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RESEARCH ARTICLE

Effects of long-term sucrose overfeeding on rat brown adipose tissue: a structural and immunohistochemical study

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ABSTRACT

The aim of this study was to determine the effects of long-term sucrose overfeeding on functional capacity and ultrastructural characteristics of the rat brown adipose tissue (BAT). For the study, 16 male Wistar rats, chow-fed and kept under standard laboratory conditions, were divided into 2 equal groups. The rats from a control group drank tap water, whereas those from a sucrose overfed group were allowed to drink 10% sucrose solution for 21 days. Structural changes of BAT were analysed at the level of light and electron microscopy on routinely prepared tissue sections or using immunohistochemical staining, in combination with stereological methods. Obtained results have shown that the significantly increased energy intake in sucrose overfed rats did not result in a higher gain of body mass compared with controls. The light microscopy analysis revealed that the BAT acquired the appearance of a thermogenically active tissue, with intensified vascularisation, reduced size of brown adipocytes and increased multilocularity. At the ultrastructural level, mitochondria of brown adipocytes became more abundant, enlarged and contained more cristae in comparison to control animals. The immunoexpression of uncoupling protein 1 (UCP1) and noradrenaline, as markers of BAT thermogenic status, was increased, whereas the pattern of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) was slightly modified. Taken together, the results of this investigation indicated that BAT possesses the ability to increase thermogenic capacity/activity in response to high energy intake and to prevent body mass gain. These findings are particularly relevant in view of recent reports on the existence of functional BAT in adult humans and its potential use to combat obesity.

KEY WORDS: Brown adipose tissue, Rat, Sucrose overfeeding, Immunohistochemistry, Ultrastructure

INTRODUCTION

Traditionally, the brown adipose tissue (BAT) has been recognised as a highly specialised heat-producing organ in mammals of small body mass, including human newborns (Himms-Hagen, 1986; Nechad, 1986). Findings on the presence of brown-like adipocytes susceptible to regulation by cold in different depots of adipose tissue in human adults renewed interest in BAT biology and raised the possibility that chronical upregulation of its energy-dissipating activity could potentially be used as a strategy to combat obesity (Cypess et al., 2012, 2014; Jespersen et al., 2013; Nedergaard et al., 2007; van Marken Lichtenbelt et al., 2009).

The main structural units of BAT are brown adipocytes, characterised by a centrally positioned nucleus, multiple lipid droplets and numerous mitochondria bearing uncoupling protein 1 (UCP1) incorporated in the mitochondrial cristae membrane. The main physiological role of UCP1 is to mediate thermogenic processes occurring in brown adipocytes. When activated, UCP1 dissipates the proton motive force normally used to drive the synthesis of cellular ATP, thus enabling heat production in the process of nonshivering thermogenesis (Nicholls and Rial, 1999; Symonds, 2013).

Beside brown adipocytes, the most numerous cells in BAT are endothelial cells (brown adipocytes and endothelial cells together constitute almost 90% of the cell population), whereas the rest of the cells are mainly preadipocytes and interstitial cells that are able to differentiate into brown adipocytes (Bukowiecki et al., 1986; Goglia et al., 1992). The existence of a rich capillary network in the BAT allows efficient supply of brown adipocytes with substrates and oxygen, but also serves to remove produced heat and transport it throughout the body (Cannon and Nedergaard, 2004).

The main regulator of BAT thermogenesis is noradrenaline, released from the sympathetic nerve endings that richly innervate the tissue. Noradrenaline acts through α- and β-receptors, and activates different signalling pathways, ultimately leading to the production of heat (Bachman et al., 2002; Bartness et al., 2010; Seydoux and Girardier, 1978; Yeh et al., 1993).

A large body of literature has investigated structural aspects of BAT activation during cold-induced thermogenesis (CIT). Depending on the intensity and duration of stimulus, the response of BAT primarily implies induction of UCP1 synthesis (Klingenspor, 2003; Puigserver et al., 1996; Ricquier et al., 1984; Ricquier and Bouillaud, 1986; Suter, 1969), mitochondriogenesis – dominantly regulated through the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)-related pathway network (Jornayvaz and Shulman, 2010; Puigserver et al., 1998) – and increased multilocularity and lipid turnover in brown adipocytes (Petrović et al., 2008; Rabi et al., 1977; Suter, 1969), followed by recruitment of new brown adipocytes from interstitial cells and preadipocytes (Cannon and Nedergaard, 2004; Klingenspor, 2003; Ricquier et al., 1984). Enhanced innervation, blood flow and angiogenesis go in line with these changes (Gélöen et al., 1992; Klingenspor, 2003; Korac et al., 2008a).

Contrary to the well-documented importance of BAT for CIT, its role in diet-induced thermogenesis (DIT), the form of heat production caused by voluntary overeating, is still under debate (Cannon and Nedergaard, 2011; Kozak, 2010; Rothwell and Stock, 1997). However, findings from several classical experiments postulated that a metabolic activation of BAT can be achieved by high-calorie diets, in turn preventing the development of obesity

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(Rothwell and Stock, 1979; Rothwell et al., 1983; Stirling and Stock, 1968). So far, the involvement of BAT in DIT was demonstrated not only in rodents but also in humans (Camps et al., 2013; McCracken and McAllister, 1984; Rothwell and Stock, 1997; Symonds, 2013; Westerterp et al., 1999). A considerable amount of literature highlighted similarities between mechanisms controlling CIT and DIT. Thus, it has been shown that a standard chow diet supplemented with sucrose solution causes voluntary hyperphagia and evokes BAT thermogenesis through a sympathetic nervous system (SNS) pathway (Holt et al., 1983; Saito et al., 1989; Walgren et al., 1987). Furthermore, some studies reported that a short-term sucrose overfeeding activates both sympathetic and sympathoadrenal systems (Davidovic et al., 1990; Holt et al., 1983).

Thus far, the majority of experimental models used to investigate the position of BAT in DIT were based on metabolic research, whereas a structural approach was surprisingly neglected. By contrast, structural remodelling of BAT related to its functional status has been investigated mainly in models of CIT (Bukowiecki et al., 1986; Cinti et al., 2002; Korac et al., 2008b) as well as in the peri- and postnatal period (Loncar, 1991; Symonds, 2013). For this reason, our current research was undertaken in order to investigate the effects of long-term sucrose overfeeding on structural characteristics of BAT. The remodelling of BAT was studied using light and transmission electron microscopy, in combination with immunohistochemistry and stereology. A pattern of UCP1 and PGC1α immunoexpressions served for estimation of the thermogenic potential of brown adipocytes, whereas immunodetection of noradrenaline allowed us to establish a correlation between morphological changes observed in BAT and noradrenergic stimulatory pathways. The findings of this investigation complement those of earlier studies, and strongly corroborate the idea that BAT is actively engaged in DIT.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Ethical Committee for the Treatment of Experimental Animals of the Faculty of Biology, University of Belgrade. Sixteen male Wistar rats, 8 weeks old, weighing 220–250 g at the beginning of the experiment were used. The animals were caged individually under standard laboratory conditions (21±1°C, 12 h:12 h light:dark cycles) and had free access to commercial rat food (Subotica, Serbia). Rats were randomly allocated into control or experimental group. The animals from experimental group (n=8) were provided with a 10% sucrose solution to drink *ad libitum* over 3 weeks, whereas the control animals (n=8) drank tap water. The overall energy intake was calculated on the basis of energy value of ingested food (11 kJ g^{-1}) and sucrose (16.7 kJ g^{-1}). The energy cost of body mass gain was calculated as body mass gain in grams per kJ of energy intake (Rothwell and Stock, 1997).

At the end of the experiment, the animals were weighed and killed by decapitation. The interscapular BAT was dissected, weighed and processed for microscopy.

Histological and ultrastructural analyses

A portion of each BAT sample was cut into small pieces, fixed in 2.5% glutaraldehyde in a 0.1 mol l^{-1} phosphate buffer (pH 7.2), post-fixed in 2% osmium tetroxide in the same buffer, dehydrated through a series of alcohol solutions of increasing concentration and embedded in Araldite (Fluka, Seelze, Germany). Tissue blocks were cut with diamond knives (Diatome, Biel, Switzerland) on a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany).

For the histological examination, semi-thin sections (2 µm) were mounted on glass slides, stained with 0.1% Toluidine Blue in 1% sodium borate buffer and examined with a Leica DMLB microscope (Leica Microsystems).

For the ultrastructural examination, ultrathin sections (70 nm) were mounted on grids, contrasted with standard uranyl acetate and lead citrate solutions using the Leica EM STAIN (Leica Microsystems), and examined with the Philips CM12 transmission electron microscope (Philips/FEI, Eindhoven, The Netherlands) equipped with the digital camera SIS MegaView III (Olympus Soft Imaging Solutions, Münster, Germany).

Morphometric and stereological analysis

The volume densities (V_V) of the major BAT components (brown adipocytes, capillaries and interstitium) were obtained from light micrographs using a standard point-counting technique (Weibel et al., 1966) and the ImageJ analysis system (http://imagej.nih.gov/ij/download.html). These stereological values were determined using Eqn 1:

$$V_V = \frac{P_x}{P_{total}} \quad (1)$$

(Weibel et al., 1966), where P_x is the number of points hitting the brown adipocytes, capillaries or interstitium, and P_{total} is the number of total points hitting BAT. The volume densities were expressed as percentage fractions of BAT volume. For this analysis, 50 randomly selected micrographs per group, at a final magnification of 1000×, were used.

The same micrographs were used to evaluate mean volume of brown adipocytes as well. For this purpose, the numerical density of brown adipocytes per unit area (N_{ABA}) and numerical density of brown adipocytes per unit volume (N_{VBA}) were determined. N_{ABA} (N per mm^2) was estimated using an unbiased 100×100 µm counting frame (Gundersen, 1978; Howard and Reed, 2005; Medigović et al., 2012). The counting frame was superimposed on the light micrographs at a final magnification of 1000× and the number of profiles lying at least partially within the frame, without being intersected by the arbitrarily defined exclusion lines (upper and right borderline of the counting frame) was determined. Further, the obtained value (N_{ABA}) was used to calculate the numerical density of brown adipocytes per unit volume (N_{VBA}: N per mm^3) for each micrograph, according to Eqn 2 (Weibel and Gomez, 1962):

$$N_{VBA} = {N_{ABA}}^{3/2} / {V_{VBA}}^{1/2}. \quad (2)$$

Finally, the mean volume of brown adipocytes was calculated as a ratio of V_{VBA} to N_{VBA} for each micrograph, and subsequently for both the control and experimental groups (Aherne and Dunhill, 1982; Goglia et al., 1992; Medigović et al., 2012).

Thirty electron micrographs per group at an original magnification of 2650× were used for general ultrastructural as well as morphometric and stereological analyses of mitochondria and lipid droplets. Regarding the lipid droplets, their V_V, number per cell profile and distribution based on their size were calculated. The same micrographs were used to determine V_V and number of mitochondrial profiles per unit area (N per 100 $µm^2$).

Using the same procedure, mitochondrial profile area and volume density of mitochondrial cristae were determined from 200 randomly selected mitochondria per group, at a magnification of 19,500×. The volume density of cristae was defined as total points hitting cristae divided by the total points hitting the mitochondria.

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The same electron micrographs were used to determine the number of cristae per mitochondrion.

Immunohistochemistry

A portion of the BAT was fixed in 10% buffered formaldehyde at 4°C overnight and routinely processed for embedding in paraffin. BAT sections of 5 µm thickness were deparaffinised and rehydrated, subsequently incubated in citrate buffer for 10 min at 100°C to retrieve antigens, and washed in phosphate buffered saline (PBS; pH 7.4). After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol and 3 sequential washings in PBS, the sections were incubated with rabbit polyclonal anti-UCP1 (1:1000; ab10983; Abcam, Cambridge, UK), anti-PGC1α (1:300; ab54481; Abcam) or anti-noradrenaline (1:1000; ab8887; Abcam) antibody overnight, at 4°C. For UCP1 and noradrenaline, immunodetection was performed using the Abcam Specific HRP/DAB (ABC) Detection Kit (ab64261; Abcam). The final reaction product was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich, Münich, Germany). After counterstaining with hematoxylin, slides were mounted and examined with the light microscope (Leica Microsystems). PGC1α immunodetection was performed using appropriate fluorophore-conjugated secondary antibody for 60 min (goat anti-rabbit IgG Alexa Fluor 488; A11008; ThermoFisher Scientific, Paisley, UK). After extensive washing in PBS, samples were mounted with Vectashield medium containing DAPI (Vector Laboratories, Peterborough, UK) and examined with a Nikon Eclipse 90i microscope using Velocity 5 software (http://support.identiv.com/velocity-3-5) (Improvision, Coventry, UK).

For semi-quantification of UCP1 and noradrenaline immunohistochemical stainings, 12 fields per group were randomly selected at 40× magnifications and analysed by ImageJ software. Data for UCP1 are presented as the reciprocal intensity (Nguyen et al., 2013) and data for noradrenaline expression are presented as positive area (Velickovic et al., 2014).

Statistics

Data values were checked for normal distribution and homogeneity of variances, and statistical analyses were performed by two-tailed Student's *t*-test using GraphPad Prism software (http://www.graphpad.com/scientific-software/prism). Data were reported as means±s.e.m. Statistical significance was set at $P<0.05$.

RESULTS

The results obtained from the analysis of the effects of sucrose overfeeding on energy balance, body mass gain, and absolute and relative BAT mass are presented in Table 1. Sucrose overfeeding significantly increased total energy intake in comparison to the control group, as can be seen from Table 1. Rats from the experimental group had a lower energy cost of body mass gain than the control animals. No significant differences were found in body mass gain or absolute and relative BAT mass.

Histological analysis showed that BAT from control animals (Fig. 1A) had a typical appearance, consisting predominantly of roundish adipocytes filled with rather large, uniformly sized lipid droplets. Between brown adipocytes, capillaries were visible. After 3 weeks of sucrose overfeeding (Fig. 1B), brown adipocytes became apparently smaller and some of them acquired a slightly elongated form. The multilocularity was far more pronounced and the size of lipid droplets was visibly reduced. The interstitium appeared enlarged and the widened capillaries usually contained erythrocytes.

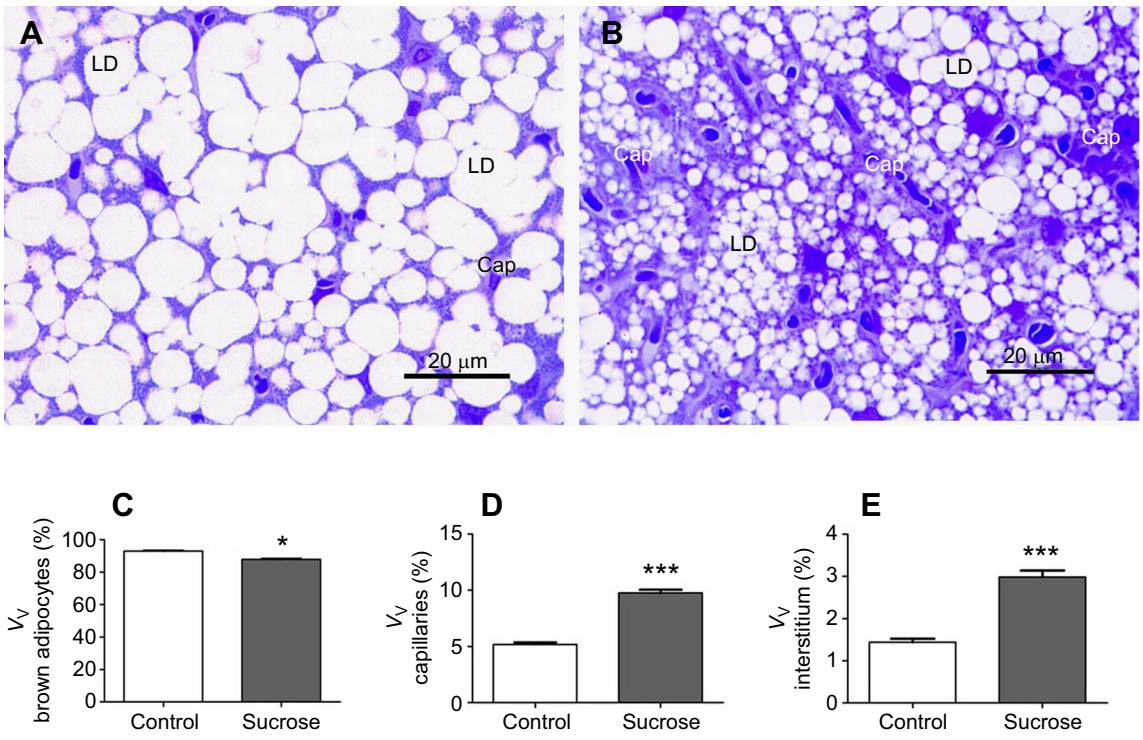

Fig. 1. Effects of sucrose overfeeding on histological and major stereological parameters of brown adipose tissue (BAT). In comparison with the control group (A), BAT of the sucrose overfed group (B) showed a reduction in brown adipocyte size accompanied by more pronounced multilocularity and a reduction of lipid droplet (LD) size. Interstitium and capillaries (Cap) were enlarged. The results of stereological analysis confirmed these observations: volume density (V_V) of brown adipocytes was decreased (C), whereas volume density of the capillary network (D) and interstitium (E) were increased. *$P<0.05$; ***$P<0.001$. All data were subjected to two-tailed Student's *t*-test. Values are presented as means±s.e.m. ($n=8$).

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Table 1. Effects of sucrose overfeeding on energy balance, body mass gain, and absolute and relative brown adipose tissue (BAT) mass

	Control	Sucrose	Statistics
Total energy intake (kJ)	6557±142.1	7734±339.4	$P<0.05$
Body mass gain (g)	135.2±1.78	144.2±9.03	n.s.
Absolute BAT mass (g)	0.362±0.0507	0.326±0.0525	n.s.
Relative BAT mass (g 100 g^{-1} body mass)	0.102±0.0133	0.090±0.0113	n.s.
Energy cost of body mass gain (g kJ^{-1})	0.021±0.0005	0.019±0.0006	$P<0.05$

Table 2. The effects of sucrose treatment on volume and numerical density of brown adipocytes

	Control	Sucrose	Statistics
Brown adipocyte numerical density ($N \times 10^3$ per mm^3)	68±5.9	99±8.3	$P<0.001$
Brown adipocytes mean volume (μm^3)	14,583±507	8972±139.5	$P<0.001$

Quantitative analysis of the main tissue components revealed that the volume density of brown adipocytes was decreased in sucrose-treated animals, whereas those of the capillaries and interstitium were increased (Fig. 1C–E).

As can be seen from Table 2, numerical density of brown adipocytes per unit area in sucrose-treated rats was significantly increased (by 46%) in comparison to the control group. In accordance with increased brown adipocyte numerical density, their volume was decreased (by 38.5%).

Electron microscopical analysis showed that the sucrose ingestion affected the fine internal structure of the brown adipocytes (Fig. 2A,B). Mitochondria seemed to be more closely packed within the cytoplasm and established close contacts with the lipid droplet surface. Further quantitative analysis showed a significant decrease in lipid droplet volume density (Fig. 2C), whereas the distribution of brown adipocytes, based on the number of lipid droplets within the cell, was shifted toward higher values compared with the control group (Fig. 2D).

Fine mitochondrial ultrastructure was further characterised using transmission electron microscopy (Fig. 3A,B) and stereological analysis (Fig. 3C–G), accompanied by PGC1α immunoexpression (Fig. 3H,I). In contrast to the control group, mitochondria from the sucrose-treated group appeared to be more abundant and larger, with a well-ordered cristae system. Indeed, quantitative methods revealed that mitochondrial volume density was significantly increased, as well as the mean mitochondrial profile area and the number of mitochondria per 100 μm^2 of cell profile. The volume density of mitochondrial cristae was increased, as well as the number of mitochondria containing 6 or more cristae per profile. PGC1α immunofluorescent staining revealed a

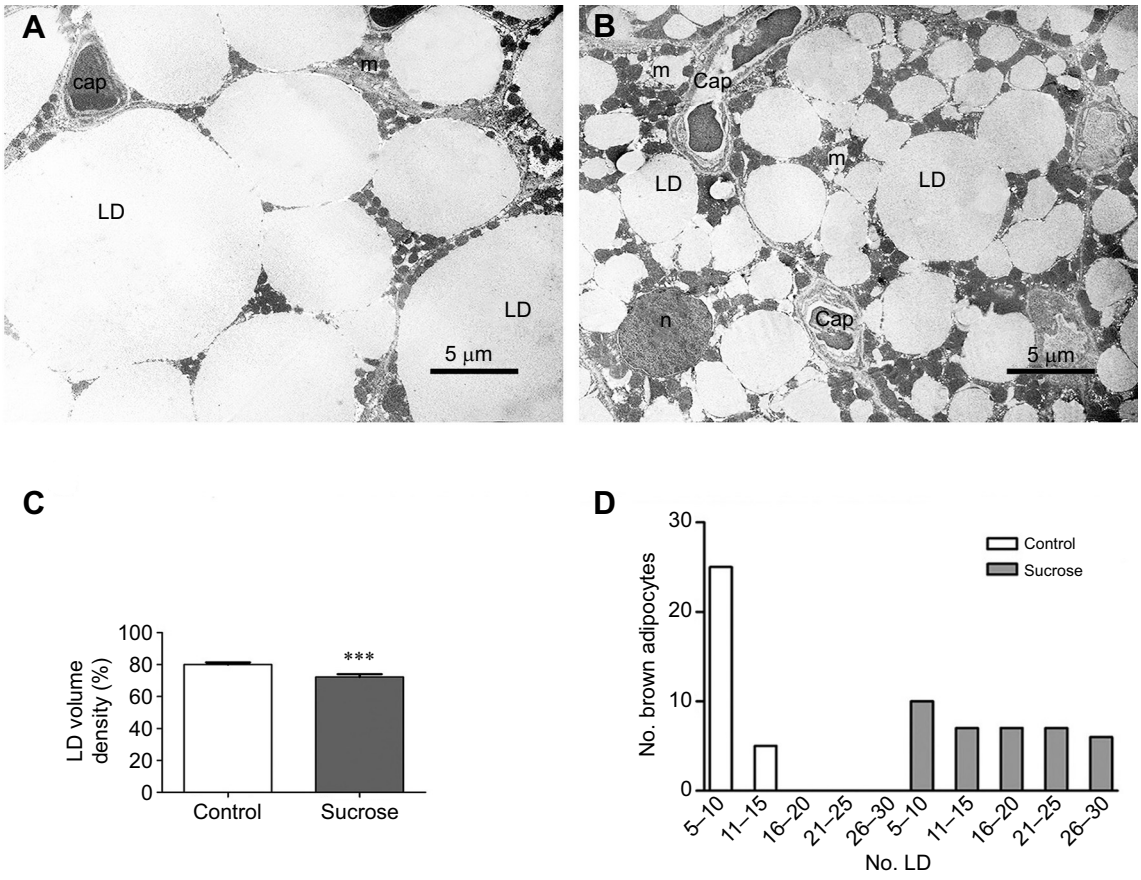

Fig. 2. Effects of sucrose overfeeding on the ultrastructure of brown adipocytes and morphometric–stereological parameters of lipid droplets. Brown adipocytes from the control animals were characterised by several large lipid droplets (LD; A), in contrast to the sucrose overfed group (B), where numerous small lipid droplets were noticed. In the sucrose overfed group, the volume density of lipid droplets was decreased (C). The histogram showing the number of brown adipocytes containing a defined number of lipid droplets (D) demonstrates a shift to a higher number of lipid droplets per cell after sucrose ingestion. ***$P<0.001$. All data were subjected to two-tailed Student's *t*-test. Values are presented as means±s.e.m. ($n=8$). LD, lipid droplet; Cap., capillaries; m, mitochondria; n, nucleus.

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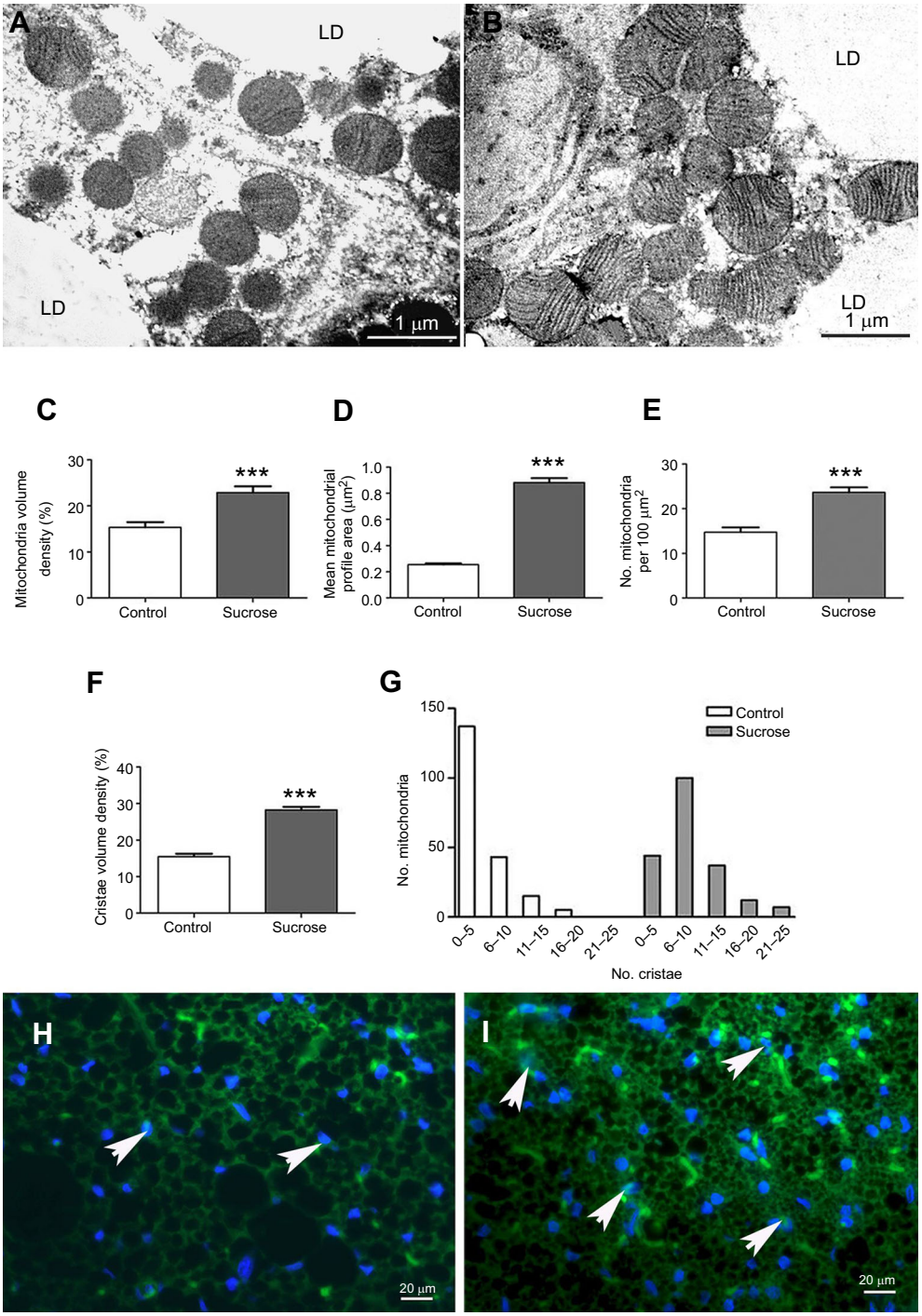

Fig. 3. Effects of sucrose overfeeding on the ultrastructure and morphometric–stereological parameters of mitochondria and PGC1α immunoexpression. Representative micrographs showing mitochondrial ultrastructure in the control (A) and sucrose overfed (B) groups. The sucrose treatment increased volume density of mitochondria (C), mean area of mitochondrial profile (D), number of mitochondria per 100 μm^2 of cell profile (E) and the volume density of cristae (F). The distribution histogram of number of mitochondria with designated number of cristae demonstrates a shift to higher values after sucrose treatment (G). The PGC1α immunopositivity was more intense in the sucrose overfed group (I) compared with the control group (H). Nuclei (blue) of brown adipocytes positive for PGC1α (green) are indicated by white arrows. ***P<0.001. All data were subjected to two-tailed Student's t-test. Values are presented as means±s.e.m. (n=8). LD, lipid droplet.

mainly cytoplasmic reaction in the control group, whereas, in sucrose-treated rats, a positive reaction was detected in the cytoplasm and in some nuclei.

Immunohistochemical detection of UCP1 was used to assess thermogenic capacity of brown adipocyte mitochondria (Fig. 4A, B). UCP1 immunopositivity was strongly enhanced in the sucrose-treated group in comparison to the control. The reaction was not uniform in the whole section. Namely, intensely stained brown adipocytes among less stained or completely negative ones were noticed, which is the pattern known as a 'harlequin effect' (Cinti et al., 2002). Semi-quantification of UCP1 staining (Fig. 4C) additionally supported our microscopic observation, showing a significant difference between the control and sucrose-treated groups.

In order to explore whether the SNS was stimulated after long-term sucrose consumption, immunohistochemistry for noradrenaline was performed (Fig. 5). Clearly visible noradrenaline-containing nerves were present in BAT of both the control and experimental group of animals. In the control group (Fig. 5A), a weak immunopositive reaction was observed mainly at the level of blood vessel wall and connective tissue stroma. Only thin and short nerve fibres were seen among brown adipocytes, mostly those of smaller size. In the sucrose overfed rats, the general pattern of noradrenaline immunopositivity was similar to that in the control group, but noradrenaline-

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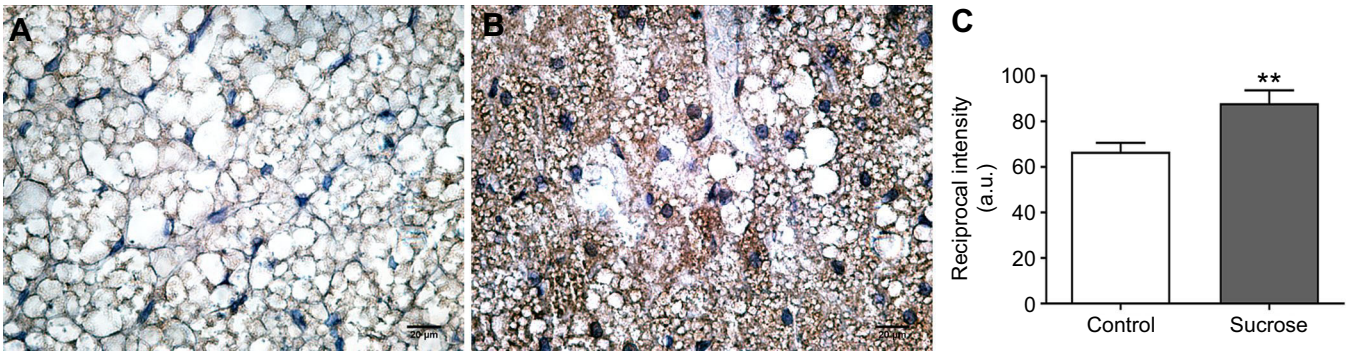

Fig. 4. Effects of sucrose overfeeding on UCP1 immunoexpression in BAT. UCP1 immunopositivity was more intense in the sucrose overfed group (B) compared with the control group (A). Data obtained after quantification of UCP1 reciprocal intensity by ImageJ software (***P*<0.01) (C). All data were subjected to two-tailed Student's *t*-test. Values are presented as means±s.e.m. (*n*=8). Scale bars: 20 μm.

containing nerve fibres were more abundant and more intensively stained, both around blood vessels and brown adipocytes (Fig. 5B). Semi-quantification of noradrenaline staining (Fig. 5C) confirmed visual observation.

DISCUSSION

Several pioneering studies performed years ago have shown that increased caloric intake caused by voluntary ingestion of highly palatable food rich in carbohydrates and/or fat may lead to thermogenic activation of BAT (Arbuthnott, 1989; Himms-Hagen, 1984; Mercer and Trayhurn, 1987; Rothwell and Stock, 1979; Saito et al., 1989). Despite some controversies that emerged afterwards, today it is widely accepted that DIT depends on the thermogenic capacity of BAT (Cannon and Nedergaard, 2010).

The animal model systems designed to study the involvement of BAT in DIT usually use the so-called 'cafeteria' diet originally introduced by Sclafani and Springer (1976). In order to investigate changes in structural, immunohistochemical and stereological characteristics of BAT caused by voluntary hyperphagia, we used the simplest form of the 'cafeteria' diet, offering rats a 10% sucrose solution to drink instead of water. The applied dietary regimen resulted in significantly increased energy intake, primarily on the basis of ingested sucrose solution, since intake of solid food was diminished (data not shown). However, the results of this study did not show any significant increase in body mass gain compared with controls, meaning that sucrose overfed rats had a comparatively decreased energy cost of body mass gain because they had increased energy expenditure (Rothwell and Stock, 1997). These findings were in line with several human and animal studies published previously, according to which excessive sucrose consumption did not cause an increase in body mass gain despite increased total energy intake (Anderson and Woodend, 2003; Martinez et al., 2010; Ramirez, 1987; Ruzzin et al., 2005; Woodend and Anderson, 2001). The protection from obesity during excessive calorie intake may have resulted, at least in rodents, from the thermogenic activation of BAT. Thus, we proceeded with detailed morphological, immunohistochemical and stereological analysis of BAT, searching for further evidence that would support or reject its involvement in DIT.

Based on similarities between mechanisms controlling CIT and DIT (Stock and Rothwell, 1986), we expected that our treatment would lead to an increase in absolute and relative BAT mass due to tissue hyperplasia (Bukowiecki et al., 1986; Petrović et al., 2005). However, this study did not show changes in BAT absolute and relative mass. Taking into account earlier reports published by others and the experimental models used therein, we assume that sucrose concentration and/or duration of the treatment were insufficient to provoke statistically recognizable effects on BAT mass. For example, Bukowiecki et al. (1983) have shown that a similar concentration of sucrose (12%) to that used in the present study (10%) doubled BAT mass after 9 weeks of treatment, whereas 32% sucrose concentration was needed for a 1.5-fold increase in BAT mass after 4 weeks of treatment (Kuroshima et al., 1995). By contrast, we found that significant changes in the relative proportion

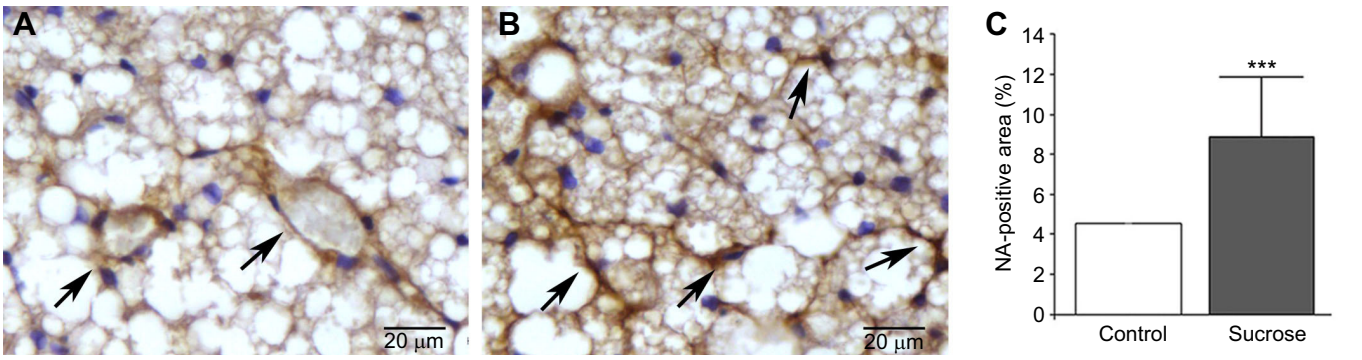

Fig. 5. Effects of sucrose overfeeding on noradrenaline immunoexpression in BAT. In the control group, moderate parenchymal noradrenergic innervation was evident among brown adipocytes (A). After sucrose consumption, the density of parenchymal noradrenergic nerves was greater, and staining was stronger (B). Noradrenergic nerves in connective tissue stroma, around blood vessels and brown adipocytes are indicated by black arrows. Data obtained after quantification of noradrenaline (NA)-positive area by ImageJ software, ****P*<0.001 (C). All data were subjected to two-tailed Student's *t*-test. Values are presented as means±s.e.m. (*n*=8).

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of main BAT structural components were shifted toward increased interstitial and vascular components, whereas the relative contribution of brown adipocytes was diminished, pointing to tissue remodelling within the existing tissue volume. This result can be explained by the fact that sucrose treatment selectively affected different tissue components, stimulating vascularisation rather than an increase in cellularity. Increased vascularisation could be the first line of tissue adaptation for facilitating heat transfer into the blood stream, as necessary under conditions of increased energy release. Considering the increase in relative abundance of interstitium, apart from carrying blood vessels, we cannot exclude the possibility of intensified proliferation and differentiation of adipocyte precursor cells localized therein, although earlier papers reported that sucrose solution concentration as high as 32% was necessary to promote BAT hyperplasia (Westerterp et al., 1999). Even if we assume that some hyperplasia took place in the sucrose-treated group, newly formed brown adipocytes did not affect BAT mass, due to their small size.

Our stereological analysis revealed that volume density of brown adipocytes was significantly decreased after sucrose treatment. In order to identify the reason for this, we calculated brown adipocyte numerical density per unit area and mean volume. The results clearly showed that numerical density of brown adipocytes in the sucrose-treated group was increased, at least in part, due to a decrease in their volume (hypotrophy). We also showed that the volume of brown adipocytes was reduced mostly on account of lipid depletion, with the remaining lipids being redistributed within the brown adipocytes so that there were more numerous lipid droplets. Amplified lipid droplets create a higher surface-to-volume ratio that is more convenient for lipolysis and mitochondrial uptake of fatty acids. These profound changes emphasised the dynamic nature of lipid droplets, which is primarily based on lipid-droplet-associated proteins (Yu et al., 2015). In addition, we have shown that, after 3 weeks of sucrose consumption, the number of lipid droplets per brown adipocyte profile was increased, as demonstrated by the higher number of brown adipocytes containing 15 or more lipid droplets. It is well known that thermogenesis cannot be evoked without simultaneously evoking lipolysis (Klingenspor, 2003; Rabi et al., 1977; Seydoux and Girardier, 1978; Suter, 1969). As a result of this activation, fatty acids are released, serving not only as the main substrate for mitochondrial oxidation but also for providing the signal to uncouple mitochondria in BAT. More intimate contacts established between mitochondria and lipid droplet surface noted in this study support ‘fuelling’ of mitochondria by fatty acids.

Chronic ingestion of 10% sucrose solution strongly affected the mitochondria within brown adipocytes, as manifested by a remarkable induction of UCP1 immunopositivity together with an increase in all mitochondrial parameters related to their enhanced thermogenic capacity. These findings are in agreement with those of previous studies reporting that an immediate BAT response on physiological stimulation relies on pre-existed thermogenic machinery, which is, after prolonged stimulation, followed by mitochondriogenesis accompanied by UCP1 synthesis (Cannon and Nedergaard, 2004; Trayhurn et al., 1987).

In terms of activated thermogenesis, the results regarding mitochondrial dynamics are very important. It is well known that the prompt response of brown adipocytes to a thermogenic stimulus is due to the activation of UCP1 already embedded in the inner mitochondrial membrane, followed by the synthesis of new amounts of UCP1, enlargement of mitochondria, formation of the new cristae and finally an increase in mitochondrial number (Golic et al., 2014; Jacobsson et al., 1994; Klingenspor, 2003; Petrović et al., 2008; Trayhurn et al., 1987). Thus, one of the objectives of this study was to analyse mitochondrial architecture and dynamics, and to interpret them in view of possible alterations in mitochondrial functional capacity/activity. In our study, sucrose overfeeding was found to enhance UCP1 immunoreactivity in comparison to the control group. As far as we know, this is the first evidence from an *in vivo* histomorphological study in support of upregulation of UCP1 expression by sucrose overfeeding. In addition, all quantitative data obtained from the analysis of the mitochondrial population (volume density, mean profile area, number per 100 μm^2 of cell area, cristae volume density and their mean number per mitochondrion) suggest functional activation of mitochondria in the sucrose overfed group. Observed changes in brown adipocyte mitochondria may be viewed as a part of their adaptation to high nutrient availability and caloric content, in which the highly calorific oxidative substrate itself controls thermogenesis and increases its own oxidation (Liesa and Shirihai, 2013).

It is well known that UCP1 expression is driven by several transcriptional mediators, including the coactivator PGC1α, a master regulator of mitochondrial biogenesis (Barbera et al., 2001). Additionally, PGC1α regulates oxidative metabolism and is strongly induced by cold exposure and/or β-adrenergic signalling (Puigserver et al., 1998). The pattern of PGC1α immunoexpression, increased mitochondriogenesis and UCP1 expression in the sucrose-treated rats in this study, together with the previous finding that PGC1α protein, along with many other regulatory factors, has an extremely short half-life (Adamovich et al., 2013), point to the importance of PGC1α for the early phases of BAT activation in DIT.

Because brown adipocytes receive direct sympathetic innervation (Bachman et al., 2002; Bamshad et al., 1999; Korac et al., 2008a), the demonstration of noradrenergic nerves within BAT is very important with respect to thermogenesis. A stimulatory effect of sucrose feeding on the activity of the SNS was recognised a long time ago (Young and Landsberg, 1977). Moreover, Davidovic et al. (1990) have shown that short-term sucrose overfeeding resulted in the activation of the sympathoadrenal system and enhanced catecholamine release in rats. Although interplay between dietary carbohydrates and SNS activity was already recognised, to the best of our knowledge, expression of noradrenaline in sucrose-treated rats was not investigated so far. The current study showed enhanced noradrenaline immunopositivity in sucrose overfed rats, not only around brown adipocytes but also around blood vessels. Observed close associations of nerves and blood vessels may be important for the transport of chemical messengers (Lever et al., 1986), but also for dilatation of blood vessels, which was observed in BAT of sucrose overfed rats. Strong noradrenaline immunopositivity in sucrose overfed rats was correlated with enhanced UCP1 immunoexpression and suggests positive regulation of BAT thermogenic capacity by the SNS.

Although results presented herein confirmed previously proposed similarities between CIT and DIT, our findings may be somewhat limited because they cannot elucidate either specific signal transduction pathways or type of receptors involved in signal transduction. This issue is important especially in light of data indicating that the β_1 signalling pathway mediates most of the SNS stimulation of DIT (Ueta et al., 2012), in contrast to β_3 signalling in CIT (Cannon and Nedergaard, 2004). Further studies will be required to clarify the mechanisms underlying this sympathetic response. Namely, Young et al. (2004) proposed that SNS stimulation by fructose probably originates from peripheral chemoreceptors located in the gastrointestinal tract and suggested

an important role of insulin in this process. The latter is not unexpected given that insulin could activate BAT depending on applied dose and duration of treatment (Markelic et al., 2011; Porras et al., 2003). It also should be noted that rats in our study, as in most other studies, were housed at room temperature (21±1°C). Given that the temperature of thermoneutrality for rat is 28°C, animals in the present study were under chronic thermal stress, which affected the 'basal' capacity and activity of BAT (Feldmann et al., 2009; Himms-Hagen, 1986; Silva, 2003). For this reason, an experiment at thermoneutral conditions should be carried out to better visualize the net effects of the applied treatment.

To summarise, the results of the presented study showed that, at room temperature, the BAT of long-term sucrose overfed rats retained the ability to protect the body from excessive body mass gain. Data obtained using morphological, stereological and immunohistochemical methods all support the activation of BAT. The findings derived from this study are relevant in view of the presence of functionally responsive BAT in adult humans (Hibi et al., 2016; Symonds et al., 2012). Further investigations will need to be undertaken to shed more light on the molecular mechanisms regulating DIT in thermogenic adipocytes before extrapolation to humans will be possible.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.D.V., M.M.U., M.M.C.M.; Methodology: K.D.V., M.M.U., M.M.C.M.; Validation: K.D.V., M.M.U., R.M.G., M.M.C.M.; Formal analysis: K.D.V., R.M.G.; Investigation: K.D.V., M.M.U., M.M.C.M.; Resources: M.M.U., M.M.C.M.; Data curation: K.D.V.; Writing - original draft: K.D.V., M.M.C.M.; Writing - review & editing: K.D.V., M.M.U., R.M.G., M.M.C.M.; Visualization: K.D.V., M.M.C.M.; Supervision: K.D.V., M.M.C.M.

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