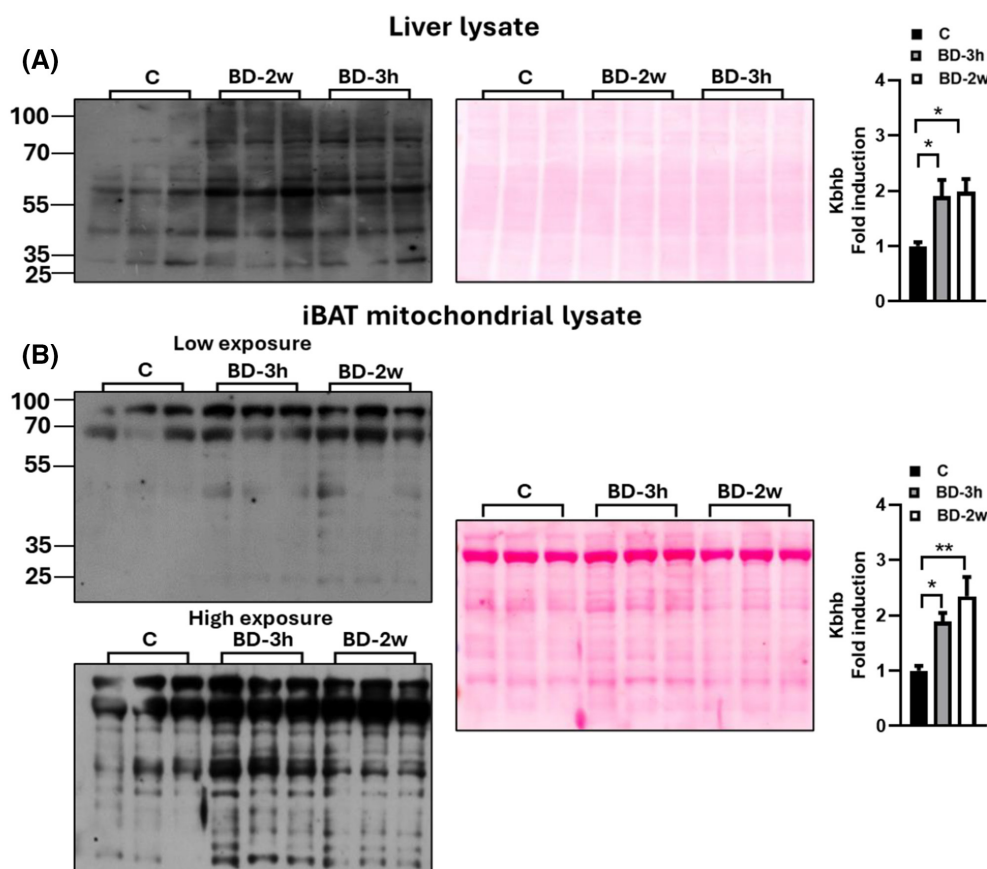


FIGURE 7 Effect of BD on lysine residues beta-hydroxybutyrylation. Western blot analysis of total lysine beta-hydroxybutyrylation in the liver lysate (A) and isolated mitochondria (B) using a pan-K-bhb antibody. For iBAT mitochondria lysates, two different exposure times were represented. Histograms represent the quantification of data. Ponceau staining was used as a loading control (25 μ g of protein/rat/lane). Data were normalized to the value obtained for control animals, set as 1. Values represent the mean $\pm$ SEM of 5–6 different rats. * p < .05, ** p < .01.

TABLE 2 List of the proteins presenting β -hydroxybutyryl lysine, detected in iBAT mitochondria extracts from C, BD-3h and BD-2w samples.

Gene name	Protein description	Peptide sequence	Peptide modifications	C	BD3h	BD 2w
Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	SKAVGEKEVR	2 beta-hydroxybutyryl (K)		●	
Ndufa10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	KGDPHEMKVTSAYLQDIEDAYKK	beta-hydroxybutyryl (K)			●
Ndufs4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	NNMQSGVNNTKKWK	2 beta-hydroxybutyryl (K)		●	
Sdhb	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	MAAVGVSLK	beta-hydroxybutyryl (K)		●	
Uqcrls1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	SSKESSEAR	beta-hydroxybutyryl (K)		●	
Uqcrc2	Cytochrome b-c1 complex subunit 2, mitochondrial	GGLGLAGAKAK	beta-hydroxybutyryl (K)	●	●	
Atp5f1c	ATP synthase subunit gamma, mitochondrial	AEIKGPEDKK	beta-hydroxybutyryl (K)			●
Atp5f1c	ATP synthase subunit gamma, mitochondrial	GLCGAIHSSVAKQMKNMAALTAAGK	beta-hydroxybutyryl (K)			●
Ucp1	Mitochondrial brown fat uncoupling protein 1	GVLGTITTLAK	beta-hydroxybutyryl (K)		●	●
Ucp1	Mitochondrial brown fat uncoupling protein 1	KELMKSR	beta-hydroxybutyryl (K)		●	

Note: The symbol • indicates the presence of the modified peptide in the samples.

mitochondria being a target. In fact, the exposure of the tissue to elevated levels of β -OHB for 2 weeks leads to an increase in tissue weight/body weight ratio and a change in tissue morphology, characterized by the presence of smaller adipocytes, that are indicative of tissue thermogenesis activation. These findings are in line with results in the literature, reporting that other treatments able to induce ketosis (ketogenic diet or administration of ketone esters) were able to affect the functionality of such tissue.^{27,29,40} However, in studies using ketone ester to induce exogenous ketosis, the treatment resulted in β -OHB levels about six-fold the control values.^{29,40} In our study, BD treatment for 2 weeks was able to induce “mild” ketosis, given the evidence that β -OHB plasma levels in this group were about 0.5 mM, i.e. a value half of those observed following 16 h of food removal (see Figure S4). This suggests that variations in β -OHB levels within the physiological range are effective in activating iBAT, although circulating β -OHB concentrations alone provide limited information without the understanding of the tissue's clearance.

The ketogenic diet and ketone ester treatments, above cited, have been reported to enhance the levels of proteins related to respiratory complexes and UCP1 in total BAT lysate, as well as proteins involved in mitochondrial biogenesis,^{27,28,40} thus suggesting an increase in mitochondrial mass. Starting from this knowledge, we decided to give further insight into the effect of β -OHB on mitochondria by evaluating its influence on the biochemical and bioenergetic parameters of these organelles. Thus, we examined alterations in mitochondrial proteomic patterns associated with changes in mitochondrial functionality. In addition, for the first time, we reported that some of the effects induced by BD on iBAT manifest within 3 h from its in vivo administration. This suggests the direct role of β -OHB on the tissue, independent of the pleiotropic effects associated with physiological or pathological conditions that increase its levels, such as fasting, ketogenic diet, physical exercise, or diabetes.

Functional data along with Western blot and the proteomic approach revealed that the primary adaptation

associated with BD-induced exogenous ketosis is the activation of mitochondrial respiratory chain functionality and UCP1-mediated proton leak. Concerning the respiratory chain, BD administration affected the levels of subunits belonging to each of the five respiratory complexes as revealed by western blot and confirmed by proteomic analysis. The last suggested that the regulation of specific subunits within each complex was not uniform, indicating changes in complex composition and suggesting critical roles for certain subunits. Notably, the response of individual subunits to BD administration was highly dynamic, with modulation of some subunits observed within 3 h from its administration. Although the upregulation of specific subunits persisted or increased after 2 weeks of BD treatment, the down-regulation of subunits belonging to complexes I and IV was only evident in the short term (BD-3h group) and disappeared after 2 weeks of treatment. This highlights the markedly dynamic nature of the response of the BAT mitochondrial respiratory chain to elevated levels of β -OHB. The dynamic up-and-down-regulation of specific respiratory complex subunits appears to be a common process involved in triggering the rapid remodeling of the mitochondrial respiratory chain. In fact, this phenomenon also occurs following *in vivo* administration of succinate, known to rapidly influence the functionality and proteomics of BAT, in a time interval between 30 min and 3 h.⁴¹

Another important data emerging from our research is that treatment with BD increased the expression of proteins associated with the structural organization of the mitochondrial respiratory chain, among these, succinate dehydrogenase assembly factor (Sdhaf1), which facilitates the assembly of complex II,⁴² and Cox7a2l protein, involved in the stabilization of complexes III and IV, by promoting their supramolecular organization in III₂+IV supercomplex.⁴³

BD administration induces post-translational Kbbh of mitochondrial proteins, as revealed by Western blot analysis (Figure 7). In line with this, proteomic data allowed us to identify Kbbh modifications in three subunits of complex I (Ndufa9, Ndufa10, Ndufs4, important for the assembly and the stability of the complex), one subunit of complex II (Sdhb, which is part of the catalytic core of the complex), one subunit of complex III (Uqcrrf1, part of the catalytic core of the complex), one subunit of complex V (Atp5f1c or gamma subunit, involved in rotatory catalysis),⁴² as well as in UCP1. Similar to other mitochondrial post-translational modifications involving acylation process, Kbbh modifications could influence protein function and protein-protein interactions.⁴⁴ Therefore, the Kbbh modifications discussed here may be part of the mechanism involved in the

dynamic responsive behavior of the respiratory chain and UCP1 to β -OHB.

The mitochondrial functional analysis reported here is in line with and corroborated by Western blot and proteomic analysis. In fact, by evaluating mitochondrial respiration rate, we were able to demonstrate the ability of BD to rapidly increase it, with the activation of the respiratory chain and UCP1 being involved. Indeed, when iBAT mitochondrial respiration is evaluated in the basal condition, its control is shared between proton leak (in BAT mainly mediated by UCP1) and the overall reactions involved in the oxidation of the substrate and in the generation of proton motive force (among these the respiratory chain). However, in the presence of GDP, which abolishes the contribution of UCP1-mediated proton leak, the control exerted by the respiratory chain on respiration becomes more relevant.⁴⁵ The ability of BD to enhance mitochondrial respiration rate both under basal conditions and in the presence of GDP, reported here, indicates an improvement in respiratory chain activity. This is further supported by the experiment aimed at determining the overall kinetics of the reactions involved in substrate oxidation and proton motive force generation, which was enhanced by BD treatment. This activation also contributes to mitochondrial thermogenesis, since BAT UCP1 dissipates the proton motive force, generated by the respiratory chain, as heat.

Furthermore, despite observing a BD-dependent activation of the respiratory chain, in mitochondria respiring under basal conditions we did not find a higher mitochondrial inner membrane potential. This indicates that the proton motive force is rapidly dissipated through the proton leak process (see Figure 5B).

The increase in UCP1 proteins, observed within 3 h from BD administration and even more prominent after 2 weeks of treatment, together with BD-induced enhancement in UCP1-dependent respiration, supports the involvement of such protein in the activation of mitochondrial respiration and thermogenesis.

Another aspect emerging from our data is that BD administration promoted an increase in mitochondrial levels of proteins involved in succinyl-CoA formation from propionyl-CoA, whereas reducing the levels of proteins involved in the formation of Acetyl-CoA from Propionyl-CoA. This suggests that BD treatment enhances the formation of succinyl-CoA and thus succinate, whose utilization as mitochondrial respiratory substrate was increased by the same treatment, as revealed by increased succinate-supported respiration (Figure 6B). Detailed metabolic fluxes analysis will be necessary to validate this possibility.

A further important point to be considered is that the formation of succinyl-CoA plays also an important role

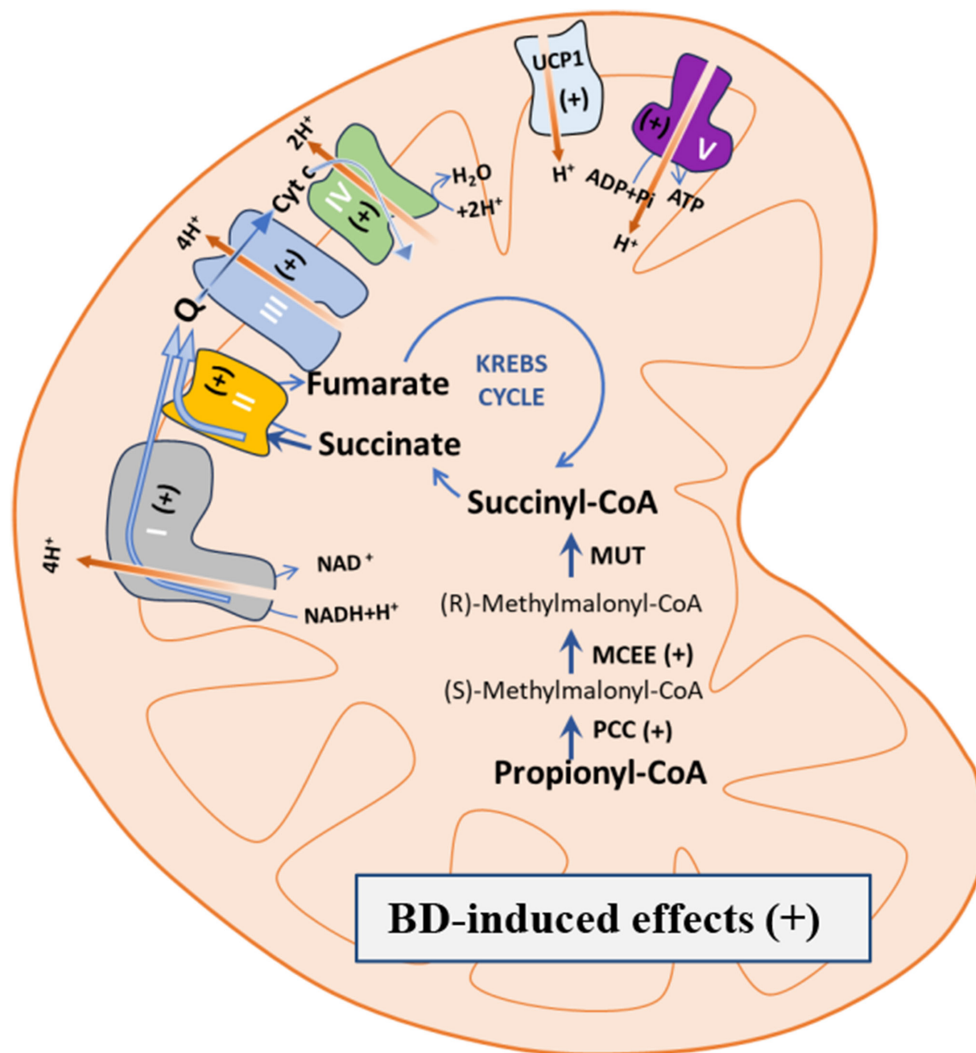


FIGURE 8 Schematic representation of the events underlying the effect of BD on iBAT mitochondrial functionality. BD treatment stimulates BAT mitochondrial respiration by improving the activity of the respiratory chain and UCP1. Increases in specific subunits of the five respiratory complexes and UCP1 underlie the BD effect on mitochondrial respiration. BD also increases the levels of proteins involved in the anaplerotic pathway that forms succinyl-CoA from propionyl-CoA. Succinyl-CoA is the precursor of succinate, the substrate of complex II of the respiratory chain, and BD enhances the use of succinate as a respiratory substrate. The symbol +, when under parentheses, indicates that one or more subunits of the specific respiratory complexes or other indicated proteins were upregulated by the BD treatment.

in ketolysis in extra-hepatic tissues, since it provides CoA for the formation of acetoacetyl-CoA from the ketone body acetoacetate. This reversible reaction is catalyzed by succinyl-CoA:3-ketoacid CoA transferase^{10,46}; subsequently, a mitochondrial 3-ketoacyl-CoA thiolase converts acetoacetyl-CoA to two molecules of acetyl-CoA, which can enter the tricarboxylic acid cycle. Therefore, the ability of BD to increase the levels of proteins involved in the pathways leading to succinyl-CoA may also be useful for β -OHB metabolism. This possibility agrees with our results showing that BD treatment increased succinyl-CoA:3-ketoacid CoA transferase (observed within 3 h) and slightly 3-ketoacyl-CoA thiolase levels.

It should be mentioned that our study has some limitations. First, all the results reported here referred to male

rats, and we did not investigate for potential sexual dimorphism response of BAT to BD administration.

Concerning proteomic analysis, pooling the samples does not allow us to consider the variability between biological samples and that for the proteins, whose levels were not validated by western blot, there is a possibility that an outlier sample drives their variation.

In the present paper, we did not investigate whether the variation in BAT activity could have affected the whole animal energy balance and to which extent the reduction in food intake, due to BD treatment, could have contributed to the decrease in body weight gain and even more in gonadal WAT mass. We limited our observation to the whole animal oxygen consumption of a subset of animals, which was stimulated (Figure S1). Further detailed studies

are needed to address this fundamental aspect, given the evidence that in the literature discordant opinions are presented on the ability of exogen-induced ketosis to affect whole animal energy expenditure.^{29,40,47}

In conclusion, by taking advantage of the ability of BD to increase plasma levels of β -OHB, we suggest a role for β -OHB as a signaling molecule that can rapidly affect iBAT physiology/thermogenesis. The findings reported in the present paper provide valuable insights into the molecular mechanisms by which β -OHB influences iBAT mitochondrial function and shed light on its effects. In particular, the mitochondrial respiratory chain, UCP1, and pathways involved in propionyl-CoA metabolism are targets of β -OHB. A schema/picture of the events underlying the mechanism by which BD affects iBAT mitochondrial functionality is reported in Figure 8. The ability of β -OHB to rapidly induce Kbh of mitochondrial proteins suggests a direct effect of the molecule on the organelle.

This finding opens new opportunities for further studies aiming to assess the actual impact of Kbh on mitochondrial protein function and BAT mitochondrial bioenergetics, as well as the ability of BD to influence the response of BAT to cold exposure and the whole animal energy balance.

AUTHOR CONTRIBUTIONS

Assunta Lombardi, Paola Venditti, Angela Amoresano, and Pieter de Lange designed research. Paola Venditti, Gabriella Pinto, Giuliana Panico, Rita De Matteis, Vincenzo Migliaccio, Gianluca Fasciolo, Gaetana Napolitano and Stefania Serpico performed researches. Assunta Lombardi, Pieter de Lange founding acquisition, Paola Venditti, Rita De Matteis, Lillà Lionetti, Pieter de Lange, Claudio Agnisola, Angela Amoresano, and Assunta Lombardi analyzed and interpreted data. Assunta Lombardi, Paola Venditti and Gabriella Pinto wrote the original draft. All authors were involved in revising the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available in the article and the [supplementary material](#).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Mild and short-term caloric restriction induces coordinated changes that promote lipid deposition while maintaining thermogenesis capacity in interscapular Brown adipose tissue of male rats.

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ABSTRACT

Calorie restriction (CR) without malnutrition improves metabolic health, slows the aging process, and prolongs longevity. Brown adipose tissue (BAT), which specializes in burning energy to produce heat, also contributes to metabolic health; however, the effects of CR on BAT function are only partially understood, and even less is known about those exerted by mild CR for a short period of time. We studied the multifaceted relationship between mild short-term CR (15% applied for two weeks) and intrascapular BAT (iBAT) in rats, and provided insights into the adaptations occurring in the tissue through multiple complementary approaches: histology, mitochondrial respirometry, comprehensive quantitative proteomics, and targeted metabolomics. Our data indicate that mild, short-term CR induces structural and metabolic adaptations in iBAT aimed at the deposition of triglycerides within the adipocyte, which appear paucilocular. CR increases the levels of enzymes involved in glycolysis while simultaneously reducing glycolytic intermediate metabolites. It also improved the levels of enzymes involved in de novo lipogenesis, in providing reducing power for lipid synthesis, and in lipid droplet dynamics. Although CR reduced mitochondrial iBAT content, tissue thermogenic potential was maintained, as it increased mitochondrial activity, compensating for their lower abundance.

Overall, our data indicate that mild CR, within 2 weeks, affects BAT metabolism inducing physiological adaptation that preserve its thermogenic capacity under conditions of energy deficit. These adaptations may represent a key mechanism underlying the systemic benefits of CR, offering new insights into the role of even mild and short-term CR in maintaining overall health.

INTRODUCTION

Caloric restriction (CR) without malnutrition has become a promising intervention for improving metabolic health and extending lifespan. It is considered the most effective non-pharmacological approach to tackling metabolic diseases, such as obesity and type-2 diabetes, as well as reducing age-related disorders such as neurodegeneration and cancer [1–5]. Preclinical studies indicate that CR may produce its beneficial effects by potentially preventing or delaying key hallmarks of ageing [4,5], and human research has shown its effectiveness in reducing biomarkers of cellular senescence in healthy young to middle-aged individuals without obesity [6].

One of the key features of CR is the significant reduction in adiposity; this effect may be important in the mechanisms of CR, given the endocrine role of white adipose tissue (WAT). In fact, extensive research on the impact of CR on WAT has shown that this intervention counteracts age-related adiposity and metabolic issues by promoting weight loss and restoring tissue function, including lowering metabolic, hormonal, and inflammatory risk factors [7,8].

In contrast to WAT, which mainly stores energy as triglycerides, brown adipose tissue (BAT) depots display a distinct profile characterized by a high number of mitochondria and the expression of uncoupling protein 1 (UCP1) [9]. The presence of UCP1 enables BAT to perform non-shivering thermogenesis, primarily through the uncoupling of oxidative phosphorylation from ATP production, resulting in energy being dissipated as heat and providing a mechanism for maximising mitochondrial substrate oxidation [10]. Brown adipocytes have unique features, including UCP1-mediated proton leak processes, alongside the simultaneous occurrence of de novo fatty acid synthesis, fatty acid oxidation, and glycolysis [11,12]. Indeed, upon activation, brown adipocytes increase lipolysis of stored triglycerides (TG) to support thermogenesis. Additionally, the cells utilize extracellular fatty acids originating from WAT lipolysis [13] or the metabolism of TG-rich lipoproteins [14]. Within thermogenic adipocytes, both extracellular and intracellular fatty acids can be fully metabolised through mitochondrial β -oxidation. At the same time, activation of glycolysis and de novo fatty acid synthesis can replenish part of the stored TG pool [11].

BAT exhibits the highest branched-chain amino acids (BCAA) catabolism [15] and branched-chain fatty acids synthesis fluxes [16]. Following cold exposure, BAT promotes systemic BCAA clearance in mice and humans [11]. In this context, a BAT-specific defect

in BCAA catabolism has been shown to attenuate systemic BCAA clearance, oxidation of BAT substrates, and thermogenesis, leading to diet-induced obesity and glucose intolerance [17]. BCAA degradation is essential for the formation of branched-chain alpha-ketoacids, whose metabolism could lead to the formation of succinyl-CoA from Propionyl-CoA, and of acetyl-CoA, thus feeding the Krebs cycle. Alternatively, BCAA can be addressed to the synthesis of branched-chain fatty acids [16].

These unique characteristics establish BAT as a key player in regulating energy balance and metabolic homeostasis, and have prompted investigations into strategies to modulate its activity for therapeutic purposes [18] and for maintaining metabolic health, given evidence that its enhanced activity and functionality protect against obesity and diabetes [19,20], as well as cardiovascular disorders [21–23]. Furthermore, a reduced amount of BAT is associated with obesity and glucose intolerance in humans [24]. Conversely, individuals with higher BAT activation tend to maintain lower body weight and experience healthier ageing [25]. Therefore, BAT has the potential to support healthy longevity; however, to evaluate its real potential in humans, additional methods are needed beyond those currently used, which exploit the absorption of ¹⁸F to determine its presence and abundance [26–29].

CR, with its widespread effects on energy metabolism, emerges as a particularly interesting modulator of BAT function. However, the influence of CR on BAT physiology has been only partly understood, and research outcome may vary depending on the intensity and duration of the applied CR, the species of rodent considered, the animal's initial condition, particularly whether it is young or old, and its initial physical state, such as whether it is overweight or lean. In most studies CR administration varied between 30% and 50%, and was applied over extended periods of time. In this context, some studies on rats as animal model suggest that a long term CR shifts BAT function from an energy-consuming system to an energy-conserving one, promoting lipogenesis and reducing levels of proteins markers of mitochondrial activity and UCP1 expression [30]. Other studies indicates that CR increased mitochondrial respiration [31], and when applied for 12 months prevents the decline in mitochondrial function associated with aging, due to a lesser reduction in mitochondrial biogenesis [32].

In addition, what observed in rats not ever is reproduced in mice. Indeed, studies employing mice as animal model report that CR reduced BAT lipid accumulation within the tissue [33], prevent the aging induced-enlargement of lipid droplet and the “whitening”

of BAT [34,35], and reduces BAT thermogenesis lowering UCP1 levels and impairing β -oxidation [35] as well as mitochondrial respiration [36].

Interestingly a recent study indicates that a metabolic shift occurs in mice BAT with varying levels of CR (10% -40% for 3 months), with changes in BAT metabolites even when caloric intake decreases by only 10% [37].

Although CR is vital for maintaining metabolic health and longevity, long-term CR can be challenging to sustain and is often discontinued. In contrast, better adherence is expected with mild CR practised for a short period. Studying the effects of a mild CR in the short term allows us to understand how small changes can lead to metabolic adaptation without excessive risks, improving the quality of life and the prevention of diseases. Currently, our understanding of the complex relationship between mild short-term CR and BAT is limited, and further research is necessary to explore the intricate interactions involved and to gain insights into tissue adaptations.

Thus, our primary aim was to investigate the metabolic adaptation caused by a mild CR (15%) applied for a short period of time (2 weeks) on interscapular BAT (iBAT) physiology through multiple complementary methods, such as histology, mitochondrial respirometry (given the vital role of these organelles in tissue thermogenesis), along with a comprehensive quantitative proteomics approach using liquid chromatography with tandem mass spectrometry (LC-MS/MS), as well as a targeted metabolomics method.

The results of the present work may be useful to further clarify the mechanisms through which BAT can contribute to the benefits of CR and in particular will allow us to evaluate whether these adaptive mechanisms are also activated by low-level and short-term caloric restriction.

METHODS

Animals

In the present study, we used male Wistar rats obtained from Envigo RMS Srl, Udine, Italy, and housed in a temperature-controlled room at $22 \pm 1^\circ\text{C}$ under a 12:12-h light–dark cycle. At approximately 10 weeks of age, the rats were housed individually and randomly assigned to two different groups, each consisting of 12 rats, with a similar mean body weight (approximately 280 g) and with the body weights normally distributed within each group. The control (C) group included rats fed ad libitum with a standard diet

(4RF21, Mucedola, Italy). The amount of food consumed in the control group was determined every day. The caloric restricted group (CR) comprised rats that received an amount of chow equivalent to 85% of that consumed by the control group. The food was provided at 10:00 am daily, with the exception of the day before the euthanasia, during which half of chow was provided at 10 a.m. and half was provided at 19.00 p.m. just before the dark light. All rats had free access to water.

This study was conducted in accordance with the recommendations of the EU Directive 2010/63 on the Care and Use of Laboratory Animals. Every effort was made to minimise the suffering and pain of the rats. All animal protocols used throughout this study were approved by the Committee on the Ethics of Animal Experiments at the University of Naples Federico II and by the Italian Minister of Health (Authorization n° 776/2021-PR).

At the end of the treatments of 2 weeks, rats were anesthetized by i.p. administration of a single dose of sodium Tiopental (40 mg/Kg b.w.) and euthanized by decapitation. Tissues were excised and weighed. iBAT was immediately processed for homogenate preparation, mitochondrial isolation and histological analyses or frozen at -80 °C for later analysis.

Histological analyses

Samples of iBAT were fixed Bouin solution (overnight at 4°C). The samples were dehydrated in ethanol, cleared, and embedded in paraffin blocks. The tissues were cut into serial 5-µm-thick sections; hematoxylin–eosin staining allowed morphological examination. To detect immunolocalization of such specific antigens, immunohistochemical staining was performed by using Leica System kit (Novolink Polymer Detection System). The procedure was performed according to the manufacturer. The primary antibody and dilutions used for analyses were VDAC (1:300 in PBS1X, ab GTX114187 GeneTex); HSP60 (1:300 in PBS1X, GTX110089 geneTex) and UCP1 (1:500 in PBS1X, AB1426; Merck Life Science).

The sections were examined using a Nikon Eclipse 80i light microscope (Nikon Instruments, Milan, Italy). Images were captured with a Sony DS-5M camera linked to an ACT-2U image analyser. Collagen molecules have a parallel structure, giving them natural birefringence. The use of picosirius red, a specific histological dye for collagen, enhances the visibility of collagen fibres. When viewed under polarized light, tissues

stained with picosirius red show colour variations that directly relate to the degree of collagen polymerisation and their three-dimensional organization.

In-solution digestion for Proteomics Analysis

Tissue lysates were prepared in RIPA buffer by pooling samples from five rats per experimental condition: C and CR. Protein concentrations were determined using the Bradford assay [38]. A protein aliquot (100 µg) from each lysate was processed for proteomic analysis. Proteins were precipitated by the addition of four volumes of ice-cold acetone, followed by centrifugation at 10,000 rpm for 10 minutes. The resulting pellets were air-dried and resuspended in a denaturing buffer consisting of 6 M urea and 25 mM ammonium bicarbonate. Cysteine residues were reduced with 20 mM dithiothreitol (DTT) at 60 °C for 1 hour and subsequently alkylated with 40 mM iodoacetamide in the dark at room temperature for 1 hour. Excess iodoacetamide was quenched with 20 mM DTT for an additional hour. Proteolytic digestion was carried out overnight (16–18 hours) at 37 °C using sequencing-grade trypsin at an enzyme-to-substrate ratio of 1:50. The digestion was stopped by acidification with 1% formic acid before peptide desalting using C18 StageTips (three layers of 3M Empore C18 discs). Tips were equilibrated and washed with 100 µL of 0.1% formic acid, and peptides were eluted sequentially with 50 µL of 50% acetonitrile (ACN) and 80% ACN, both containing 0.2% formic acid. The eluates were dried in a vacuum concentrator (SpeedVac) and reconstituted in 2% ACN with 0.2% formic acid for subsequent LC–MS/MS analysis.

LC–MS/MS Analysis

Peptides were analyzed on an LTQ Orbitrap XL (Thermo Fisher Scientific) coupled to a Proxeon nanoEasy-II nanoLC system. Separation was performed on a C18 reverse-phase capillary column (75 µm ID × 200 mm length, 5 µm particles) with a flow rate of 250 nL/min. A linear gradient from 5% to 60% of buffer B (0.2% formic acid, 95% ACN) in buffer A (0.2% formic acid and 2% acetonitrile in MilliQ water) was applied over 200 min. The mass spectrometer operated in Data-Dependent Acquisition (DDA) mode, scanning from 300–1800 m/z. The five most intense precursor ions per scan were selected for fragmentation via collision-induced dissociation (CID), applying a dynamic exclusion window of 40 seconds.

Bioinformatics Processing

Raw mass spectrometry data were analyzed using the Andromeda search engine embedded in the MaxQuant software suite (version 1.6.8.0) [39]. Peptide and protein identification was performed against the *Rattus norvegicus* reference proteome, retrieved from the UniProt database. Search parameters included trypsin specificity allowing up to three missed cleavages, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation, N-terminal pyro-glutamate formation (from glutamine), and β -hydroxybutyrylation of lysine as variable modifications. The minimum peptide length was set to six amino acids, and at least one unique or razor peptide was required for protein identification. Mass tolerances were set to 10 ppm for precursor ions and ± 0.02 Da for the fragment ions. A false discovery rate (FDR) threshold of 1% was applied at both the peptide and protein levels.

Quantitative data analysis was performed using Perseus software (version 1.6.8.0) [40]. Technical replicates were merged, and entries corresponding to potential contaminants, reverse identifications, or proteins detected solely by site were excluded from further analysis. Label-free quantification (LFQ) intensity values were $\log_2$ -transformed, and proteins with at least two valid measurements across experimental conditions were retained. Missing values were imputed using a normal distribution-based approach (width = 0.3; downshift = 1.8). The resulting dataset included 488 proteins, annotated with UniProt accession numbers, gene and protein names, identification scores, and sequence coverage. Differential protein expression was determined based on LFQ intensity ratios between CR and control conditions, with thresholds set at fold change > 1.3 for upregulation and < 0.77 for downregulation.

The STRING software was used to integrate all known and predicted associations between regulated proteins, including both physical interactions and functional associations [41]. A full STRING network (the edges indicate both functional and physical protein associations), a medium confidence of 0.4, and k-means clustering (the network is clustered to a specified number of clusters) were set.

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichments were obtained by String and by the KEGG database [42] by converting Uniprot id into KEGG annotation (genome annotation database).

Metabolomics Analysis

Sample preparation

Tissue lysates were subjected to protein precipitation and metabolite extraction by the addition of four volumes of an organic solvent mixture (1:1, v/v, acetonitrile:methanol). The resulting supernatants were subsequently diluted 1:10 with an aqueous solution containing 0.1% formic acid and 5 mM ammonium formate. Metabolite extracts were then analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using Multiple Reaction Monitoring ion mode for targeted analyses.

LC-MRM/MS analysis

The analyses were performed using Sciex 5500 QTrap mass spectrometry system equipped with exionLCTM. Chromatographic separation was performed by a Kinetex C18 column (5 µm particle size, 100 Å pore size, 100 × 2.1 mm; Phenomenex) at 35 °C. Separation was carried out over an 8-minute run at a flow rate of 0.2 mL/min, employing a binary gradient from 2% B to 98% B. Mobile phase A was composed of ultrapure water supplemented with 0.1% (v/v) formic acid and 5 mM ammonium formate, whereas mobile phase B consisted of acetonitrile containing 0.1% (v/v) formic acid. Electrospray Ionization (ESI) was carried out in positive ion mode for amino acid detection and in negative ion mode for organic acids. The ESI source was operated under the following conditions: curtain gas (CUR) at 20 psi, collision gas (CAD) set to medium, ion spray voltage (IS) at 5.5 kV, and a source temperature (TEM) of 500 °C.

Instrumental parameters for the MRM method, including precursor and product ion m/z values, collision energies, and dwell times, were configured according to previously published protocols [43,44]. Data acquisition files (.wiff) were processed using Skyline software [45]. It was employed to verify the co-elution of MRM transitions corresponding to each target analyte and to extract chromatographic peak areas for quantitative assessment across biological samples.

Determination of mitochondrial respiration rate in whole BAT homogenate and isolated mitochondria.

Immediately after excising the tissue, iBAT was free of any visible WAT contamination. It was immersed in an ice-cold isolation buffer (220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA, and 0.5% fatty acid-free bovine

serum albumin BSA, pH 7.4). The tissue was homogenised in 10 volumes of isolation buffer using a Potter-Elvehjem homogeniser and centrifuged at $8000 \times g$ for 10 minutes at 4°C . The floating fat was removed, and the pellet was re-suspended in the supernatant. The fat-free homogenate was stored at 4°C until required for respiratory measurements.

To isolate mitochondria, BAT was homogenised in 10 volumes of isolation buffer, and the entire homogenate was centrifuged at $10.000 \times g$. The supernatant was discarded, and the resulting pellet was re-suspended in the original volume and centrifuged at $700 \times g$. The resulting supernatant was then centrifuged at $8.000 \times g$ to pellet the mitochondria. Mitochondria were washed twice and re-suspended in a minimal volume of isolation medium lacking BSA, kept on ice until use.

The mitochondrial respiration rate was evaluated polarographically using a Clark-type electrode (Hansatech Instruments Ltd, United Kingdom). Fat-deprived homogenate or isolated mitochondria were incubated in a respiration medium composed of 80 mM KCl, 50 mM HEPES (pH 7.0), 1 mM EGTA, 5 mM K_2HPO_4 , and 0.5% fatty acid-free BSA (w/v). and maintained at 37°C . Respiration was started by adding one of the following substrates: Succinate + rotenone (10 mM, $3,7 \mu\text{M}$), Pyruvate + malate (5 mM, 2 mM). Successively, guanosine diphosphate (GDP) (2 mM) was added to the respiration medium.

To evaluate the ability of mitochondria to use Glycerol-3-phosphate (G3P) as substrate for respiration, mitochondria were incubated in the above respiration medium, and respiration was started by adding G3P (5 mM) to the respiration medium.

All reagents used for mitochondrial isolation and respiration rate detection were from Merck Life Science S.r.l.

Western blot

BAT was homogenized in 10 vol of RIPA buffer supplemented with antiprotease cocktails (Merck Life Science). Homogenates were left on ice for 1 h, and then centrifuged at $17,000 g$ for 30 min at 4°C . The resulting supernatants were collected, and the protein concentration was determined using Bio-Rad's DC assay (Bio-Rad Laboratories, Hercules, CA). Isolated mitochondria, prepared as described above, were diluted in RIPA buffer supplemented with antiprotease cocktails and treated as described for the homogenate.

Primary antibodies used throughout the study were the following: anti-uncoupling protein 1 (UCP1, AB1426; Merck Life Science), a cocktail of antibody used to detect CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP VA subunits of oxidative phosphorylation complexes (Total OXPHOS Rodent WB Antibody Cocktail, ab110413, Abcam, Cambridge UK). Anti-voltage dependent anion channel (VDAC, ab GTX114187 GeneTex Inc. USA), anti-heat shock protein 60 (HSP-60, GTX110089 GeneTex Inc.), anti-vinculin (PA5-80216 In vitrogen, Thermofisher Scientific, Italy),

Anti pyruvate dehydrogenase (PDH 3205 Cell Signalling Technology, Danvers Massachusetts, USA, anti hexokinase II (Hexokinase II 2024 Cell Signalling Technology), anti platelet type phosphofructokinase (PFKP 8164 Cell Signalling Technology), anti glyceraldehyde 3 phosphate dehydrogenase (GAPDH 5174 Cell Signalling Technology), anti phosphoglycerate mutase 1 (PGAM1 12098 Cell Signalling Technology), anti pyruvate dehydrogenase Kinase (PDHK1 3820 Cell Signalling Technology), anti Enolase (ENO 3810, Cell Signalling Technology), anti mitochondrial pyruvate carrier 2 (MPC2 46141 Cell Signalling Technology), anti-Superoxide dismutase 1 (SOD1 4266 Cell Signalling Technology), anti Superoxide dismutase-2 (SOD2 ab13533 Abcam, Cambridge), anti hydroxynonenal adduct (HNE adduct ab46545 Abcam,); Cluster of Differentiation 68 (CD68, sc-59103 SantaCruz Biotechnology. Inc. USA); Galectin 3 (GAL3, sc-20157 SantaCruz Biotechnology. Inc.).

The primary antibodies were diluted 1:1000, with the exception of total OXPHOS, CD68 and galectin that were diluted 1:500

Lipid hydroperoxide levels and susceptibility of BAT to oxidative insult

BAT lipid hydroperoxides (Hps) levels were measured in tissue homogenates (0,01 mg proteins) by assessing the decrease in NADPH absorption at 340 nm, which results from the oxidation of NADPH by the glutathione peroxidase – glutathione reductase system in the presence of GSH [46], using a multi-plate reader (Synergy™ HTX Multimode Microplate Reader, BioTek). The amount of Hps was expressed as NADPH oxidised nmol min⁻¹ mg protein⁻¹.

The susceptibility of BAT homogenates to an in vitro oxidative stress insult was assessed as reported by Fasciolo et al. (2023) [47]. The changes in Hps levels induced by treatment of the BAT homogenate (0,01 mg protein) with Fe and ascorbate (Fe/As 100/1000 µM) for 10 min at room temperature were assessed. The reaction was stopped

by adding 0.2% 2,6-di-*t*-butyl-*p*-cresol. The hydroperoxide levels were evaluated as described above. Reagents used were from Merck Life Science.

Cytosolic and mitochondrial glycerol-3-phosphate dehydrogenase activities

To evaluate the activity of cytoplasmic glycerol 3-phosphate dehydrogenase (GPD1), BAT homogenate (0,01 mg of protein) was incubated with 4 mM NAD⁺ and 5 mM G3P, and the production of NADH was monitored at 340 nm using a multiplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek) [48]. One unit of enzyme activity is defined as 1 μmol NAD⁺ reduced per minute per mg of protein.

The Activity of mitochondrial glycerol-3-phosphate dehydrogenase (GPD2) was assayed as described in Pecinová et al (2020) [49] in 0,01 mg of proteins as glycerol-3-phosphate: 2,6-dichlorophenolindophenol oxidoreductase. The assay medium contained 50 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, 1 mg/mL BSA and 1 mM KCN, pH 7.4. The reaction was started by adding 25 mM G3P and 100 μM DCPIP and the change of the absorbance was monitored for 60 s at 30 °C at 610 nm ($\epsilon_{610} = 20.1 \text{ /mM/cm}$). The Enzyme activity was expressed as nmol min⁻¹ x mg protein⁻¹ and measured with a multiplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek). Reagents used were from Merck life Science S.r.l.

Statistical analysis

Data were analysed with the GraphPad Prism 6 software system. An unpaired *t*-test determined the statistical significance of differences between the two experimental groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

CR reduces body weight gain and affects iBAT morphology

At the start of treatment, the rats had similar body weights ($279 \pm 8 \text{ g}$ and $286 \pm 7 \text{ g}$ in C and CR, respectively $n=12$) and after two weeks of treatment the CR groups showed a reduction in body weight gain (50 ± 5 and 33 ± 3 grams in C and CR, respectively $n=12$). CR did not affect BAT wet weight and its contribution to the total body weight (Fig 1A).

iBAT morphology showed changes in lipid droplet size and organisation in cells. In fact, haematoxylin and eosin stain revealed that the CR animal group had an increase in lipid droplet diameter compared to C group, indicating fat accumulation in the tissue. In CR group, many cells underwent morphological changes, appearing paucilocular compared to the multilocular adipocytes in the C group (Fig 1B).

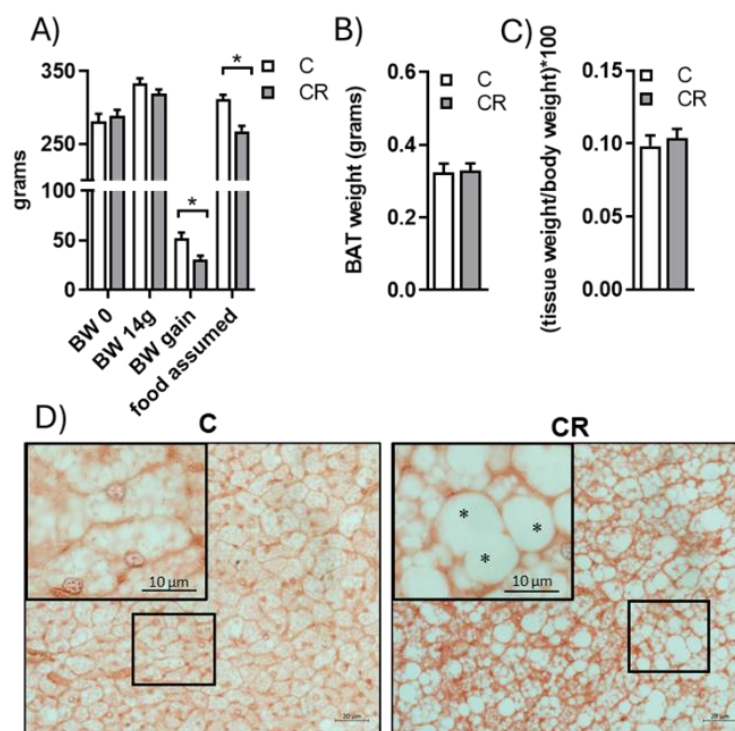


Figure 1 Effect of CR on rats body weight gain, energy assumption, iBAT weight (A-C) and morphology (D). Histological Images are representative of BAT sections. Hematoxylin and eosin staining, showed different cellular shapes between the two groups within BAT parenchyma, and a different size of lipid droplets inside the multilocular adipocytes. CR group showed a lot of paucilocular adipose cells (*). Inset represent high magnification of the frame areas. Magnifications used: 40X or 100X (insert); scale bars applied: 20 μm and 10 μm (insert). In the panel A-C, histograms represent the mean ± SEM of 12 different rats *P<0.05 vs C.

CR affects the whole iBAT and mitochondrial proteomic pattern

We performed a proteomic analysis of iBAT to examine the molecular changes caused by treatments. iBAT proteins underwent traditional in-solution digestion for a large-scale shotgun bottom-up approach using Liquid Chromatography-Tandem Mass Spectrometry LC-MS/MS analysis. In the entire iBAT lysate, we identified 486 proteins, with 30% being dysregulated by at least 1.3-fold following CR treatment; of these, half were upregulated and half downregulated (Fig. 2A).

The proteins upregulated more than 2-fold by CR include Aldo-keto reductase family 1, member C15 (*Akr1c15*); Keratin, type I cytoskeletal 10 (*Krt10*); member RAS oncogene family Rab1b (*RAB1B*); SH3 domain-binding glutamic acid-rich-like protein (*Sh3bgrl1*);

Malic enzyme (*Me1*); Decorin (*Dcn*); Long-chain-fatty-acid--CoA ligase (*Acs15*); Caveolae-associated protein 2 (*Cavin2*); Keratin, type II cytoskeletal 1 (*Krt1*); S-formylglutathione hydrolase (*Esd*); ATP-citrate synthase (*Acly*); Alpha-1,4 glucan phosphorylase (*Pygm*); IF rod domain-containing protein (AABR07001416.1); Acetyl-coenzyme A synthetase, cytoplasmic (*Acss2*); Isocitrate dehydrogenase [NADP] cytoplasmic (*Idh1*); UTP--glucose-1-phosphate uridylyltransferase (*Ugp2*); Calcium-transporting ATPase (*Atp2a1*); Glucose-6-phosphate 1-dehydrogenase (*G6pdx*); Cytochrome c oxidase subunit 6C-2 (*Cox6c2*) (fig 2A). The proteins that were downregulated include: Omega-amidase NIT2 (*Nit2*); NADH dehydrogenase [ubiquinone] 1 alpha subcomplex Ndufa5; Fatty acid-binding protein (*Fabp3*); Mitochondrial Medium-chain acyl-CoA ligase (*Acsf2*); Leucine-rich repeat-containing protein 59 (*Lrrc59*); 6-phosphogluconolactonase (*Pgls*); Mitochondrial import inner membrane translocase subunit (*Timm50*); Acyl-CoA thioesterase 11 (*Acot11*); RAB5C, member RAS oncogene family (*Rab5c*); Calreticulin (*Calr*); Sodium/potassium-transporting ATPase subunit beta-3 (*Atp1b3*); Methylmalonyl CoA epimerase (*Mcee*); Monocarboxylate transporter 1 (*Slc16a1*); Moesin (*Msn*).

To gain further insights into the processes and pathways regulated by CR, KEGG pathway analysis was performed, focusing on proteins dysregulated by at least 1.3-fold due to CR (Fig 2B).

When considering the up-regulated proteins, the primarily enriched pathways are Metabolic pathways, Carbon metabolism, Glycolysis / Gluconeogenesis, Biosynthesis of amino acids, Galactose metabolism, Glycogen metabolism, Pentose phosphate pathway, and Cardiac muscle contraction (Fig. 2 B green bars).

The pathways that are principally enriched in proteins down-regulated by CR are Oxidative phosphorylation, Metabolic pathways, Thermogenesis, Prion disease, Non-alcoholic fatty liver disease, Huntington disease, Parkinson's disease, Valine, leucine, and isoleucine degradation (Fig. 2B, blue bars).

Proteins involved in Glycolysis / Gluconeogenesis, Glycogen metabolism, Oxidative phosphorylation and Thermogenesis dysregulated by CR are also reported in Fig 2C.

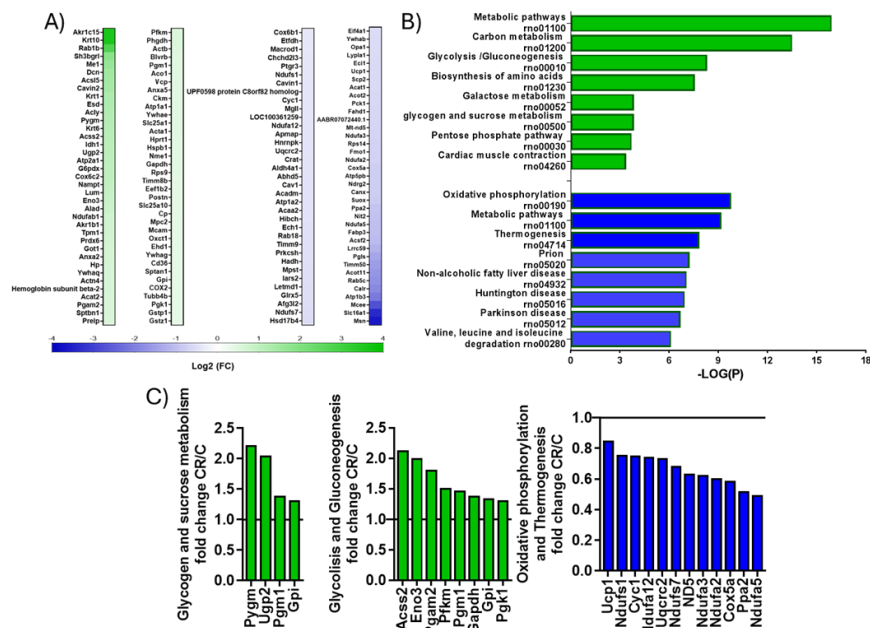


Figure 2 Effect of CR on the BAT proteome. A) heatmap depicting proteins dysregulated by CR of at least 1.3- fold. Data are reported as Log2 (fold change, FC). The names of the genes of the dysregulated protein are reported. B) KEGG pathway analysis of the top KEGG enriched gene pathway, in green are reported the upregulated pathways, in blue the downregulated ones. C) relative abundance of proteins involved in pathways up and down-regulated by CR. The continue line represents the values of the control set to 1. Each value is the result of two technical replicates obtained by a pool of 5 rats.

CR influences levels of enzymes and metabolites involved in glucose metabolism pathways.

As described above, according to KEGG enrichment analyses, some proteins up-regulated by CR belong to the network of glycogen metabolism and the glycolytic pathway (Fig 2B, 2C).

Concerning proteins involved in glycogen metabolism and affected by CR, they participated in both glycogenolysis and glycogenesis. Indeed, both Uridine diphosphate-glucose pyrophosphorylase (UPG2), a key enzyme in glycogenesis, and glycogen phosphorylase (PYGM), a key enzyme involved in glycogenolysis, were enhanced by about 2-fold. In addition, CR also increased the levels of Phosphoglucomutase 1 (PGM1), which catalyses the reversible isomerization of alpha-D-glucose 1-phosphate to alpha-D-glucose 6-P (Fig 2A,2C). Histochemical staining of iBAT sections with periodic acid Schiff (PAS), which visualizes molecules with a high percentage of carbohydrate content, such as glycogen, indicates a reduction in staining intensity of iBAT sections from CR treated rats (Fig. 3). These data indicate a lower glycogen accumulation within the tissue and suggest the predominance of glycogenolysis over glycogenesis in BAT of CR treated rats.

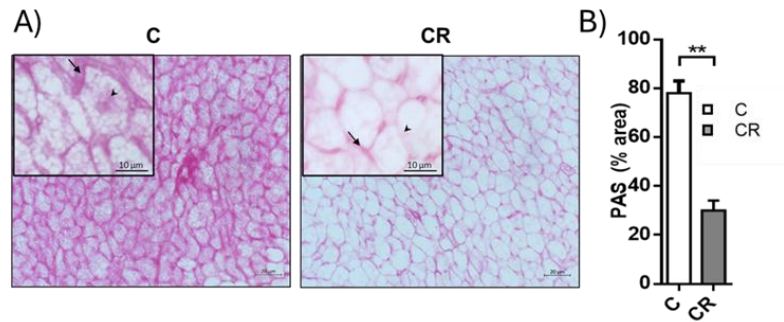


Figure 3 Effect of CR on glycogen levels. (A) Representative iBAT sections stained with periodic acid Schiff (PAS); insert represents high magnification of the frame area. Arrows indicate extracellular stained regions, whereas arrowheads indicate PAS-positive intracellular areas. Magnifications used: 40X or 100X (insert); scale bars applied: 20 μ m and 10 μ m (insert). (B) Quantification of PAS staining. Histograms represent the mean $\pm$ SEM of 4 different rats.

As shown in Fig. 2B, KEGG enrichment analysis indicated that CR elevates the protein levels of enzymes involved in the glycolytic pathway. This was verified by Western blot analysis, which revealed increased levels of hexokinase, phosphofructokinase (PFKP), enolase, and glyceraldehyde dehydrogenase (GAPDH) (Fig. 4A, B).

In addition, the levels of mitochondrial pyruvate carrier isoform 2 (MPC2) are significantly increased, confirmed by Western blot (Fig. 2 and Fig. 4A, B). CR also enhanced pyruvate dehydrogenase (PDH) levels, while not significantly affected pyruvate dehydrogenase kinase (PDK1) (Fig. 4A, B).

To further examine the impact of CR on the glycolytic pathway, through target metabolomics we measured the levels of certain intermediate metabolites (Fig. 4C). CR did not influence glucose levels in iBAT, but it decreased the levels of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and dihydroxyacetone phosphate. Pyruvate levels were below the detection limits of the equipment.

Interestingly, since CR opposite regulates two proteins involved in the first and second steps of the pentose phosphate pathway (PPP), such as glucose-6-phosphate dehydrogenase (G6PD), which was upregulated, and 6-phosphogluconolactonase (PGLS), which was downregulated, (Fig. 2A), we measured the levels of D-ribose 6-phosphate, a product of the PPP pathway. D-ribose 6-phosphate tended to be reduced in iBAT from CR rats; however, the differences did not reach statistical significance (Fig. 4C).

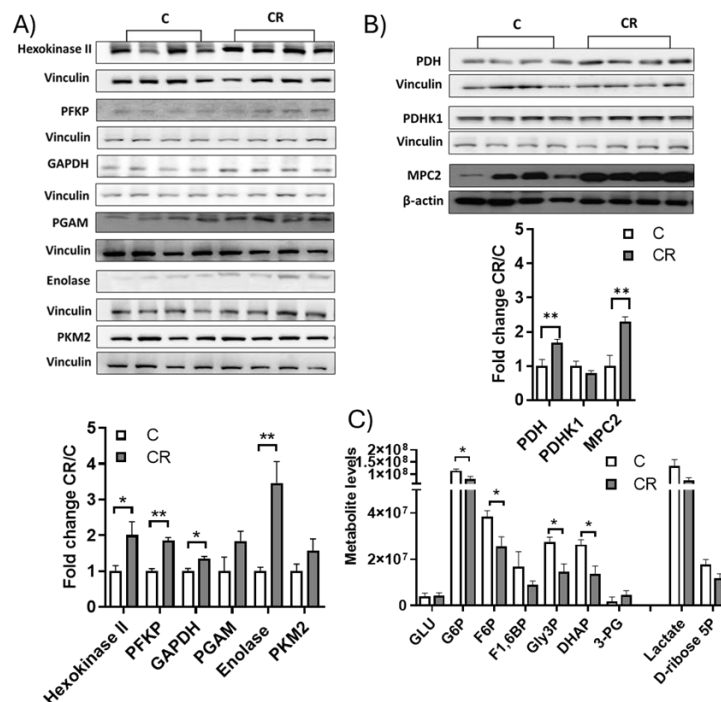


Figure 4 Effect of CR on Glycolysis pathway. Western Blots of proteins involved in glycolysis/gluconeogenesis pathways and pyruvate metabolism. Vinculin or β actin was used as the loading control. (A) Histograms represent quantification of the western blot data, which were normalized to the value obtained for C animals, set as 1 (B). Levels of glycolysis intermediates obtained with targeted metabolomics (C). Values are represented as mean $\pm$ SEM of at least 4 different rats * $P < 0.05$ vs C ** $P < 0.01$ vs C. Abbreviation of the panel C are: GLU: Glucose; G6P: Glucose-6-phosphate, F6P: Fructose-6-phosphate, F1,6BP: Fructose-1,6-bisphosphate, Gly-3P: Glyceraldehyde-3-phosphate DHAP: Dihydroxyacetone phosphate,, 3PG: 3-Phosphoglycerate

CR enhances mitochondrial respiration when detected in isolated mitochondria but not in total tissue lysate.

Given the evidence that iBAT mitochondria are the principal site of thermogenesis, we investigated the ability of CR to affect their functionality by integrating proteomics, western blot, immunohistochemistry and respirometry data.

KEGG analysis of dysregulated proteins detected by the proteomic approach indicated that oxidative phosphorylation is one of the mainly enriched pathways in proteins down-regulated by CR (FIG 2 C). Consistent with the proteomic data, Western blot analysis of specific subunits belonging to the five mitochondrial respiratory complexes—namely NDUFB8 (complex I), SDHB (complex II), UQCRC2 (complex III), MTCO1 (complex IV), and ATP5A (complex V)—showed that these proteins were reduced in iBAT lysate (Fig. 5 A, C).

Interestingly, when detected in the isolated mitochondria, the respiratory complexes' subunits mentioned above were all upregulated by CR (Fig. 5 B, C).

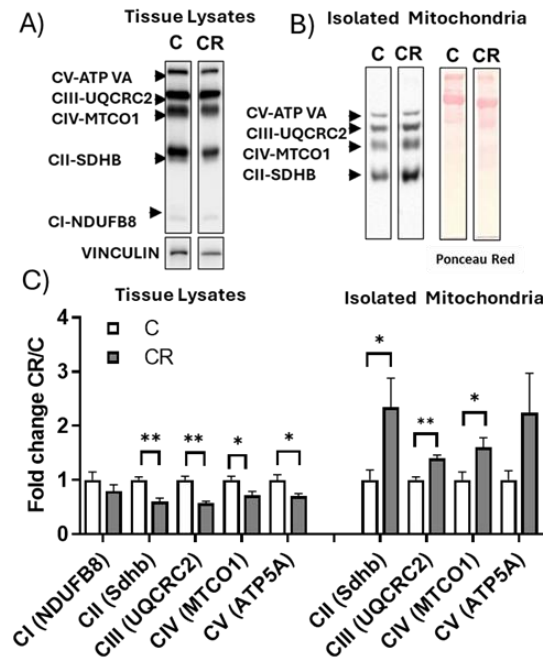


Figure 5 Effect of CR on respiratory complexes subunits. Representative Western blots of respiratory complexes subunits (CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATPVA), detected in the whole iBAT lysate (A) and isolated mitochondria (B). Vinculin or Ponceau staining was used as a loading control (25 µg of protein/rat/lane). Histograms represent quantification of the western blot data, which were normalized to the value obtained for C animals, set as 1 (C). Values represent the mean ± SEM of 6 different rats. * $P < 0.05$, ** $P < 0.01$ vs C

Regarding UCP1, proteomic analysis (Fig 2 A) and Western blotting (Fig 6) indicated that its levels were downregulated by CR in BAT lysate (Fig 6A), while an increase in the protein was observed when examining isolated mitochondria (Fig 6B). Furthermore, iBAT staining with UCP1 evidenced that in BAT from CR animals higher immunopositivity in some paucilocular cells where localize mitochondria (Fig 6D) (arrows), in agreement with WB analyses conducted in mitochondrial enriched fraction.

The observation that CR effectively increased the levels of SDHB, UQCRC2, MTCO1, ATP5A, and UCP1 in isolated mitochondria, while simultaneously decreasing their abundance in whole tissue lysates, strongly suggests a reduction in total mitochondrial content within BAT of CR-treated rats.

To clarify this possibility, we evaluated the levels of two protein markers of mitochondrial abundance, such as HSP60 and VDAC.

Indeed, immunohistochemical analyses of iBAT sections showed that CR rats exhibited weak VDAC or HSP60 staining compared to the Control group (Fig 6D, VDAC-HSP60, black arrows). These findings align with Western blot data, which indicate a reduction in VDAC and HSP60 levels (-48% and -50%, respectively, in CR vs. C) in total tissue lysate (Fig. 6 A and C). Interestingly, when detected in isolated mitochondria, the levels of

HSP60 and VDAC remained unaffected by CR treatment (Fig 6 B and C). These data further support the conclusion that CR reduces BAT mitochondrial content.

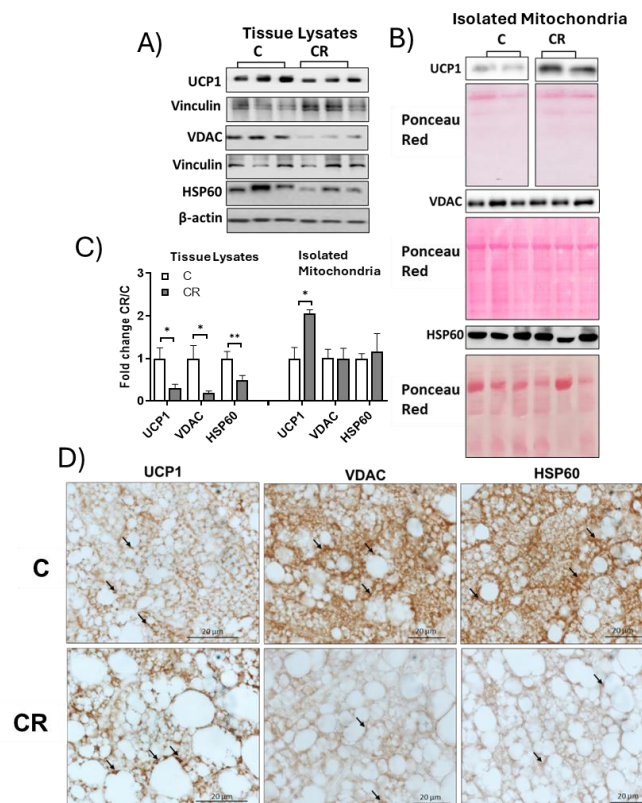


Figure 6 Effect of CR on UCP1, VDAC, and HSP60. Representative Western blots of UCP1, VDAC, and HSP60 detected in BAT total lysate (A) and isolated mitochondria (B). Vinculin or Ponceau staining was used as a loading control (25 μ g of protein/rat/lane). Histograms represent quantification of the western blot data, which were normalized to the value obtained for C animals, set as 1 (C). Values represent the mean $\pm$ SEM of 4 different rats. *P<0.05, **P<0.01 vs C. Representative iBAT sections stained with immunohistochemistry for UCP1, VDAC and HSP60 (D). Arrows indicate immunoreactive areas where localize mitochondria. Magnification used: 100X; scale bar applied: 10 μ m.

We then measured mitochondrial respiration rates in the whole fatty acid-deprived iBAT homogenate and isolated mitochondria. This choice is fundamental since measuring respiration rate in iBAT homogenates provides a powerful readout of both mitochondrial content and functionality, whereas in isolated mitochondria, it reveals only their intrinsic functional capacity (Fig.7).

Two different respiratory substrates were used, namely pyruvate plus malate and succinate plus rotenone, which involve complex I- and complex II-linked respiratory pathways, respectively. Respiration was detected both in the presence and absence of GDP, the inhibitor of UCP1.

When analysed in the entire iBAT homogenate, no significant differences in respiration rates were observed between the CR and C groups (Fig. 7A), regardless of the substrate used. Adding GDP to the respiration medium, which is intended to inhibit UCP1 activity,

reduced mitochondrial respiration rate; however, no differences in GDP-inhibited respiration rate were found between the C and CR groups (fig 7A).

When isolated mitochondria were analysed, CR tends to increase the pyruvate-supported respiration rate; however, the differences did not reach statistical significance (Fig 7B).

In succinate-energized mitochondria, we observed a significant increase in respiration rate induced by CR, both when it was detected in the presence and in the absence of GDP (Fig 7B).

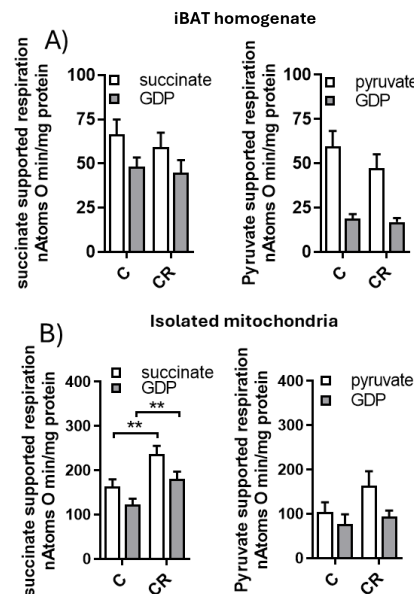


Figure 7 Effect of CR on mitochondrial respiration rate observed in the whole homogenate (A) or in isolated mitochondria (B). Respiration was measured using succinate (+rotenone) or pyruvate (+malate) as substrates, both in the absence and presence of GDP. Values represent the mean $\pm$ SEM of 6-8 different rats. ** $P < 0.01$ vs C

Effect of CR on oxidative stress.

To assess whether CR could influence oxidative stress in iBAT, we examined some markers of oxidative stress and lipid peroxidation, such as the mitochondria's ability to release H_2O_2 (as an index of mitochondrial superoxide formation) (Fig 8A), tissue lipid hydroperoxides levels (Fig 8 B), 4-hydroxynonenal (HNE) protein adducts levels (Fig 8C), the susceptibility of BAT to oxidative stress, as well as superoxide dismutase 1 and 2 protein levels (SOD1 and SOD2).

The ability of isolated mitochondria to release H_2O_2 , when energized with either succinate or glycerol -3-phosphate (G3P), was unaffected by CR. Additionally, no changes were observed in tissue' lipid hydroperoxide levels or HNE protein adducts between the C and CR samples. Similarly, the response of BAT to an oxidative insult was not altered by CR. CR did not influence SOD1 levels but down-regulated SOD2 levels.

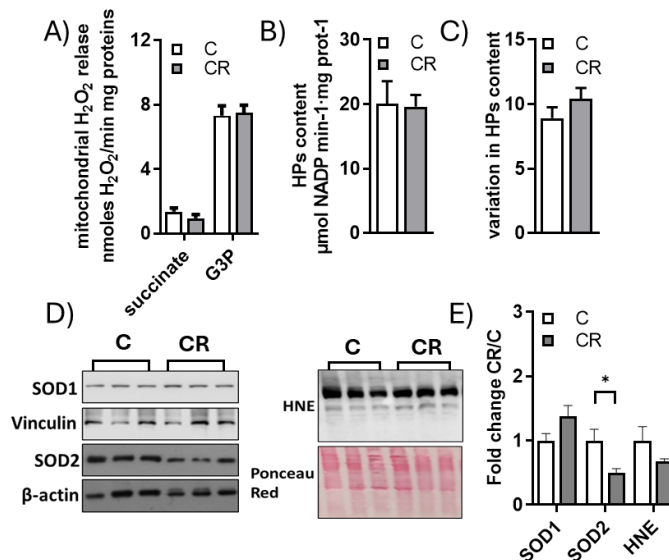


Figure 8 Effect of CR on oxidative stress. Mitochondria H_2O_2 release (A), lipid hydroperoxides tissue content (B), susceptibility of tissue to an oxidative insult (C), western blot of SOD1, SOD2, and HNE protein adduct. (D) Histograms represent quantification of the western blot data, which were normalized to the value obtained for C animals, set as 1 (E) Values are the mean $\pm$ SEM of 6 or 8 different rats * $P < 0.05$ vs C

CR affects the activity of glycerol-3-phosphate shuttle components.

The G3P shuttle is a metabolic hub, linking glycolysis, lipid synthesis, and oxidative phosphorylation [50]. In the G3P shuttle, cytosolic glycerol-3-phosphate dehydrogenase-1 (GPD1) converts DHAP into G3P by reducing NAD^+ to NADH. G3P diffuses into the mitochondrial intermembrane space and is oxidized by mitochondrial glycerol-3-phosphate dehydrogenase-2 (GPD2), which, by transferring electrons to the coenzyme Q, acts as a non-conventional component of the electron transport chain (ETC). Therefore, we assessed the activities of GPD1 and GPD2 and the ability of mitochondria to oxidize. CR significantly enhanced GPD1 activity (Fig 9 A) without affecting GPD2 activity (Fig 9 B); furthermore, CR did not affect the ability of isolated mitochondria to use G3P as a respiratory substrate (Fig 9 C).

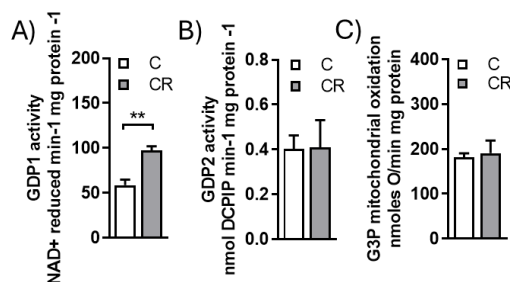


Figure 9 Effect of CR on components of glycerol phosphate shuttle. Activity of cytosolic Glycerol-3-phosphate dehydrogenase (GPD1) (A), activity of mitochondrial glycerol-3-phosphate dehydrogenase (GPD2) (B), and glycerol-3-phosphate mitochondrial oxidation (C). Values are the mean $\pm$ SEM of 4 different rats. ** $P < 0.01$ vs C.

CR affects the levels of enzymes involved in amino acid synthesis and the BCAAs degradative pathway, without affecting their tissue and serum levels.

Since KEGG enrichment pathway analysis indicated that more down-regulated proteins, identified through a proteomic approach, belong to the BCAAs degradation pathway, we explored the potential effects of CR treatment on tissue and serum BCAAs levels. Interestingly, through targeted metabolomics, we found no significant changes in the levels of BCAAs and other amino acids in both iBAT tissue and serum between the control and CR groups (Fig. 10A and B).

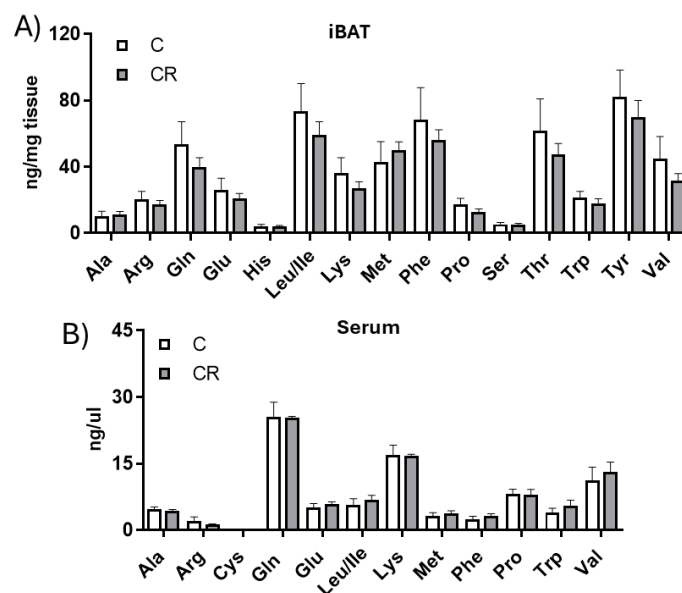


Figure 10 Amino acid levels detected in iBAT and sera amino acid levels detected in CR and C rats. Amino acid levels were detected in whole iBAT (A) and sera (B) extracts. Values represent the mean $\pm$ SEM of 4 different rats.

CR influences the collagen deposition in the perivascular area and intermediate filaments proteins.

Among CR's most upregulated proteins detected in the iBAT lysate are lumican and decorin (Fig 2A), i.e., two extracellular matrix proteins involved in forming collagen fibrils; thus, we evaluated whether CR could influence collagen deposition.

For this purpose, we used histological sections of iBAT stained with picrosirius red and observed them under optical and polarized light microscopy (Fig. 11 A and B respectively). The quantification of collagens stained red in the intercellular spaces in iBAT indicates a significant increase in peri-adipocyte collagen accumulation in CR group. In addition, when analysed by plane-polarized light, the images of the stained sections from CR rats indicate variations in collagen fibres appearing green (newly synthesized collagen III thin fibres, arrowhead), yellow/orange or red (collagen I thick fibres, white

arrow), especially around the perivascular area. iBAT section from CR rats showed an enhanced green signal and lower red signal compared to controls, thus suggesting that CR induces the formation of new thin fibers (Fig 11, A).

Since fibrillogenesis could be associated with macrophage infiltration within the tissue, we evaluated some markers of their presence, such as Galectin-3 (Gal-3) and CD38, in BAT. Western blot analysis and immunohistochemistry revealed no changes in the levels of these proteins between CR and C rats (Fig. 11C and D, respectively), thus excluding macrophage infiltration into the tissue. Indeed, immunoreactive cells were found localized in vessels and not in adipose tissue parenchyma around adipocytes (Fig. 11, D, arrows).

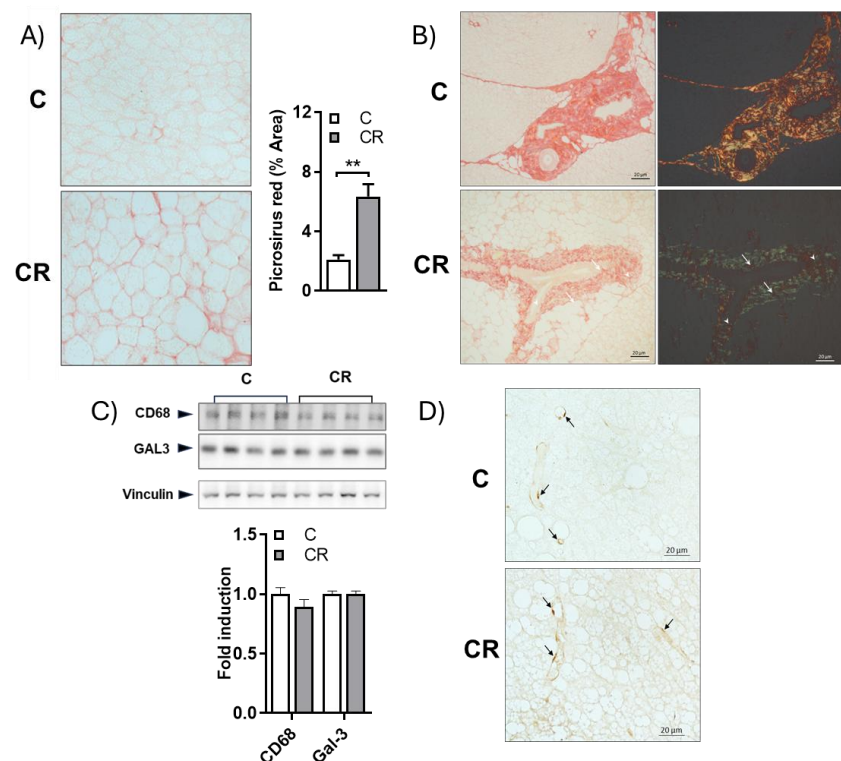


Figure 11 Effect of CR on collagen fibres and macrophage presence in the tissue. Histological section of iBAT stained with picrosirius red observed under optical light microscopy. Histograms represent the quantification of Picrosirius red stained area (A). Histological section of iBAT stained with picrosirius red observed polarized light microscopy (B). Arrows indicate the newly synthesized collagen III thin fibres, that appears green; arrowhead evidence yellow/orange or red collagen fibres (collagen I, thick fibres). Magnifications used: 10X or 20X; scale bar applied: 20 μ m. Representative Western blotting analysis of CD68 and galectin (Gal3) protein levels (C). Each lane contained 30 μ g of protein of total iBAT lysate from a single rat. Vinculin was used as the loading control. Histograms show quantification of the signals expressed relative to the value obtained from the control, and represent means $\pm$ SEM for 4 different rats. Histological section of iBAT stained with immunohistochemistry for CD68 (D). Arrows indicate macrophages localization in the tissue. Magnification used: 40X; scale bar applied: 20 μ m

DISCUSSION

The manipulation of BAT physiology holds the potential for metabolic benefits and CR has been reported as an important intervention able to improve metabolic health. Given the low long-term adherence to strict caloric restriction, the effects of moderate caloric restriction applied over shorter durations may offer metabolic benefits while being more feasible, and our study reports that a mild CR of 15% applied for only two weeks is able to induce coordinated metabolic and structural changes in the BAT, offering further insight into the metabolic processes modulated by CR.

CR effects on iBAT de novo lipogenesis and TG deposition.

In iBAT, CR promotes TG accumulation, as evidenced by increased lipid droplet size; this effect is consistent with previous studies on rat models, which applied a 30% CR over 6 month-day periods [30]. Notably, our findings extend this concept, demonstrating that even a milder 15% CR applied for just two weeks can elicit a comparable response, underscoring the significant short-term metabolic impact of mild CR.

In BAT, support for thermogenesis through glucose metabolism is limited, whereas glucose is crucial for providing fuel for de novo lipogenesis (DNL) [12]. Additionally, the carbohydrate storage as glycogen and its metabolism play a vital role in BAT physiology and lipid droplet (LD) deposition [51]. Our findings indicate that a mild and short-term CR influences glucose metabolism by affecting glycogen turnover and glucose oxidation. Proteomic analysis showed that enzymes involved in both glycogen synthesis and breakdown (UPG and PYGL, respectively) increased with CR treatment, yet tissue staining revealed reduced glycogen levels, suggesting that iBAT adapts by supplying substrates for glycolysis (see schema Fig. 12, green box). Additionally, iBAT from CR treated-rats show increased glycolytic enzymes, stable tissue glucose, and decreased glycolytic intermediates, indicating enhanced glycolytic flux, which fuels lipogenesis (Fig. 12, blue box). The reduced level of the glycolysis intermediate G6P, known to activate glycogen synthetase, could have also contributed to the CR-induced reduction in BAT glycogen levels. Our data regarding the ability of short-term mild CR to induce an increase in glycolytic protein levels are consistent with literature findings obtained in mice subjected subject to CR 30% for 8-weeks, showing an increase in the mRNA expression of the proteins involved in this process [33] and that obtained in rats.

It is known that to sustain high glycolysis rate, NADH produced by GAPDH has to be promptly oxidized to regenerate NAD⁺ [41,52]. Our data indicate CR facilitates this regeneration by enhancing the activity the cytosolic GPD1, a component of glycerol-3-phosphate shuttle, which converts glycolysis derived- DHAP into G3P. Our data also suggest that the formed G3P serves as the structural backbone for TAG synthesis [53], rather than being used as mitochondrial respiratory substrate, since CR does not affect the mitochondrial GDP2 activity and the mitochondrial ability to utilize G3P as a respiratory substrate

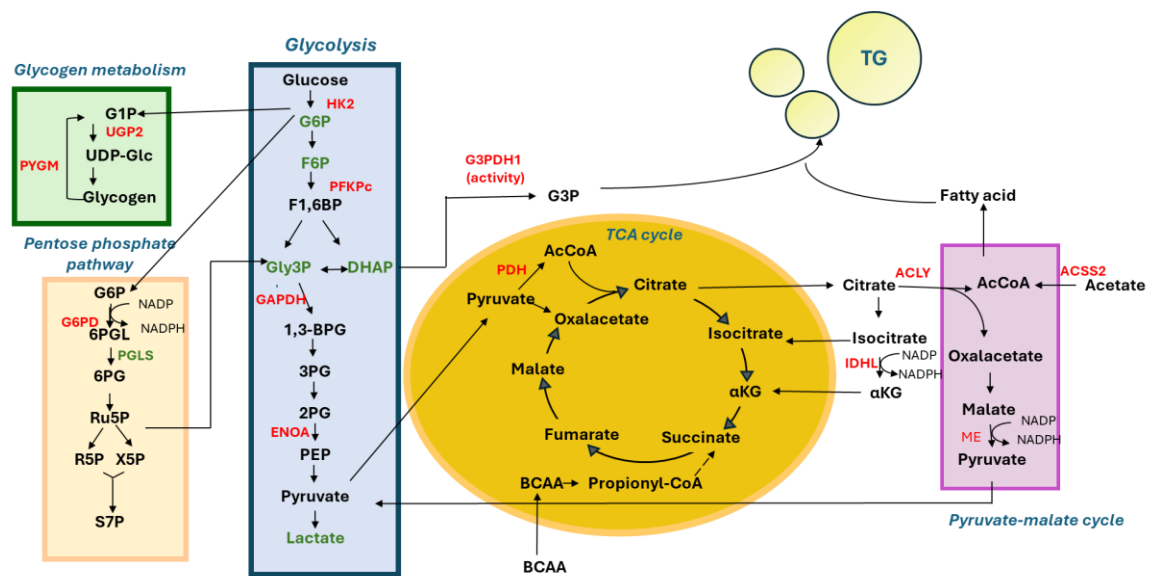


Figure 12 Scheme depicting the metabolic pathways influenced by a mild and short-term CR. The enzymes upregulated by CR are coloured in red, the metabolites and the enzymes downregulated are coloured in green. Abbreviation used: G1P: glucose-1-phosphate, UDP-Glc : Uridine Diphosphate Glucose; G6P: Glucose-6-phosphate, F6P: Fructose-6-phosphate, F1,6BP: Fructose-1,6-bisphosphate, Gly3P: Glyceraldehyde-3-phosphate DHAP: Dihydroxyacetone phosphate, 1,3-PG: 1,3-Phosphoglycerate 3PG: 3-Phosphoglycerate, 2PG: 2-Phosphoglycerate 6PGL: 6-Phosphoglucono-δ-lactone,, 6PG: 6-Phosphogluconate Ru5P: Ribulose-5-phosphate R5P: Ribose-5-phosphate, XuP: Xylulose-5-phosphate S7P: Sedoheptulose-7-phosphate, G3P: alpha glicero phosphate,, AcCoA Acetyl-CoA BCAA: branched chain amminoacid; α-KG: alpha ketoglutarate. TG: triglycerides

G6P also plays a key role in the pentose phosphate pathway (PPP), where it is converted to 6-phosphogluconolactone by G6PD, an enzyme whose expression is increased by CR, as revealed by the proteomic approach. The reaction also produces NADPH, which is essential for cellular processes such as fatty acid synthesis (see also below), antioxidant defence, and ribose-5-phosphate formation, a precursor for nucleotide synthesis. However, despite higher G6PD protein levels, the PPP does not seem to be stimulated by CR; this can be due to CR induced PGLS downregulation, which is involved in the second step of the PPP, thus suggesting the presence of a bottleneck in this second step

of the pathway, which limits the PPP flow. Supporting this, ribose-5-phosphate levels are not significantly affected by CR - treated rats (Fig.12, yellow box).

Data reported here suggest the ability of CR to induce the import of pyruvate into the mitochondria and its decarboxylation to Acetyl-CoA, as indicated by an enhancement in the mitochondrial pyruvate carrier subunit MCP2 and PDH levels. In the matrix, Acetyl-CoA reacts with oxaloacetate to produce citrate (see brown box Fig. 12). Pyruvate-derived citrate can be utilized in the tricarboxylic acid (TCA) cycle or exit the mitochondria to reach the cytosol and be converted back into acetyl-CoA, through the activity of ATP-citrate lyase (ACLY), a key player connecting glucose and fatty acid metabolism. Cytosolic Acetyl-CoA can be directed toward de novo fatty acid synthesis, since it serves as the primary carbon source for this process. Neo-formed fatty acids can undergo activation to Acyl-CoA, a crucial step that enables their incorporation into TG for storage [54].

Our data indicate an increase in ACLY levels, thus highlighting the role of this protein in the effect of CR on de novo fatty acid synthesis. CR also enhances the levels of ACSS2, which is unique in its ability to produce Acetyl-CoA from acetate for lipid biosynthesis, and complements ACLY in the production of Acetyl-CoA via the pyruvate route [55]. CR-induced enhancement in ACLY levels is in line with literature data, which applied 30% CR for 30 days [30]. De novo lipogenesis also requires the power of NADPH to polymerize acetyl-CoA into fatty acid chains, and CR is effective in enhancing the levels of cytosolic enzymes catalyzing reactions that generate NADPH, such as ME, G6PDH (already described above), and IDH. (see schema)

The activation of the glycolytic pathway, the upregulation of de novo lipogenesis, and the enlargement of LD are also consistent with the CR-induced decrease in ACOT11 protein levels (also known as THEM1). Indeed, some recent evidences show that, in BAT, beyond its role in hydrolyzing Acyl-CoA esters into free fatty acids and CoA, under cold exposure or following the integrated stress response, ACOT11 accumulates in the nucleus. There, it interacts with ChREBP and reduces the expression of target genes by limiting the induction of genes that control glucose oxidation and its conversion to lipids [56].

Regarding LD enlargement induced by CR, more proteins dysregulated by CR are involved in LD formation and degradation, which is mediated by lipolysis and/or fusion with lysosomes or autophagosomes [57]. Among the most upregulated proteins are

ACSL5, AKR1C15/17- β HSD, and RAB1B, while RAB5 is among the most downregulated proteins by CR. ACSL5 catalyzes the formation of fatty acyl-CoAs from long-chain fatty acids (C16–C20) that can be used for TG synthesis [58]. RAB1B targets diacylglycerol acyltransferase, one of the enzymes crucial for TG synthesis, from the endoplasmic reticulum to the LDs, thus promoting their growth [59]. AKR1C15, has recently been reported as an LD-associated protein involved in LD dynamics, capable of preventing lipolysis [60]. Endosomal trafficking participates in lipid droplet catabolism, and recent studies suggest that inhibiting endosome maturation through RAB5 downregulation or by expressing its dominant-negative mutant results in LD accumulation and increased TG levels [57]. Therefore, the reduction of RAB5 may also have contributed to CR-induced LD accumulation.

KEGG analysis of dysregulated proteins, individuated by proteomic approach, showed that one of the pathways mainly enriched in proteins down-regulated by CR is BCAAs degradation; in addition, one of the most down-regulated proteins by CR is MCEE, which is involved in the formation of succinyl-CoA from Propionyl-CoA. However, associated with a reduction in proteins involved in BCAA degradation, targeted metabolomics did not reveal any significant changes in BCAA and other amino acids levels, both in iBAT and serum samples. On a speculative level, it is possible to suggest that the reduction in enzymes involved in the degradation of BCAA could be functional to their use for lipogenesis and the synthesis of branched-chain fatty acids. Further detailed studies, specifically aimed at investigating metabolic flux, will be required to elucidate this aspect.

Effect of CR on BAT thermogenic capacity

Mitochondria are the primary site of thermogenesis in iBAT. Some studies primarily based on the analysis of mRNA and protein expression levels involved in thermogenesis indicate that in mice and rats long term CR was effective in declining BAT thermogenesis [30,35].

At first glance, some data reported in this article suggest that CR reduced the thermogenic capacity of the tissue. Indeed, KEGG analysis indicated that the pathways and processes primarily enriched in proteins downregulated by CR in the whole iBAT lysate were oxidative phosphorylation and thermogenesis, as also confirmed by Western blot analysis. Furthermore, CR reduced the mitochondrial content of the tissue, as evidenced by the levels of two mitochondrial markers, VDAC and HSP60, which were reduced by CR treatment in the total tissue lysate but not in isolated mitochondria

samples. A reduction in mitochondrial content is in agreement with data present in literature obtained in rats subject to CR 30% for 6 months.

These findings seem to conflict with the unchanged mitochondrial respiration rate detected in the whole BAT homogenate. The choice of detecting respiration rate both in iBAT homogenate and in isolated mitochondria allow us to assert that this inconsistency is more about appearance than reality. Indeed, when detected in whole homogenate mitochondria respiration rate reflects both mitochondrial content and function, while its evaluation in isolated mitochondria provide insights only into organelle functional capacity. Our data suggest that an increase in mitochondrial functionality could compensate for the CR- induced reduction in iBAT mitochondrial content, thus leading to the unchanged tissue's thermogenic capacity. This suggestion is supported by the evidence showing that i) CR enhances the mitochondrial respiration rates in isolated organelles, ii) Western blot analysis performed on isolated mitochondria reveals that CR elevates the levels of proteins of respiratory chain complexes as well as UCP1.

Thus, the brown adipocytes could benefit from a cost-saving adaptation, requiring fewer organelles to maintain cellular functions and fewer resources, i.e., such as amino acids, lipids, and ATP, necessary for supporting the mitochondrial network structure in a condition of energy restriction.

Our data also indicate that an enhancement of the respiratory chain activity, as well as UCP1, underlie the ability of CR to enhance mitochondrial respiration. Indeed, when detected in the basal condition (i.e, in the presence of succinate alone), the control of respiration is shared between the activities of the reactions involved in substrate oxidation, among these the respiratory chain, and proton leak. In the presence of GDP, the control exerted by the respiratory chain on respiration becomes more relevant, due to its inhibition of UCP1-mediated proton leak [61]. The stimulatory effect of isolated mitochondrial respiration rate by CR, observed both under basal conditions and in the presence of GDP, suggests an improvement in respiratory chain activity, as corroborated by the associated enhancement of respiratory chain complexes' subunits.

In addition, our respirometry data indicate that CR was more effective in enhancing complex II-linked respiratory pathways rather than complex I-linked respiratory pathways, since a significant effect was observed only in the presence of succinate as substrate.

Mitochondria are the primary source of cellular reactive oxygen species (ROS). Increases in mitochondrial oxygen consumption or mitochondrial membrane potential, driven by electron transport chain activity, can lead to enhanced ROS production [62]. Our data indicate that, although CR increases mitochondrial respiration rate, the release of H₂O₂ by these organelles (used as an index of mitochondrial ROS production) remains unchanged. It is plausible that the rise in mitochondrial UCP1 levels which dissipate proton motive force across the inner mitochondrial membrane and reduce membrane potential [63] could compensate the higher activity of the respiratory chain and thereby maintain ROS output at a constant level.

Effect of CR on iBAT structural remodelling

In iBAT, CR-induced metabolic changes are associated with structural and morphological remodelling, involving cytoskeleton and the extracellular matrix (ECM), aimed at supporting new functional demand and necessary to accommodate larger LDs. EMC remodelling has been described in physiological and pathological conditions, such as cold exposure and obesity, and has been considered essential to sustain BAT activation [64]. The collagen network fibers surrounding brown adipocytes are integral components of the tissue's remarkably dynamic extracellular matrix, which maintains tissue integrity and elasticity and allows BAT to expand and contract during thermogenesis [64]. Our data indicate that CR induces EMC remodelling involving the collagen network, as suggested by histological analysis of picrosirius red-stained iBAT, indicating increased collagen deposition in the preadipocyte area and evidence of increased collagen III deposition in perivascular areas. The effect of CR in increasing the levels of decorin and lumican, two proteoglycan proteins involved in collagen fibrillogenesis [65,66], may have played a significant role in this process. Together with the enhanced collagen deposition, we did not find markers of macrophage infiltration into the tissue, as revealed by unchanged levels of the proteins Galectin-3 and CD-68, observed by Western blot and Immunohistochemistry, thus excluding the occurrence of inflammation and fibrosis processes associated with CR-induced fibrillogenesis. This is in line with the evidence of CR-induced decorin overregulation, the role of which extends beyond matrix structural proteins. Indeed, it has been shown that decorin interacts with several ECM components and neutralizing or repressing the activity of TGF- β contrasts the fibrotic scar formation [67].

Intermediate filament (IFs) networks may reorganize to accommodate larger LDs or redistribute tension within the cell [68] and our proteomic analysis indicates that proteins forming IFs, such as KRT10, KRT1, and AABR07001416.1, are upregulated by CR by more than 2- fold (Fig. 2), and that tubulin was downregulated.

CR also downregulates moesin, a protein belonging to the Ezrin/radixin/moesin (ERM) family, which cross-links cortical actin filaments and plasma membranes. Moesin also affects signaling by sequestering Rho guanosine 5-triphosphatase (GTPase) and protein kinase A (PKA)-anchoring proteins, both involved in regulating cytoskeletal organization [69]. In addition, recent studies indicate that actomyosin-mediated tension is critical for the thermogenic capacity of adipocytes [70], suggesting that the significant downregulation of moesin induced by CR could influence not only the morphological change of brown adipocytes but also their mechanical response to adrenergic stimulation.

CONCLUSION

Taken together, the data indicate that CR, even when mild and applied for only 2 weeks, is able to profoundly influence iBAT metabolism and morphology, which may represent a key mechanism underlying its broad systemic effects, offering new insights into the role of CR in maintaining overall health.

Our data allow to suggest that TG accumulation within adipocytes, induced by CR, may serve as an energy reserve, enhancing BAT resilience, in conditions that demand increased heat production during energy scarcity, particularly when circulating free fatty acids from WAT lipolysis and triglyceride-rich lipoproteins are utilized to sustain the energy requirements of tissues other than BAT. Moreover, CR-induced mitochondrial content reduction, associated with increased mitochondrial respiration, provides advantages in terms of cellular energetic costs for maintaining thermogenesis under energy restriction.

These results open new opportunities for further studies aimed at evaluating the actual impact of mild and short-term CR on BAT response to cold exposure.

Furthermore, since our analyses are limited to male rats, further studies will be needed to evaluate the potential sexually dimorphic response of BAT to mild CR applied for a short term, and if the patterns observed in rats are similarly found in mice.

Overall, considering the limited long-term adherence typically associated with hypocaloric diets, the finding that even short-term, mild CR can induce in BAT metabolic adaptations highlights a potentially impactful avenue for translational research.

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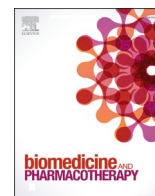
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1,3-butanediol administration as an alternative strategy to calorie restriction for neuroprotection – Insights into modulation of stress response in hippocampus of healthy rats

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ABSTRACT

Ketogenic diet has a wide range of beneficial effects but presents practical limitations due to its low compliance, hence dietary supplements have been developed to induce ketosis without nutrient deprivation. The alcohol 1,3-butanediol (BD) is a promising molecule for its ability to induce ketosis, but its effects on brain have been investigated so far only in disease models, but never in physiological conditions. To support BD use to preserve brain health, the analysis of its activity is mandatory. Therefore, we investigated, in healthy rats, the effect of a fourteen-days BD-administration on the hippocampus, an area particularly vulnerable to oxidative and inflammatory damage. Since BD treatment has been reported to reduce energy intake, results were compared with those obtained from rats undergoing a restricted dietary regimen, isoenergetic with BD group (pair fed, PF). Reduced pro-inflammatory signaling pathways and glial activation were revealed in hippocampus of BD treated rats in comparison to control (C) and PF groups. ROS content and the extent of protein oxidative damage were lower in BD and PF groups than in C. Interestingly, higher amounts of nuclear factor erythroid 2-related factor 2 (Nrf2), decreased level of lipid hydroperoxides, lower susceptibility to oxidative insult, higher amounts of superoxide dismutase-2, glutathione reductase and glutathione peroxidase (GPx), and increased GPx activity were observed in BD animals. BD administration, but not dietary restriction, attenuated endoplasmic reticulum stress, reduced autophagic response activation, and was associated with an increase of both the neurotrophin BDNF and pre-synaptic proteins synaptophysin and synaptotagmin. Our results highlight that BD plays a neuroprotective role in healthy conditions, thus emerging as an effective strategy to support brain function without the need of implementing ketogenic nutritional interventions.

1. Introduction

Ketosis, occurring in conditions of low carbohydrate availability, prolonged fasting or undertaking ketogenic diets, is characterized by a state of elevated blood ketones [1]. The ketone bodies (KBs), acetoacetate and β -hydroxybutyrate (β HB), are organic compounds produced endogenously from free fatty acids oxidation in the liver and represent an alternative fuel source to glucose for the brain as well as other

peripheral tissues, when glucose is short in supply [2]. Moreover, KBs have been demonstrated to act as hormone-like signalling molecules and exert pleiotropic effects in multiple organs by modulating substrate utilization, inflammation, oxidative stress, catabolic processes, and gene expression [1–3].

Interestingly, ketogenic diet has been proposed as an effective strategy for the treatment of epilepsy, psychological disorders, brain injury, and neurodegenerative diseases [4–7]. Also, nutritional ketosis is

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considered a promising strategy to improve health status, and there has been growing research interest in the last years in its potential application for ameliorating exercise performance and age-related diseases [7,8]. Indeed, it was demonstrated that dietary interventions based on ketosis ameliorate brain network stability [9], and prevent or rescue brain energy deficit [10], thus protecting the aging brain. However, this nutritional regimen presents practical limitations, especially in the long-term, due to its low compliance [5,11], so the effectiveness of oral exogenous ketone supplements in inducing ketosis without dietary restriction has been investigated [12–14]. Exogenous ketone supplements, including ingestible ketone salts and ketone esters, were demonstrated to elevate circulating β HB, affecting glucose, free fatty acids and triglyceride concentrations [8,15]. So, their global market has drastically grown in the last decade essentially for their properties such as increased mental clarity, enhanced athletic performance, and appetite control [14]. In this context, it is worth mentioning that the secondary alcohol 1, 3-butanediol (BD), used as component of ketone esters [16,17], represents an alternative method to induce ketosis without nutrient deprivation. Indeed, after ingestion, BD undergoes a series of oxidation steps in the liver leading to the production of β HB and consequent increase of plasma β HB concentration [13,18]. It was previously shown that BD acts as an endothelium-dependent vasodilator [19], enhances the antioxidant capacity of the gonadal white adipose tissue in rat [20], and exerts an anti-inflammatory and neuroprotective effect in rats after spinal cord injury [21]. Furthermore, it plays a leptin-sensitizing effect in the hypothalamus of diet-induced obese mice [22]. However, our understanding of the impact of β HB on the brain in physiological conditions remains limited since recent data showing the beneficial effect of β HB signalling in this organ mainly refer to processes involved in disease conditions, as proceeding from studies in animal models of pathologies [4,6,14]. To fill this gap, a study in which the increase in β HB levels is induced by the administration of its precursors in healthy animals, other than contributing to give further insight into the mechanism by which β HB affects brain physiology, would help to clarify whether this intervention can be considered a valid treatment to prevent or delay the onset of neurological pathologies. The hippocampus is crucial for learning and memory and is particularly vulnerable to dietary influences and/or oxidative and inflammatory damages, thus alterations of its functional or structural integrity can prelude the onset of neurodegenerative events [23]. Considering the central role of hippocampus and the deleterious consequences of perturbations of its homeostasis, it is important to identify molecules or treatments that could play a neuroprotective role on this brain area just using healthy animals to point out the effects detectable in physiological conditions. To this end, we evaluated the effect of exogenous ketosis induced by BD, focusing, in healthy adult rats, on mechanisms involved in the ability of cells to respond to stress, namely inflammation, redox balance, autophagy and endoplasmic reticulum stress. Further, since BD is known to induce a reduction in energy intake [20,24] we compared its effect with those observed in rats undergoing a restricted dietary regimen, isoenergetic with BD group (pair fed, PF), to further unveil the effects of this specific molecule.

2. Materials and methods

2.1. Materials

Bovine serum albumin fraction V (BSA), prestained protein ladder, salts and buffers were purchased from DelTek (Naples, Italy). Rabbit anti-human Haptoglobin and mouse anti-human actin IgGs were from Sigma-Aldrich (St. Louis, MO, USA). The dye reagent for protein titration was from Bio-Rad (Hercules, CA); polyvinylidene difluoride (PVDF) and nitrocellulose membranes were from GE Healthcare (Milan, Italy). Fuji Super RX 100 film, developer and fixer were from Laboratorio Elettronico di Precisione (Naples, Italy). 1,3 BD was purchased by Merck Millipore.

2.2. Experimental design

All experiments were carried out with male Wistar rats (90 days old; about 270 g) purchased from Envigo RMS Srl (Udine Italy). Animals received a standard diet (4RF21 Mucedola) during the experimental phase (14 days). BD treatment was chosen given its ability to significantly increase β HB serum levels [20].

Rats were randomly divided into three groups: 1) control group (C), composed of rats receiving a single i.p. administration of physiological solution at the beginning of the treatment. Rats had free access to water and food; 2) BD treated group (BD), composed of rats that received a single i.p. administration of BD (2.5 mmol/100 g body weight) followed by the oral administration of the compound in drinking water (10 % v/v) for 14 days. Rats had free access to BD drinking solution and food; 3) pair fed group (PF), composed of rats that received a single i.p. administration of a physiological solution at the beginning of the treatment and that were pair fed on an isoenergetic basis to the BD group, in order to guarantee equal amounts of energy intake between BD treated and PF group, during the two weeks of treatments. In detail, since BD treatment reduced animal energy intake by 15 % compared to C, the PF group received a daily amount of chow calibrated to match the energy intake assumed daily by BD group. Rats had free access to water and food.

For the evaluation of the energy intake of BD group, the energy of BD assumed through drinking solution was considered.

During the experimental phase, drinking BD solution (10 % v/v) was prepared and replaced daily. Food and BD drinking solution intake were monitored daily, while animal body weight was monitored three times a week. Rats of BD group assumed on average of 20 mL of BD drinking solution daily.

At the end of the 14 days treatment, the rats were anesthetized, and from a small cut on the tail a drop of blood was collected to detect β HB by using a ketone body meter (Glucomen Areo β -ketone sensor). The animals were then euthanized by decapitation, brains were quickly removed and hippocampus was harvested and dissected as previously described [25]. Aliquots were immediately processed or snap frozen in liquid nitrogen and stored at -80°C for further analyses. Blood samples were also collected, serum was isolated by centrifugation (1400 g, 10 min, 4°C), and then stored at -20°C until used for determination of inflammatory markers.

This study was carried out under the recommendations in the EU Directive 2010/63 for the Care and Use of Laboratory Animals. All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health (Authorization n° 776/2021-PR.). Every effort was made to minimize animal pain and suffering.

2.3. Protein extraction

The homogenization of aliquots of frozen hippocampus (about 40 mg) was carried out in seven volumes (w/v) of cold RIPA buffer (150 mM NaCl, 50 mM Tris- HCl pH 8.0, 0.5 % sodium deoxycholate, 0.5 % NP-40, 0.1 % SDS pH 8.0) containing 1 % Protease Inhibitor Cocktail and 1 % Phosphatase Inhibitor Cocktail (Euroclone, Milan, Italy). Homogenates were incubated (30 min) at 4°C and then centrifuged (14,000 g, 45 min, 4°C) [26]. Nuclear extracts used for detecting Nrf2 were isolated as previously reported [27]. In detail, aliquots of frozen hippocampus (about 30 mg) were homogenized in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl_2 , 0.15 % Igepal CA-630. The homogenates were centrifuged at $700 \times g$ for 5 min. The nuclear pellets were resuspended in half of the volume of nuclear homogenization buffer and were centrifuged as before. This step was repeated twice. The nuclear pellet was extracted in buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25 % glycerol (30 min on ice, followed by 10 min at room temperature). The extract was then centrifuged at $20,000 \times g$ (4°C , 30 min) and the supernatant was collected.

Protein concentration of supernatants was finally evaluated by the colorimetric Bio-Rad Bradford protein assay, using a commercial kit (Bio-Rad, Hercules, CA, USA).

2.4. Western blotting

Electrophoretic fractionation of hippocampal proteins (30 µg) was carried out under denaturing and reducing conditions [28] on 12.5 % polyacrylamide gels (to quantify glial fibrillary acidic protein [GFAP], nuclear factor erythroid 2-related factor 2 [Nrf2], superoxide dismutase-2 [SOD-2], catalase, glutathione peroxidase [GPx], glutathione reductase [GR], synaptophysin, synaptotagmin, brain derived neurotrophic factor [BDNF], beclin, and P62-sequestosome-1 [p62]) or on 10 % polyacrylamide gels (to assay toll-like receptor-4 [TLR4], nuclear factor kappa-light-chain-enhancer of activated B cells [NFkB], eukaryotic initiation factor 2α [eIF2α], post-synaptic density protein 95 [PSD-95], glucose-regulated protein [GRP-78], calnexin). Proteins were then transferred onto PVDF or nitrocellulose membrane, washing and blocking steps were performed according to previously published procedures [29,30].

After blocking the membranes were incubated with primary antibodies (overnight, at 4 °C), washed and then treated (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary antibodies. The specific dilutions of primary and secondary antibodies are reported in Supplementary Table 1. As the amount of phosphorylated proteins (NFkB, eIF2α) was expressed as relative to the total NFkB or eIF2α, after revelation of the immunocomplexes, the membranes were stripped [28] and then incubated with the specific antibody for the total form of the protein (Supplementary Table 1). For loading control, after detection of each antigen, the membranes were stripped and incubated (overnight, 4 °C) with mouse anti-β-actin IgG (1:1000 in 0.25 % v/v non-fat milk) followed by goat anti-mouse horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; 1:30,000 in 0.25 % v/v non-fat milk; 1 h, 37 °C). For loading control of Nrf2 nuclear content, histone H3 levels were used. In detail, membranes were incubated with rabbit anti-Histone H3 (1:1000, in 1 % v/v non-fat milk) followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; 1:200,000 in 1 % v/v non-fat milk; 1 h, 37 °C). Signal detection was carried out using the Excellent Chemiluminescent Kit Westar Antares (Cyanagen s.r.l., Bologna, Italy). Densitometric analysis of chemidoc or digital images of X-ray films exposed to immunostained membranes was performed with Un-Scan-It gel software (Silk Scientific, UT, USA).

2.5. Analysis of serum and hippocampal tumor necrosis factor alpha (TNF-alpha) and interleukin 6 (IL-6)

The concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in both plasma and hippocampus were evaluated by sandwich enzyme-linked immunosorbent assay (ELISA) using the Duo-Set ELISA kit specific for rats (R&D, DBA Italia; TNF-alpha, catalog number: DY510; IL-6, catalog number: DY506).

In detail, serum samples were diluted 1:15 (in PBS containing 1 % BSA, 0.05 % Tween 20) and used in the assay according to the manufacturer's instructions. TNF-alpha and IL-6 concentrations were reported as pg per mL.

For cytokines quantification in hippocampus, proteins were extracted by homogenizing slices of frozen hippocampus in lysis buffer (100 mM Tris/HCl, pH 7.0, 1 M NaCl, 4 mM EDTA, 2 % Triton X-100, 0.1 % sodium azide) containing 1 % Protease Inhibitor Cocktail and 1 % Phosphatase Inhibitor Cocktail (Euroclone, Milan, Italy). Homogenates were then centrifuged (14,000 g, 30 min, 4 °C), and TNF-alpha and IL-6 concentrations were assessed in the homogenate supernatants diluted 1:20 [31]. Data were reported as pg per mg of total proteins.

A seven point standard curve with recombinant rat TNF-alpha (4000–62.5 pg/mL) or IL-6 (8000–125 pg/mL) was used in the

respective assay, according to manufacturer's instructions.

2.6. Evaluation of nitro-tyrosine and haptoglobin (Hpt)

Nitro-tyrosine (N-Tyr) concentration in hippocampus homogenates was measured by ELISA essentially as previously described [32]. In particular, samples were diluted (1:9000, 1:18000, 1:40000) with coating buffer (7 mM Na₂CO₃, 17 mM NaHCO₃, 1.5 mM NaN₃, pH 9.6), and aliquots of each dilution (50 µL) were incubated in the wells of a microtitre plate (Immuno MaxiSorp; overnight, 4 °C). Washing and blocking were performed according to Mazzoli et al. [33]. The wells were then incubated (1 h, 37 °C) with 50 µL of rabbit anti-N-Tyr IgG (Covalab, distributed by VinciBiochem, Vinci, Italy; 1: 1000 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween, pH 7.4, containing 0.25 % w/v BSA) followed by 60 µL of goat anti-rabbit horseradish peroxidase-conjugated IgG (GAR-HRP IgG; Immunoreagents, Raleigh, NC, USA; 1:9000 dilution; 1 h, 37 °C). Peroxidase-catalyzed color development from o-phenylenediamine was measured at 492 nm. Data were reported as OD per mg of total proteins.

Haptoglobin (Hpt) was titrated by ELISA in both serum and hippocampus. Samples were diluted (plasma = 1: 20000–1:60000–1:120000; hippocampus = 1: 700, 1:2100, 1:7000) with coating buffer and Hpt was immunorevealed by incubation 50 µL of rabbit anti-human haptoglobin (1:800 in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween, pH 7.4, containing 0.25 % w/v BSA), followed by 60 µL GAR-HRP IgG (1:12000 dilution, 1 h, 37 °C). Peroxidase-catalyzed color development from o-phenylenediamine was measured at 492 nm. The calibration curve was obtained by assaying the immunoreactivity of 0.8, 0.4, 0.2, 0.1, 0.03, 0.01, 0.003 ng of purified haptoglobin.

2.7. Evaluation of lipid oxidative damage and in vitro susceptibility to oxidants

The hydroperoxides (HPs) content in hippocampus homogenates was used to evaluate the extent of lipid oxidative damage [34] by measuring the stoichiometrically correlated reduction in NADPH absorbance, at 340 nm, due to the coupled reactions catalysed by the glutathione peroxidase and glutathione reductase enzymes in the presence of GSH. Briefly, hippocampus homogenate (0.1 mg of protein) was added to 250 µL of 0.2 mM EDTA, 0.124 M Tris-HCl buffer, pH 7.6 supplemented with glutathione peroxidase (0.025 U/mL), GSH (0.425 mM), glutathione reductase (0.025 U/mL) and NADPH (1.5 mM). Variation in the Absorbance was detected at 340 nm by a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). HPs content was expressed as nmol NADPH oxidized•min⁻¹•mg⁻¹ proteins.

To estimate the susceptibility of the hippocampus to oxidative stress we evaluated the change in the hydroperoxide levels induced by the treatment of tissue homogenates (0.01 mg) with Fe and ascorbate (Fe/As), at a concentration of 100/1000 µM, for 10 min at room temperature. In such system ascorbate reduced iron, and Fe²⁺ reacted with samples' lipid hydroperoxides producing the hydroxyl radical that, if not scavenged by the antioxidants of the sample, triggered lipid peroxidation. The reaction was stopped by the addition of 0.2 % 2,6-di-*t*-butyl-*p*-cresol (BHT) and the hydroperoxide levels were evaluated as previously described [34].

2.8. Determination of reactive oxygen species (ROS) content

ROS content was evaluated by determining the conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent dichlorofluorescein (DCF) induced by ROS [35]. In brief, an amount of hippocampus homogenate (25 µg of proteins) was added to 250 µL of nonobasic phosphate buffer (0.1 M, pH 7.4) containing 10 µM DCFH-DA. After the addition of 100 µM FeCl₃, the mixture was incubated for 30 min. The ROS- induced conversion of DCFH-DA to the fluorescent product DCF was evaluated by a microplate reader (Tecan

Infinite 200 pro plate reader, $\lambda_{\text{EX}}485$, $\lambda_{\text{EM}}530$). The conversion of DCFH to DCF in the absence of homogenate was detected to evaluate the background. The results were expressed as Relative Fluorescent Unit (RFU)/ μg protein.

2.9. Evaluation of total antioxidant capacity (TAC)

To estimate TAC, we evaluated the decolorization of the mono-cation radical of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS $^{\bullet+}$), induced by hippocampal homogenate antioxidants, at 740 nm. Hippocampus homogenate (0.01 mg) was added to 250 μL of KH_2PO_4 0.1 M, pH 7.4 supplemented with ABTS $^{\bullet+}$ (0.437 mM), and variation in absorbance was detected with a microplate reader. ABTS $^{\bullet+}$ was obtained by the reaction between potassium-persulfate (245 mM) and ABTS (7 mM), for 16 hours at room temperature [36]. A calibration curve was obtained using 3,5-di-tert-4-butylhydroxytoluene (BHT, 0.05–2.5 mM). Total antioxidant capacity was expressed as μmol BHT Equivalents/mg protein.

2.10. Antioxidant enzyme activities

Specific activity of the enzyme superoxide dismutase was measured at 550 nm following the decrease in the rate of reduction of cytochrome c (induced by superoxide radicals, produced by the xanthine-xanthine oxidase system) in the presence of hippocampal homogenate. Briefly, hippocampal homogenates (0.1 mg protein) were added to 250 μL of a solution containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4 , pH 7.8, 20 mM cytochrome c, 5 mM xanthine, and 0.01 U of xanthine oxidase. Variations in absorbance were followed by a microplate reader. A unit of SOD activity corresponds to the enzyme concentration able to inhibit the reduction of cytochrome c by 50 % in the presence of xanthine + xanthine oxidase [37].

Glutathione peroxidase (GPX) specific activity was evaluated using H_2O_2 and reduced glutathione (GSH) as substrates and measuring the rate of NADPH oxidation at 340 nm, catalysed by glutathione reductase (GR) which reduces the oxidized glutathione (GSSG) obtained by the reaction [38], using a microplate reader. Briefly, hippocampus homogenate (0.02 mg of proteins) was added to 250 μL of 0.1 M KH_2PO_4 , 1 mM EDTA, pH 7.0 supplemented with NADPH (1.5 mM), H_2O_2 (1.5 mM), GSH (10 mM) and GR (2.4 U/mL). Glutathione reductase specific activity (GR) was evaluated assessing the oxidation rate of NADPH in the reaction of reduction of GSSG [39]. Briefly, hippocampus homogenate (0.02 mg proteins) was added to 250 μL of 0.2 M KH_2PO_4 , 2 mM EDTA, pH 7.0 supplemented with NADPH (2 mM) and GSSG (20 mM) [38], and the rate of NADPH oxidation was monitored at 340 nm, by using a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). The results were expressed as μmol NADPH oxidized $\cdot\text{min}^{-1} \cdot \text{mg}^{-1}$ proteins.

Catalase specific activity was assessed according to Aebi [40] by measuring the decomposition of H_2O_2 . In brief hippocampus homogenate (0.1 mg proteins) was added to 1 mL sodium phosphate buffer (50 mM, pH 7) supplemented with H_2O_2 (30 mM). Catalase activity was calculated by determining the changes in the absorbance at 240 nm for 2 min, using extinction coefficient of $39.4 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as μmol of H_2O_2 reduced per min per mg of proteins at pH 7.0 and 25 °C.

2.11. Statistical analysis

The sample size was defined by a priori power analysis, by using the G*Power software version 3.1.9.4 from the Heinrich Heine University of Dusseldorf (<http://www.gpower.hhu.de>). The power analysis was based on our previous investigations estimating a large effect size (Cohen's $d > 0.8$), and using a desired power of 0.80 with a probability of a Type I error of 0.05, implying that 7–8 different samples from each group are sufficient to observe statistically significant differences with the parameters set as such using one-way ANOVA.

Data were expressed as mean values $\pm$ SEM. Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. $P < 0.05$ was considered significant in the reported analyses.

3. Results

3.1. Titration of beta-hydroxybutyrate (βHB) and inflammatory markers in serum

In order to verify the ketogenic effect of BD, plasma levels of βHB were titrated in the three groups, namely the control group (C), the group of rats that received administration of BD (BD group), and the third group of rats that were pair fed on an isoenergetic basis to the BD group (PF group). βHB levels were found significantly higher in BD group respect to both control and PF ones (Fig. 1A; $p < 0.001$; F (2, 21) = 17.85) in line with results showing that BD administration increases plasma βHB concentration [13,18].

Plasma inflammatory status was assessed by measuring the concentrations of cytokines (TNF-alpha and IL-6) and Hpt, an inflammatory marker sensitive to nutritional stress [41]. As reported in Fig. 1 B-D, the levels of these markers were significantly lower in plasma of BD than C rats ($p < 0.01$), with no differences between PF and BD group (Hpt, F (2, 21) = 169.3, $P < 0.0001$; TNF-alpha, F (2,21) = 17.52, $P < 0.0001$; IL-6, F (2,21) = 27.62, $P < 0.0001$).

3.2. Evaluation of hippocampus inflammatory status

Neuroinflammation represents one of the pathological mechanisms underlying aging [42] and neurodegenerative diseases [43,44], and recent studies also demonstrated the involvement of pro-inflammatory conditions in the onset of depressive-like behaviors and anxiety symptoms [45,46]. To verify whether BD treatment influences molecular mechanisms involved in the modulation of hippocampal inflammatory status, we evaluated pro-inflammatory signaling pathways by measuring the amount of TLR4, the degree of NFkB phosphorylation, and the levels of TNF-alpha, IL-6, Hpt and GFAP.

As shown in Fig. 2A-B, TLR4 amount and the ratio between phosphorylated and total NFkB (p-NFkB/NFkB), which is an index of protein pathway activation, were found decreased in BD-treated rats compared to both control and PF groups, while no differences were detected between C and PF (TLR4, F (2,21) = 13.33, $P < 0.0001$; -NFkB/NFkB, F (2,21), F (2,21) = 17.93, $P < 0.0001$). In line with results obtained in plasma, both TNF-alpha and IL-6 concentrations were lower in hippocampus of BD rats compared to C group (TNF-alpha, F (2,21) = 14.72, $P < 0.001$; IL-6, F (2,21) = 10.18, $P < 0.01$; Fig. 2C-D). Interestingly, cytokines levels did not differ between PF and BD group, suggesting that their decrease might be due to reduced energy intake, which is common to both BD and PF rats.

Further, lower amounts of GFAP, a marker of astrogliosis, and Hpt were detected in the hippocampus of BD-treated rats respect to both the control and PF rats (GFAP, F (2,21) = 29.42, $P < 0.0001$; Hpt, F (2,21) = 6.944, $P < 0.01$; Fig. 2E-F).

Altogether, these results demonstrate that BD administration did not induce inflammation, but conversely it exerts a beneficial role modulating the inflammatory response.

3.3. Oxidative status of rat hippocampus

Oxidative stress is a broad inflammation-related mechanism that has been shown to be strongly implicated in aging/neurodegeneration [47, 48]. Oxidative stress is a by-product of energy metabolism and occurs when there is an imbalance in the homeostatic processes that balance the cellular production of reactive oxygen species (ROS) and their

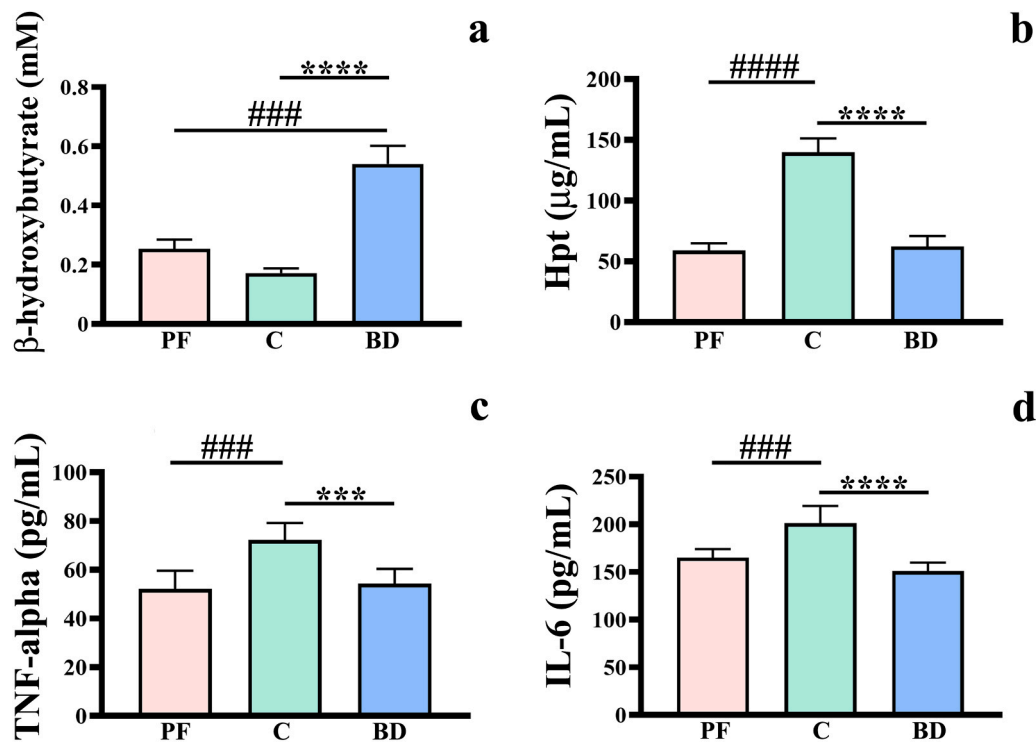


Fig. 1. Titration of beta-hydroxybutyrate (β HB) and inflammatory markers in serum. (A) β HB level in serum was measured by a ketone body meter (Glucomen Areo β -ketone sensor). (B) Haptoglobin concentration was measured by ELISA in serum samples diluted 1: 20000, 1:60000, and 1:120000. Immunodetection was carried out with rabbit anti-Hpt and GAR-HRP IgGs. Data represent means $\pm$ SEM of 8 animals from each group. (C) TNF-alpha and (D) IL-6 levels were titrated by sandwich ELISA following the manufacturer's instructions. Data are reported as means $\pm$ SEM of 8 different rats from each experimental group. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). *** $P < 0.001$, **** $P < 0.0001$ versus BD. ### $P < 0.001$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

buffering by antioxidant activity. To highlight the effect of BD treatment on redox homeostasis in the hippocampus, we evaluated ROS tissue content, N-Tyr amount, as a marker of oxidative damage to proteins induced by peroxynitrite, and lipid hydroperoxides content, as a marker of oxidative damage to lipids. Further, the total tissue antioxidant capacities (TAC), and the *in vitro* susceptibility to an oxidant insult were assessed in order to evaluate the tissue ability to counteract an oxidative attack.

As shown in Fig. 3, total ROS content was reduced to the same extent in both PF and BD groups compared to C ($F(2,21) = 7.731$, $P < 0.01$; Fig. 3A). The observed lower level of ROS was associated with a reduced oxidative damage to lipids and proteins in BD group in respect to the control (lipid hydroperoxides, $F(2,21) = 4.269$, $P < 0.05$; N-Tyr, $F(2,21) = 10.47$, $P < 0.001$; Fig. 3B-C). Interestingly, lipid hydroperoxides levels did not differ between PF and C groups (Fig. 3B). Similarly, susceptibility to oxidative insult, calculated as change in the hydroperoxide levels (Δ HPs) induced by the treatment with Fe/As, was reduced in the hippocampus of BD-treated rats in comparison to both C and PF rats ($F(2,21) = 27.58$, $P < 0.0001$; Fig. 3D). Total antioxidant capacity resulted unmodified by both treatments (Fig. 3E).

In order to get further insight into the mechanistic pathway underlying the BD-associated decrease in oxidative condition, we measured the nuclear levels of Nrf2, a fundamental player in the modulation of the antioxidant response. In line with the condition of reduced oxidative stress in the hippocampus of BD-treated rats, we found increased amount of nuclear Nrf2 in hippocampus of rats receiving BD ($F(2,21) = 7.055$) respect to both C and PF rats ($P < 0.01$ and $P < 0.05$ respectively, Fig. 4A). Further, a BD-related increase of the protein content of the enzymes SOD-2 and glutathione reductase (GR) in comparison with both PF and C rats was detected (SOD-2, $F(2,21) = 4792$, $P < 0.05$; GR, $F(2,21) = 5378$, $P < 0.05$; Fig. 4B-C). In addition, glutathione peroxidase (GPx) and catalase amounts were higher in BD than in C group

(GPx, $F(2,21) = 8.308$, $P < 0.01$; catalase, $F(2,21) = 5.911$, $P < 0.01$), with no differences between PF and BD groups (Fig. 4D-E). Notably, we found an increase in the activity of GPx in hippocampus of BD rats in comparison to C group, while SOD, GR and catalase activities did not differ between the two groups ($F(2,21) = 3.378$, $P < 0.05$; Fig. 5A-D). Further, higher activities of GR and SOD were observed in BD group respect to PF (GR, $F(2,21) = 5.349$, $P < 0.05$; SOD, $F(2,21) = 4.996$, $P < 0.05$).

Taken together these data support the hypothesis that BD plays a neuroprotective role by promoting the increase of nuclear levels of Nrf2, the master regulator of antioxidant systems, thus inducing the expression of enzymes involved in the cellular antioxidant responses. In this way, the shift of oxidative balance towards the increase of antioxidant players contributes to preserving redox homeostasis.

3.4. Endoplasmic reticulum stress and autophagy

Endoplasmic reticulum stress plays a critical role in cell homeostasis [49]. Therefore, we investigated whether BD administration modulates the level of ERS indicators, including the degree of eIF2 α phosphorylation and the amount of the two chaperones calnexin and GRP78.

As shown in Fig. 6A-B, lower amounts of both calnexin and GRP78, and reduced p-eIF2 α /eIF2 α ratio were detected in the hippocampus of BD treated rats, with no differences between control and PF groups (calnexin, $F(2,21) = 6.992$, $P < 0.01$; GRP78, $F(2,21) = 13.17$, $P < 0.001$; p-eIF2 α /eIF2 α , $F(2,21) = 10.70$, $P < 0.001$). This result suggests that ER stress activation is specifically counteracted/prevented by the compound administration and is not just a consequence of reduced energy intake associated to BD administration.

Since a dysregulation of autophagy can induce ER stress and the rise of inflammatory process, the impact of BD administration on autophagic response has been explored. Autophagy is a fundamental process for

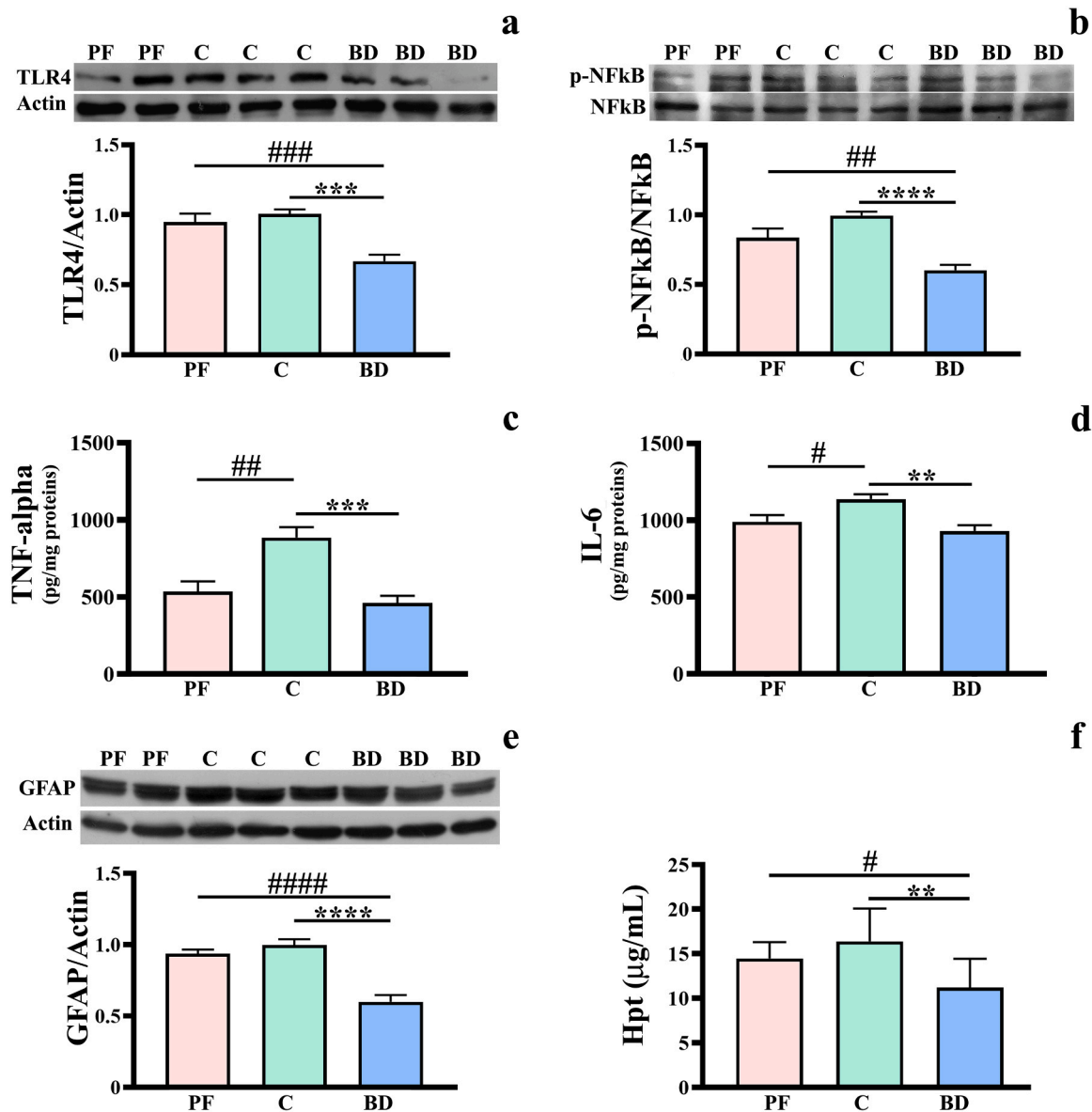


Fig. 2. Evaluation of inflammatory status. (A) TLR-4 amount (representative western blotting and densitometric analysis) in protein extracts from hippocampus of adult rats. Samples were analyzed by 10 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with mouse anti- β -actin as loading control. Values shown are means $\pm$ SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (B) p-NFkB/NFkB ratio (representative western blotting and densitometric analysis) in protein extracts from hippocampus of adult rats. Samples were analyzed by 10 % SDS-PAGE and western blotting. After revelation of the immunocomplexes (by rabbit anti-phospho NFkB and GAR-HRP IgGs), the membrane was stripped and treated with rabbit anti-NFkB and GAR-HRP IgGs. The amount of phosphorylated NFkB was expressed relative to total NFkB level. Values shown are means $\pm$ SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (C), TNF-alpha and (D) IL-6 amount was titrated by sandwich ELISA following the manufacturer's instructions. Data are expressed as pg per mg of total proteins and reported as means $\pm$ SEM of 8 different rats from each experimental group. (E) GFAP amount (representative western blotting and densitometric analysis). Samples were analyzed by 12.5 % SDS-PAGE and western blotting. After revelation of immunocomplexes (by rabbit anti-GFAP and GAR-HRP IgGs), the membrane was stripped and re-incubated with anti- β -actin. Values shown are means $\pm$ SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (F) Hpt concentration was measured by ELISA in hippocampus samples diluted 1:700, 1:2100, and 1:7000. Immunodetection was carried out with rabbit anti-Hpt and GAR-HRP IgGs. Data represent means $\pm$ SEM of 8 animals from each group. Control rats (C); 1,3-butanol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus BD; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

maintaining cells homeostasis, mostly balancing energy sources during development and nutritional stress [50]. As shown in Fig. 6C-D, the levels of beclin and p62, two key proteins involved in this mechanism, were significantly lower in BD rats than in both C and PF groups (beclin, $F(2,21) = 33.30$, $P < 0.0001$; p62, $F(2,21) = 9.036$, $P < 0.01$). Interestingly, no differences were evidenced between C and PF group, thus suggesting that the observed effect induced by BD cannot be just ascribed to the reduction in energy intake associated with its

administration.

3.5. BDNF and synaptic markers

In order to further clarify whether BD administration influences critical markers of brain functioning, we investigated whether the amount of BDNF, a neurotrophin crucial in the modulation of survival and development of the nervous system [51], is affected by the

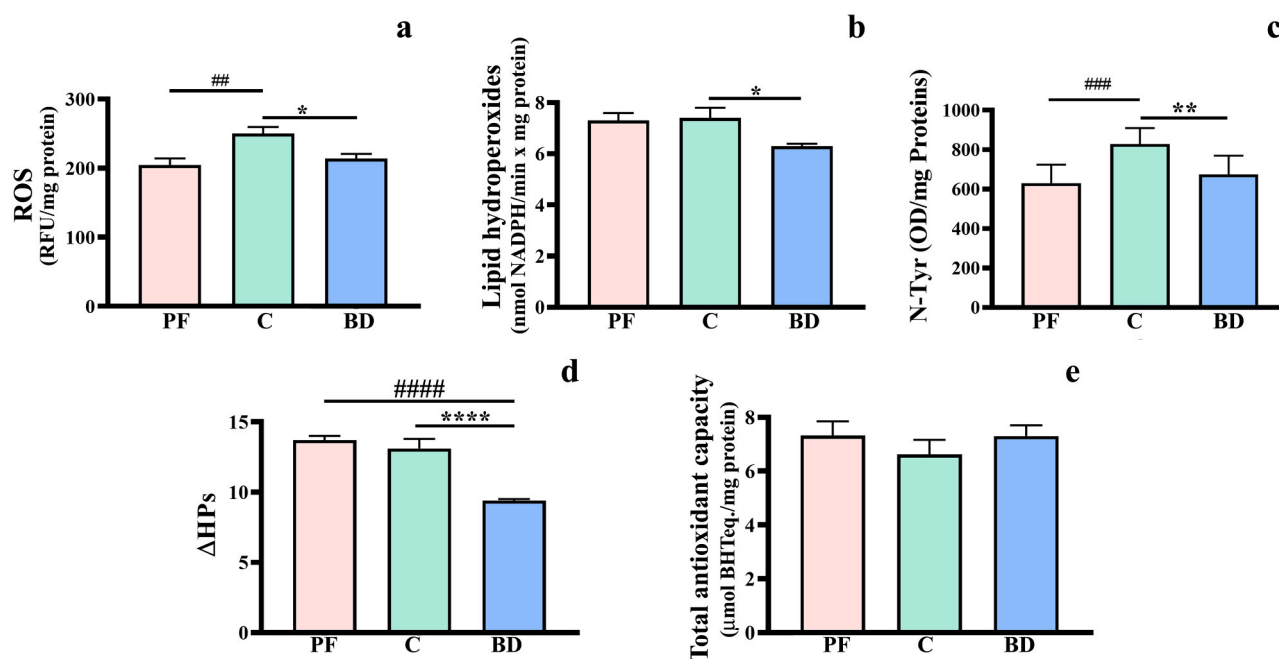


Fig. 3. Evaluation of oxidative status. Quantification of (a) total ROS; (b) lipid hydroperoxides; (c) protein oxidative damage (N-Tyr); (d) oxidative damage susceptibility (ΔHPS); (e) tissue soluble antioxidant capacity (TAC) in hippocampus of adult rats. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. Values are means $\pm$ SEM of 8 animals from each group. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ versus BD; # $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ versus PF.

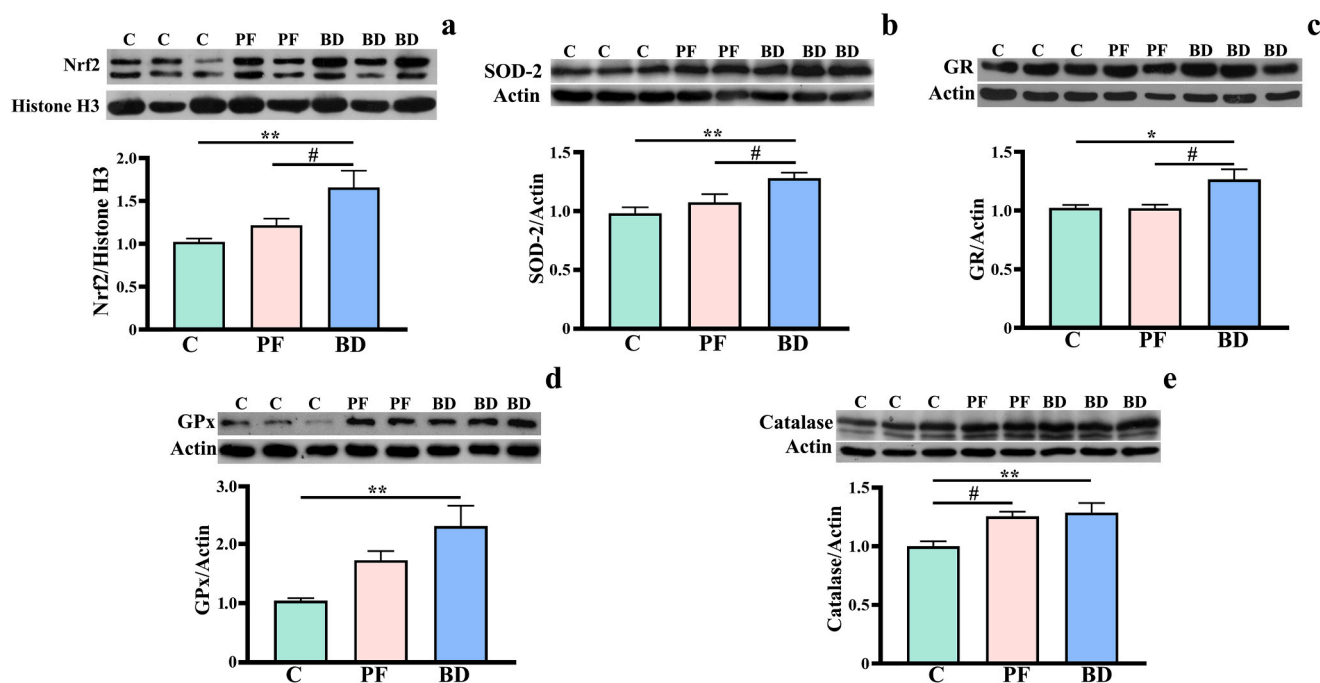


Fig. 4. Quantification of antioxidant enzymes amount. Western blotting quantification of: (a) nuclear factor-E2-related factor 2 (Nrf2), (b) superoxide dismutase (SOD), (c) glutathione reductase (GR), (d) glutathione peroxidase (GPx), (e) catalase in protein extracts from hippocampus of adult rats. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 12.5 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with rabbit anti-Histone H3 (for Nrf2 quantification) or mouse anti-β-actin (for antioxidant enzymes) as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means $\pm$ SEM of 8 different rats. * $P < 0.05$, ** $P < 0.01$ versus BD; # $P < 0.05$ versus PF (one-way ANOVA followed by Tukey post-test).

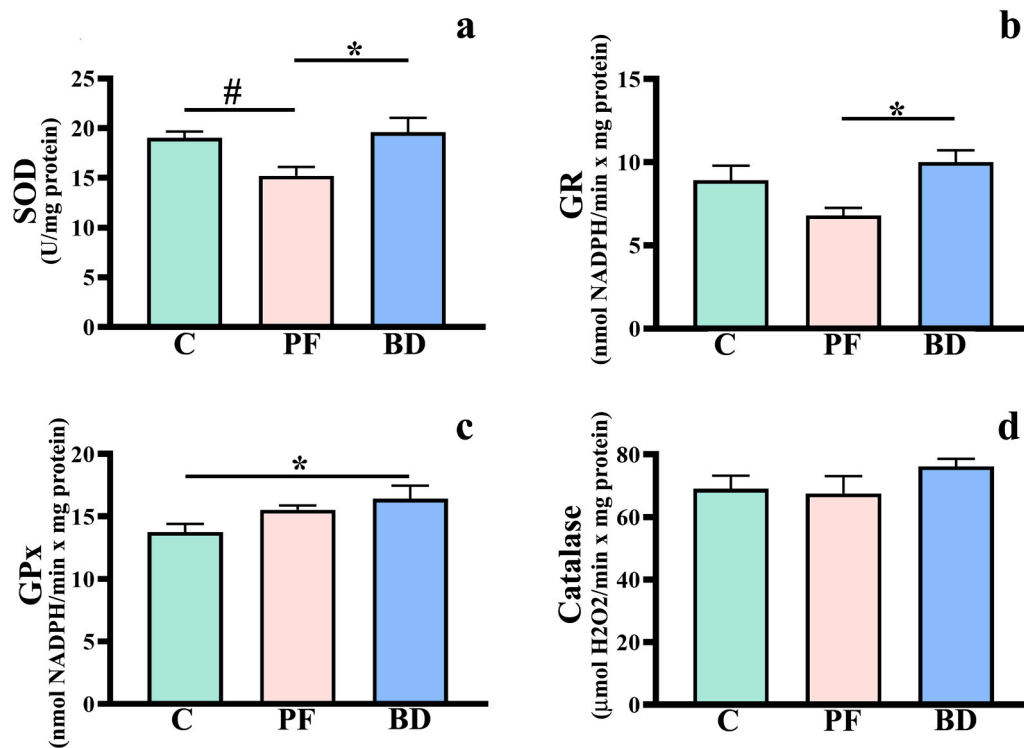


Fig. 5. Quantification of antioxidant enzymes activities The activities of (a) superoxide dismutase (SOD), (b) glutathione reductase (GR), (c) glutathione peroxidase (GPx), and (d) catalase were quantified in hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. Values are the means $\pm$ SEM of 8 different rats. * $P < 0.05$ versus BD; # $P < 0.05$ versus PF.

treatment.

As shown in Fig. 7, BDNF level was found higher in BD than in both C and PF groups ($F(2,21) = 6.641$, $P < 0.01$; Fig. 7A). Since BDNF signalling participates in the modulation of different neurophysiological processes such as synaptic function, dendritic spine maturation and stabilization [52], we evaluated the levels of presynaptic proteins synaptotagmin and synaptophysin, as well as that of the postsynaptic protein PSD-95. BD treatment was associated with a significant increase in the three proteins with respect to C group (synaptotagmin, $F(2,21) = 15.82$, $P < 0.0001$; synaptophysin, $F(2,21) = 8.04$, $P < 0.01$; PSD-95, $F(2,21) = 7.632$, $P < 0.01$; Fig. 7B-D). Notably, the amount of pre-synaptic proteins did not differ between control and PF group, thus suggesting that BD-associated increase cannot be achieved by just a 15 % reduction of energy intake as simulated in PF group.

4. Discussion

The effectiveness of ketogenic regimen in treatment of obesity and refractory epilepsy [6,7], as well as the beneficial and neuroprotective effect in neurological pathologies [4–7] have been ascribed to ability of ketone bodies to replace glucose as brain's main energy source and to act as signaling molecules [2,3]. However, ketogenic diets don't have long-term tolerability, and may present challenges in clinical application [53,54]. As a matter of fact, they may be accompanied by a temporary cluster of symptoms or longer-term effects [5,11,55]. Therefore, alternative dietary supplements have been developed to induce exogenous ketosis [13,56]. In particular, several exogenous ketogenic supplements (EKSs), such as ketone esters (KEs), ketone salts (KSs), and medium chain triglycerides (MCTs) are known to play a therapeutic role thanks to their effectiveness in increasing blood KB level [12,13,57,58,59]. As a matter of fact, KEs, KSs and MCT oils can evoke anti-seizure and anti-epileptic effects [56,60–62], and exert alleviating effects on

neurodegenerative diseases [59,60,63,64]. Nevertheless, to date their beneficial effects have been investigated only in disease models, both *in vivo* and *in vitro*.

We believe it is mandatory to clarify the role of these treatments in physiological conditions by using *in vivo* models, given the opportunity to exploit these supplements as future strategies to prevent brain aging and/or delay the development of neurodegenerative diseases. Therefore, we investigated the effect of BD-administration on the hippocampus of adult healthy rats to gain insight into the mechanisms by which BD might affect brain homeostasis in physiological conditions. Since BD administration was associated with a 15 % reduction in animal energy intake, its effect was compared to that of a restricted dietary regimen, isoenergetic with that of BD group (PF group). This allowed us to highlight the specificity of ketone supplements and to discriminate between the BD effects and those induced simply by a regime of dietary restriction.

We report here that BD treatment plays a neuroprotective role by reducing the activation of pro-inflammatory signaling pathways activation. As a matter of fact, treated animals showed lower degree of NFkB phosphorylation and reduced amount of TLR4, as well as reduced glial activation, evidenced by decreased levels of GFAP, and lower concentration of Hpt in comparison to both control and PF animals. So, we evidenced, in hippocampus, a higher protective role of BD in comparison to mild caloric restriction. It is worth mentioning that this is the first study demonstrating a beneficial BD-dependent modulation of inflammatory state in hippocampus, in healthy conditions. Notably, the levels of the inflammatory cytokines TNF- α and IL-6 were found reduced in both BD and PF groups in comparison to the control, in line with previous work showing that caloric restriction correlates with a decrease of pro-inflammatory markers [65,66]. Further, rats receiving BD administration showed lower serum levels of inflammatory markers in comparison to control animals.

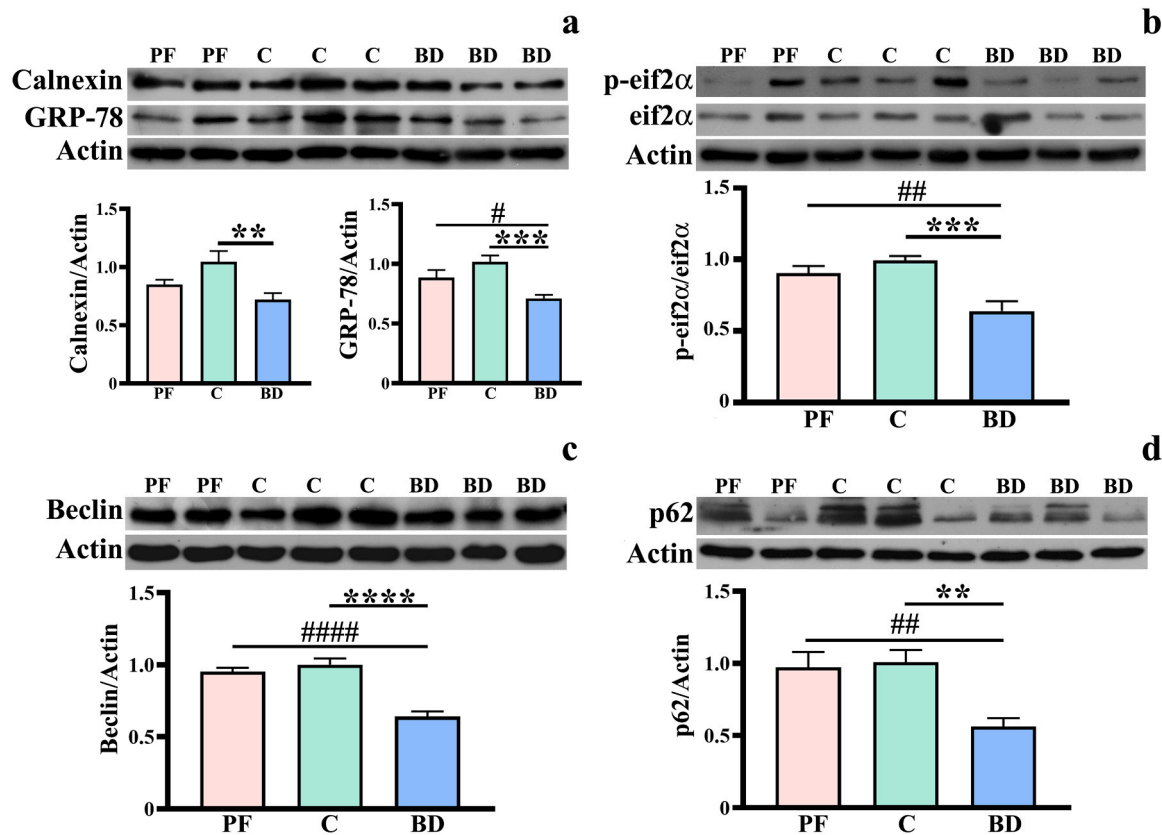


Fig. 6. Evaluation of endoplasmic reticulum stress and autophagy. (A) calnexin and GRP-78, (b) p-eif2 α /eif2 α , (c) beclin, (d) P62-sequestosome-1 (p62) were measured in protein extracts from hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 10 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with anti- β -actin as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means $\pm$ SEM of 8 different rats. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus BD; # $P < 0.05$, ## $P < 0.01$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

Our data suggest that the modulatory effect of BD is ascribable to the β HB rise, which is known to antagonize neuroinflammation [67–69]. In our experimental model, plasma β HB level was found about 3- and 1.5-fold higher in BD and PF groups with respect to C. Therefore, it can be speculated that the observed differences regarding the indices of inflammation between the three groups mainly depend on the differences in plasma levels of β HB, since ketone bodies are able to cross the blood-brain barrier and their influx into the brain has been shown to be proportional to the circulating levels [70–72]. It can be argued that the mechanism underlying the observed BD anti-inflammatory effect in our experimental paradigm might rely on the β HB-mediated inhibition of NF κ B pathway, with the consequent suppression of the pro-inflammatory cytokines IL-6 and TNF- α . Our results agree with previous works showing that EKSs-generated therapeutic ketosis may evoke beneficial effects on central nervous system diseases [58,59,73,74], thus evidencing that BD may act, through increased β HB level (ketosis), preserving hippocampus physiology.

Redox homeostasis controls multiple cellular signaling pathways and its dysregulation is implicated in the pathogenesis and progression of neurodegenerative disorders [75,76]. The central nervous system is particularly sensible to ROS injury [76] since i) neurons consume large amounts of oxygen, ii) neuronal mitochondria generate large amounts of hydrogen peroxide, and iii) neuronal membranes are rich in polyunsaturated fatty acids, which are particularly susceptible to oxidative stress. Thus, we investigated whether BD treatment affects hippocampal oxidative status, focusing on both antioxidant defense system and oxidative damage markers. A reduced ROS content together with a lower degree of oxidative damage to proteins was observed in both BD

and PF groups. Interestingly, the extent of lipid peroxidation was decreased in BD animals but not in PF rats, maybe because of the increased activity and amount of the enzyme GPx, which might be also responsible for the reduced susceptibility to oxidative insult revealed in this group. Further, BD treatment was found associated with the increase in nuclear levels of nuclear factor-erythroid 2-related factor-2 (Nrf2), the pivotal modulator of antioxidant response, together with higher concentrations of the antioxidant enzymes SOD-2, GR, and catalase. Since BD supplemented rats showed higher plasma levels of β HB, which has been suggested to activate Nrf2 pathway [77], we hypothesize that modulation of the antioxidant response in hippocampus, in our experimental model, occurs through the regulation of Nrf2. Our data agree with the evidence that an increase in ketone levels, obtained by ketogenic diet administration, was able to induce nuclear Nrf2 accumulation in both liver and hippocampus [78], and with the finding that ketogenic diet attenuates oxidative stress and inflammation through the activation of Nrf2 and the consequent suppression of NF κ B pathway, in a rat model of spinal cord injury [79]. Notably, we demonstrate, for the first time, that BD administration in physiological conditions, is more effective on Nrf2 with respect to caloric restriction. The unchanged activity of SOD-2, GR, and catalase notwithstanding the increased protein levels could result from a post-translational modification of the proteins and could have an important role in preserving the functionality of the hippocampus in animals not challenged with oxidative stress, as in our experimental conditions. This prevents a huge metabolization of ROS, which would lead to a deleterious reduction in ROS signaling. Indeed, ROS signaling is critical for neuronal development and differentiation [80] and participates in hippocampal synaptic plasticity processes such

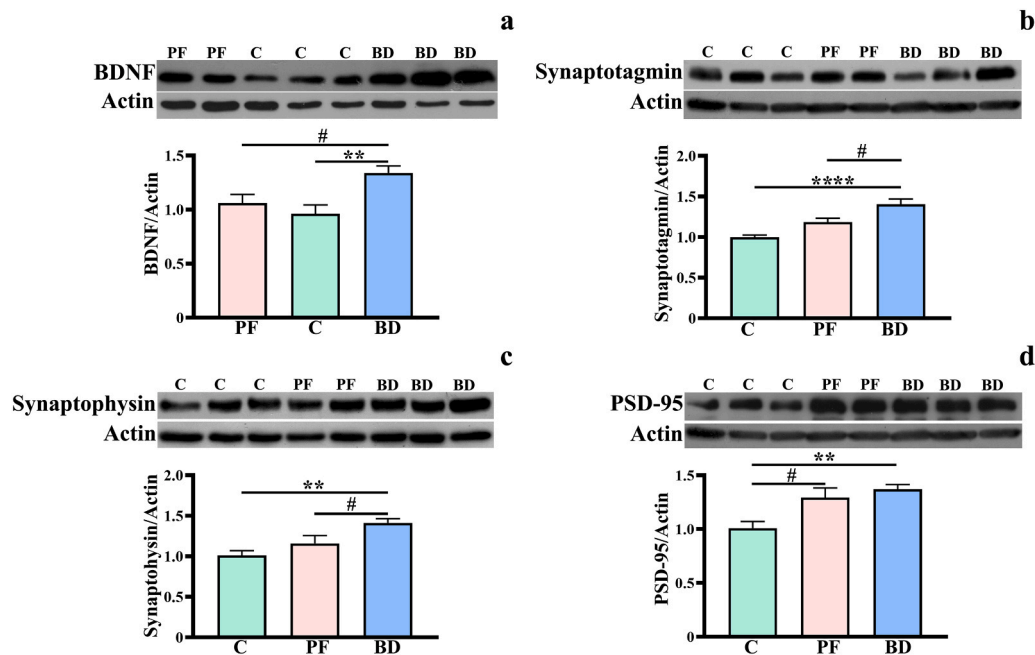


Fig. 7. Brain derived neurotrophic factor (BDNF) and synaptic proteins. (a) BDNF, (b) synaptotagmin, (c) synaptophysin, (d) post-synaptic density protein 95 (PSD-95) were quantified in protein extracts from hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 12.5 % (BDNF, synaptophysin, synaptotagmin) or 10 % (PSD-95) SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with anti- β -actin as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means $\pm$ SEM of 8 different rats. ** $P < 0.01$, **** $P < 0.0001$ versus BD; # $P < 0.05$ versus PF (one-way ANOVA followed by Tukey post-test).

as long-term potentiation [80,81]. The β HB-dependent neuroprotective modulation of redox balance has been considered implicated also in the ability of ketone bodies to suppress the unfolded protein response associated with endoplasmic reticulum stress and to correct defective autophagy in pathological conditions [82,83]. In our model, BD treatment was associated with a reduction of the degree of eIF2 α phosphorylation and the amount of the two chaperones calnexin and GRP78, in agreement with the finding that BD attenuates ER stress in the hypothalamus of diet-induced obese mice [22]. Our data also agree with the evidence that other treatments enhancing β HB, such as the ketogenic diet, protect hippocampus from epilepsy-induced activation of ER stress in rat [84], as well as from hypoglycemia-induced activation of ER stress pathway in mice [85], thus supporting the use of BD as a strategy to preserve hippocampus functionality in healthy conditions.

In addition, a reduction of the autophagy-related proteins beclin and p62 with respect to control rats was observed in BD rats but not in PF rats that underwent a mild caloric restriction. It should be highlighted the novelty of our results, since data available in the literature are limited to the description of a modulatory effect of ketone bodies in pathological conditions characterized by an abnormally prolonged or excessive ER stress response activation or by a dysregulation of autophagic flux. Notably, we report for the first time that BD plays a protective action also in physiological conditions and this could be crucial for guaranteeing the proper functioning of ER and autophagy. In this context, it should be mentioned that ER besides participating in the synthesis, folding, structural maturation, and degradation of proteins folded incorrectly [86], also impacts several cellular processes required for brain function [87], and its homeostasis contributes to neurodevelopmental processes [87]. As representing a major response pathway to cellular stress, autophagy is tightly connected to ER stress activation [49,88]. Both mechanisms operate to maintain homeostasis, but their dysregulation can induce the rise of inflammatory processes, participating in the onset or progression of several diseases [49,88]. Therefore, BD plays a neuroprotective role through the positive modulation of redox homeostasis and the reduction of the integrated

activation of the major response pathways related to stress. This beneficial action might also contribute to preserving neuronal plasticity since ER stress and neuroinflammation are associated with synaptic loss as well as the repression of the expression of a synaptic proteins cluster [89, 90]. As a matter of fact, we found that BD treatment induces an increase of the neurotrophin BDNF, the presynaptic proteins synaptophysin and synaptotagmin, and the post-synaptic protein PSD-95. Interestingly, in PF group only the amount PSD-95 was found increased, demonstrating the stronger effect of BD on the regulation of neurotrophin and pre-synaptic proteins. Our results are in line with the finding that KBs induce the production of hippocampal BDNF. Indeed, several studies demonstrated the induction of specific BDNF promoters by β HB in hippocampus neuronal cell lines [91–93], and it was shown that systemic administration of β HB in diabetic mice restored retina BDNF levels towards those of non-diabetic animals [94].

Since we observed that BD treatment induced higher β HB plasma levels than caloric restriction, our hypothesis is that differences between PF and BD group can be explained by the different circulating levels of β HB, which acts as molecular mediator of the observed effect. As a matter of the fact, β HB is known to protect the brain from oxidative stress, acting as a free radical scavenger of hydroxyl radicals and superoxide ions [5,77,95]. Further, data obtained in pathological models showed that both the ketogenic diet and β HB treatment are able to prevent or alleviate ER stress activation, both *in vitro* and *in vivo* [96–98], and to modulate autophagy [94,99]. Overall, we postulate that BD modulation of stress response in the healthy animal model occurs through mechanisms based on the regulation of NF κ B and Nrf2 pathways. In agreement with our findings, the beneficial effects described for several EKS were proposed to be induced likely through ketosis-evoked neuroprotective effects, for example, by decreased inflammatory processes and decreased oxidative stress [59,74,99,100]. Actually, our data can be also interpreted at the light of the fact that β HB, being an inhibitor of histone deacetylase, also induces an increased expression of specific genes regulating the cellular antioxidant defences, such as catalase, SOD-2, GPx [101,102], and is involved the induction of BDNF

expression [65,103,104], which in turn is a modulator of synaptophysin and synaptotagmin synthesis [105–107]. Most of our results seems to be the consequence of the increased circulating levels of β HB, but it cannot be excluded that other metabolites and/or other signaling pathways in addition to those investigated in this work may occur. This opens the way for further research into additional effects associated with BD administration.

Altogether our results highlight that BD, independently from energy intake reduction associated with its assumption, modulates Nrf-2 and NF- κ B pathway, ameliorating redox and inflammatory status, and also promoting neurotrophin expression. Therefore, our findings represent an important advance in the knowledge of BD effects just because point out that this supplement could represent an effective neuroprotective treatment to manage brain function, also in healthy conditions, with possible applications for preventing or delaying aging without the need of implementing ketogenic nutritional interventions.

Ethics approval

The study was conducted according to the guidelines of the Declaration of Helsinki, approved by “Comitato Etico-Scientifico per la Sperimentazione Animale” of the University of Naples Federico II, and authorized by Italian Health Minister (776/2021-PR).

Informed consent statement

Not applicable.

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CRediT authorship contribution statement

Gianluca Fasciolo: Writing – review & editing, Investigation, Formal analysis, Data curation. **Natasha Petecca:** Writing – review & editing, Investigation. **Francesca De Palma:** Writing – review & editing, Investigation. **Luisa Cigliano:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria Stefania Spagnuolo:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Assunta Lombardi:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paola Venditti:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Giuliana Panico:** Writing – review & editing, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors have no financial or non-financial interests to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2024.117774](https://doi.org/10.1016/j.biopha.2024.117774).

Data Availability

Data will be made available on request.

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GENERAL CONCLUSIONS

The adipose organ plays a crucial role in metabolic homeostasis, and accumulating evidences supports its designation as a key target organ for the treatment of metabolic disorders such as obesity, and associated disease among these insulin resistance, type 2 diabetes [1,2]. By taking advantage of the ability of BD to increase β -OHB plasma levels, we propose a role for β -OHB as a signaling molecule that rapidly modulates adipose organ physiology, potentially by enhancing lipolysis in WAT and thermogenesis in BAT, thereby revealing mechanisms that link energy metabolism to β -OHB. The effects of β -OHB observed are highly tissue-specific, a feature that is critical for the precise regulation of diverse metabolic pathways across different organs. One of the most striking and novel findings of this study is the demonstration that the effects induced by β -OHB emerge rapidly and are remarkably sustained even after 14 days of treatment, thus indicating that the rapid effects of β -OHB exerted on WAT and BAT are not merely transient adjustments but serve as critical initiators of physiological responses, that are progressively stabilized and maintained over the long term. These evidences highlight the fundamental role of β -OHB in both immediate and sustained metabolic regulation.

Our data also indicate that β -hydroxybutyrylation plays a pivotal role in mediating the mitochondrial actions of β -OHB, enabling rapid, nongenomic regulation of mitochondrial function and underscoring a crucial layer of metabolic control beyond transcriptional effects.

An important aspect of the effects induced by BD is its ability to reduce energy intake. It is therefore essential to distinguish between effects attributable to increased β -OHB levels following exogenous BD supplementation and those potentially arising from reduced energy intake. Data obtained from BAT and WAT indicate that the effect induced by BD are already evident as early as 3 hours after in vivo administration and persist for at least 14 days, thereby excluding any confounding effect from reduction of energy intake. Furthermore, our findings show that in BAT, a 15% reduction of energy assumption over two weeks, comparable to that observed by BD treatment, produces effects opposite to that induced by BD with respect to triglycerides accumulation within the tissue; indeed BD reduces triglycerides accumulation (reported in Research Article n.2), whereas energy restriction enhanced it (reported in Research Article n. 4). These data indicate that the action of BD-induced β -OHB appears to dominate over that of BD-induced energy restriction. Consistent with this concept, the literature reports that exogenous ketosis

induced by β -OHB precursors promotes mitochondrial biogenesis [3,4], whereas we reported that energy restriction is associated with a decrease in BAT mitochondrial content. Together, these findings reinforce the conclusion that, in BD-treated animals, β -OHB formed can effectively counteract the metabolic impact of caloric restriction.

Our findings also provide important new insights into the effects of BD-induced exogenous ketosis on hippocampus homeostasis, revealing its potential as a powerful neuroprotective agent. Remarkably, this intervention may benefit not only individuals with neurological conditions but also healthy populations, by preserving cognitive function and potentially delaying the onset of age-related cognitive decline.

Overall, this preclinical study not only contributes to the expanding body of literature on ketone bodies but also highlights a therapeutically promising avenue of exogenous ketosis for improving metabolic health, as well as brain longevity and resilience, without the need for significant dietary modifications which often require strict adherence and can be challenging to maintain.

Although the findings of the study presented here are intriguing, more aspects warrant further consideration. Our study examined only short-term BD administration (3 hours and 2 weeks) and longer interventions are needed to determine whether these effects persist and whether tissue adaptations to ketosis emerge. All experiments were performed in male rats; we did not assess sex-specific responses to BD, leaving open the possibility of sexual dimorphism in the observed effects. Thus, further studies, particularly in human populations, are necessary to evaluate the translational significance and assess clinical applicability.

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