Chronic heat stress up-regulates leptin and adiponectin secretion and expression and improves leptin, adiponectin and insulin sensitivity in mice

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Abstract

Heat stress (HS) induces adaptive responses that are responsible for alterations of carbohydrate and lipid metabolism. This study aimed to evaluate the effects of chronic heat treatment on the expression and secretion of leptin and adiponectin, important regulators of energy homeostasis, food intake and insulin action. C57BL/6 mice were subdivided into three groups (24 mice each). The first group was kept under control conditions (C: 22 ± 2 °C). The second group was exposed to HS (35 ± 1 °C). The third group was kept under control conditions and was food restricted (FR). The HS group had higher rectal temperature than the C and FR groups and lower food intake than the C group. *Hspa1* (*Hspa1a*) gene expression in adipose tissue, muscle and liver was higher under HS than FR and C. Heat treatment resulted in decreased blood glucose and non-esterified fatty acids; increased leptin, adiponectin and insulin secretion; and greater glucose disposal. Leptin, adiponectin, leptin and adiponectin receptors, insulin receptor substrate-1 and glucose transporter mRNAs were up-regulated in HS mice. This study provides evidence that HS improves leptin and adiponectin signalling in adipose tissue, muscle and liver. Heat stress was responsible for improving insulin sensitivity and glucose uptake in peripheral tissues, probably mediated by adipokines. Changes in the adipokine levels and sensitivity to them may be considered as an adaptive response to heat.

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Introduction

Under a hot environment, the body's thermoregulatory system adjusts a variety of physiological mechanisms through a combination of dry heat exchange and evaporative heat loss to attain a balance between the heat produced within the body and the heat lost to the environment. Profound alterations at the cellular level also occur upon heat stress (HS).

These alterations encompass changes in gene expression and biochemical adaptive responses, characterised by the impairment of major cellular functions and reprogramming of cellular metabolism (Lindquist 1986).

In contrast to acute HS, the effects of chronic HS on gene expression and cellular metabolism have been studied only to a limited extent (Zhang *et al.* 2002, Bhusari *et al.* 2008). Acute and chronic HS pose two distinct challenges to animals. Acute HS (animals exposed to 40–42 °C for up to 24 h) results in hyperthermia and can lead to death if subjects are exposed to it for an excessive length of time. In contrast, chronic HS (33–35 °C) can be tolerated for a longer period, and although it alters animal physiology (Gordon 1993, Horowitz 2002), it is non-lethal.

HS rodents exhibit increased basal insulin levels, greater insulin sensitivity and glucose uptake and lower plasma glucose (Torlinska *et al.* 1987, Gupte *et al.* 2009, 2011). These results agree with the findings in dairy cows that chronic HS leads to hypoglycaemia and increases basal insulin levels despite marked reductions in nutrient intake (Wheelock *et al.* 2010).

An increase in triglyceride content is observed in the liver of young rats (Ando *et al.* 1998) and dairy cows (Basiricò *et al.* 2011) exposed to a hot environment. In addition, decreased plasma cholesterol, phospholipid and non-esterified fatty acid (NEFA) contents are found in HS dairy cows (Sano *et al.* 1983, Ronchi *et al.* 1999). Ronchi *et al.* (1999) concluded that the alterations of carbohydrate and lipid metabolism in HS cattle are due to the high ambient temperature rather than indirect factors, such as the reduction of feed intake.

Two adipokines, leptin and adiponectin, are metabolically relevant in coordinating energy homeostasis (Ailhaud 2006). Leptin affects energy homeostasis by decreasing food intake and by up-regulating fatty acid oxidation and down-regulating lipogenesis in peripheral tissues (Rabe *et al.* 2008). Furthermore, leptin increases insulin sensitivity. Adiponectin regulates

feeding behaviour through peripheral and central mechanisms and serves as a 'starvation' signal (Hoyda *et al.* 2011). Studies conducted on rodents demonstrate that adiponectin increases whole-body insulin sensitivity, decreases hepatic glucose production and increases fat oxidation in liver and muscle, leading to an overall decrease in fat deposition (Ailhaud 2006, Lafontan & Viguerie 2006). In addition to its insulin-sensitising effect, adiponectin may modify glucose metabolism by stimulating pancreatic insulin secretion (Rabe *et al.* 2008).

Few data are available on the interactions between HS and adipokine expression and secretion. To our knowledge, only such studies concern the effects of HS on leptin gene expression and secretion in birds (Al-Azraqi 2008, Dridi *et al.* 2008) and in lactating pigs (Renaudeau *et al.* 2003) and on leptin and adiponectin gene expression and secretion in mouse adipocytes (Bernabucci *et al.* 2009).

On the basis of our previous results (Bernabucci *et al.* 2009), we conducted a study to evaluate whether chronic HS rather than a reduction in voluntary food intake is associated with changes in the expression and secretion of leptin and adiponectin and in insulin sensitivity and glucose metabolism in peripheral tissues.

Materials and methods

Animals and experimental design

All animal procedures were reviewed by the Animal Care Ethics Committee of the University of Tuscia, Viterbo, Italy, approved by Italian Government Authorities, and were in accordance with European Guidelines. Seventy-two male C57BL/6 mice (Charles River Laboratories, Milan, Italy), 7 weeks old, were used. After an adaptation period of 14 days under control conditions ($22 \pm 2^\circ\text{C}$; photoperiod: 12 h light:12 h darkness) and with *ad libitum* access to a standard chow diet (VRF1 diet, Charles River Laboratories), the animals were subdivided into three groups of 24 mice each. One group was kept under control conditions and fed *ad libitum* (C group; negative control). The second group was exposed to high ambient temperature (HS group; $35 \pm 1^\circ\text{C}$; photoperiod: 12 h light:12 h darkness) for 5 days continuously and fed *ad libitum*. The third group was kept under control conditions and was food restricted (FR group; positive control) for 5 days. The level of food restriction corresponded to 80% of the dietary intake by control mice, which is a normal response of mice exposed to HS.

To avoid the influence of diurnal cycling, each experiment was started between 0800 and 0900 h. Food intake, water consumption, body weight (BW) and rectal temperature (RT) were recorded daily. On 0 and 5 days, six animals from each group were

used to perform a glucose tolerance test (GTT). At the beginning (day 0) and after 2 and 5 days of the experimental period, six animals from each group were killed via diethyl ether inhalation followed by cervical dislocation, and blood and tissue samples were collected immediately. Blood was collected into vials containing heparin and then centrifuged. The plasma was stored at -20°C until analyses. Subcutaneous white adipose tissue (WAT), gastrocnemius skeletal muscle (M) and liver (L) samples were placed in RNA-Later solution (Ambion, Inc.) and frozen at -80°C until total RNA isolation. Small intestine tissue samples were fixed in glutaraldehyde and paraformaldehyde for histological examination.

HS treatment

The HS protocol lasted for 5 days and was designed to induce moderate chronic stress, i.e. mice were continuously exposed to an ambient temperature of $35 \pm 1^\circ\text{C}$. Hyperthermia was induced via an infrared ceramic heat lamp (100 W; Ceramicx Ireland Ltd., Cork, Ireland). There were four mice per cage (Metabolic cage 3700M022, Tecniplast, Varese, Italy), and the lamps were positioned above each cage at a distance of 10 cm. The air temperature within the cage was checked every hour. The RT of the mice was measured every 2 h during the daytime (from 0800 to 1800 h) using a thermistor probe (Temp JKT; Oakton Instruments, Vernon Hills, IL, USA).

The use of an infrared heat lamp to induce hyperthermia is safe: does not emit any type of light at the wavelengths to which murine retinal cells are sensitive and provides the opportunity to maintain the body temperature at any desired level for some time (Sharma 2007).

Biochemical analysis

Plasma indices of metabolic status were determined via an automatic analyser (IL-300 Plus, Instrumentation Laboratory, Lexington, MA, USA). Plasma glucose (kit from Instrumentation Laboratory) and NEFAs (NEFA-C kit; Wako Fine Chemical Industries, Dallas, TX, USA) were measured by enzymatic colorimetric methods. Plasma leptin, adiponectin and insulin were measured using commercial ELISA kits. The assays were conducted in 96-well microplates according to the manufacturer's instructions, with a Spectra-I microplate spectrophotometer (SLT Lab-Instruments GmbH, Grodig/Salzburg, Austria). Mouse leptin (sensitivity: 0.05 ng/ml) and adiponectin (sensitivity: 0.5 ng/ml) ELISA kits (Millipore, Billerica, MA, USA) were used to determine their blood concentrations. Insulin was measured using a Mouse Insulin ELISA Kit (Millipore),

which had a sensitivity of 0.2 ng/ml. Inter- and intra-assay coefficients of variability (CV, %) were 3.85 and 1.48 for leptin, 5.97 and 5.75 for adiponectin and 9.07 and 2.81 for insulin respectively. All experiments were performed in duplicate.

Glucose tolerance test

GTTs were performed on six animals per group that had been fasted overnight for 16 h. Animals were injected with 1.5 g/kg BW of glucose into the peritoneal cavity. Glucose (OneTouch Ultra Easy, Lifescan, Milan, Italy) was measured in blood collected from the tail tip immediately before and at 30, 60, 90 and 120 min after the injection. After correcting for the levels at time zero (defined as baseline level), the glucose response to the GTT was calculated as the area under the curve (AUC) using the linear trapezoidal formula. The glucose AUC was reported from 0 to 120 min.

Real-time PCR

Total RNA was isolated from the subcutaneous WAT, M and L using QIAzol Lysis Reagent (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using an ImProm-II RT System (Promega) in a total volume of 20 µl on a PCR Express thermal cycler (Hybaid, Ashford, UK). Controls without RT were performed to exclude the possibility of DNA contamination. Quantitative real-time PCR was performed following the manufacturer's recommendations using a LightCycler (Roche Applied Science) and a QuantiTect primer assay for mouse (Qiagen): leptin (QT00164360), adiponectin (QT01048047), leptin receptor (*ObRb*; QT00154133), adiponectin receptor 1 (*AdipoR1*; QT00154217), *AdipoR2* (QT00165326), insulin receptor substrate 1 (*Irs1*; QT01540840), glucose transporter type 4 (*Glut4* (*Slc2a4*); QT01538334), *Glut2* (*Slc2a2*; QT00103537) and heat-shock protein-A1 (*Hspa1* (*Hspa1a*); QT00254436). The gene expression levels were normalised using β -actin (*Actb*; QT01136772) mRNA as an internal control. PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2% agarose/Tris-borate-EDTA gel electrophoresis to confirm amplification specificity and amplicon size. To allow relative quantification after PCR, standard curves were constructed from the standard reactions for each target and *Actb* by plotting crossing point (Cp) values, i.e. the cycle number at which the fluorescence signal exceeded background vs log cDNA dilution. The Cp reading for each of the unknown samples was then used to calculate the amount of either the target or the *Actb* relative to the standard using the Second Derivative Maximum

Method with the LightCycler Analysis Software 3.5 (Roche Applied Science). All reactions were performed in duplicate.

Histological analysis

At the end of the 5-day period, small intestine tissue samples were obtained for morphological assessment of epithelial damage. The tissue was fixed in 2.5% paraformaldehyde + 2.5% glutaraldehyde in cacodylate sucrose buffer (0.1 M cacodylate, 0.1 M sucrose, 5 mM CaCl_2 and 5 mM MgCl_2 , pH 7.2) overnight at 4 °C, rinsed in cacodylate buffer at pH 7.2 (three changes for 30 min at 4 °C) and then post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C. Samples were dehydrated in an ascending series of alcohol solutions (30–100%), infiltrated with a mixture of acetone:epon resin (2:1 for 1 h at 4 °C, 1:1 for 1 h at 4 °C and 1:2 for 1 h at 4 °C), embedded in epon resin overnight and polymerised at 60 °C for 48 h. Semi-thin sections (1 µm) and ultrathin (70 nm) sections were cut using an Ultracut ultramicrotome (Reichert–Jung, Nussloch, Germany). Semi-thin sections stained with toluidine blue were observed using Axiophot optical microscope (Zeiss, Oberkochen, Germany). For transmission electron microscopy (TEM), ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate and then observed using a Jeol 1200 EX II TEM (JEOL Datum Company, Tokyo, Japan).

Statistical analysis

Data were analysed using the MIXED procedure of the Statistica-7 Software package (Stat Soft, Inc., Tulsa, OK, USA) according to the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \varepsilon_{ijkl}$$

where, Y_{ijkl} , dependent variable; μ , overall mean of the population; α_i , effect (random variable) of mice; β_j , mean effect of day of sampling (days 0, 2 and 5); γ_k , mean effect of treatment (C, FR and HS); $(\beta\gamma)_{jk}$, interaction of day of sampling $\times$ treatment; ε_{ijkl} , unexplained residual element assumed to be independent and normally distributed.

The repeated measures statement was used to analyse RT and GTT data. Each variable analysed was subjected to three covariance structures: compound symmetry, autoregressive order one and unstructured covariance. The covariance structure that had the largest Akaike's information criterion and Schwarz's Bayesian criterion was considered the most desirable. For all parameters tested, the best covariance structure was compound symmetry. Least-square means were separated with the predicted difference (PDIF) procedure. Significance was declared at $P < 0.05$.

Results

Effects of HS on BW, food and water intake, RT and tissue *Hspa1* gene expression

At the beginning of the trial, the overall mean ($\pm$ s.d.) BW for the C, HS and FR groups was 19.9 ± 2.6 , 20.7 ± 2.4 and 20.0 ± 2.2 g respectively. After 5 days of the experiment, the BW of C mice increased by 0.31 g/day, while in FR and HS mice, the BW decreased by 0.84 and 0.14 g/day respectively. The overall food intake was higher in the control group (0.40 ± 0.05 g/g BW^{0.75}) compared with the FR (0.25 ± 0.03 g/g BW^{0.75}) and HS groups (0.29 ± 0.04 g/g BW^{0.75}). Water consumption was lower in the C and FR groups (0.61 ± 0.05 ml/g BW^{0.75} and 0.55 ± 0.09 ml/g BW^{0.75} respectively) than in the HS group (0.70 ± 0.12 ml/g BW^{0.75}).

The temporal responses of RT and *Hspa1* mRNA expression are shown in Fig. 1. RT was higher ($P=0.0010$) in HS mice compared with C and FR mice. No differences in RT were observed between C and FR mice during the 5-day experimental period (Fig. 1A). In HS mice, the RT peaked after 1 day of heat exposure and then started to decrease and remained slightly higher compared with C and FR until the end of the experiment.

Plasma metabolite and hormone concentrations in HS mice

Blood glucose was lower in the HS ($P=0.0234$) and the FR groups ($P=0.0383$) than the C group (Fig. 2A). The maximum decrease in plasma glucose was observed after 2 days in the HS group. In the FR mice, NEFA increased compared with C ($P=0.0384$) and HS ($P=0.0184$). HS mice had the lowest NEFA ($P=0.0456$; Fig. 2B).

The plasma concentrations of the adipokines and insulin in HS, FR and C mice are shown in Fig. 3. Compared with C and FR, plasma leptin (Fig. 3A) and adiponectin (Fig. 3B) were higher in the HS group after 2 days ($P=0.0245$) and 5-day of HS ($P=0.0313$). At day 2 of FR, adiponectin also increased ($P=0.0413$) when compared with C. The concentration of insulin (Fig. 3C) increased markedly ($P=0.0219$) in HS mice, while FR induced a decrease when compared with C, both on day 2 ($P=0.0462$) and day 5 ($P=0.0471$).

Effects of HS on glucose tolerance

As shown in Fig. 4A, at the beginning of the experiment (day 0), there were no detectable differences in glucose or behaviour during the GTT between the experimental (HS) and the control (FR and C) groups. On day 5 of the experimental period, glucose level, 30 min after the i.p. injection, was lower in the HS mice compared with the FR ($P=0.0369$) and C ($P=0.0401$)

Figure 1 Kinetics of rectal temperature (A) and *Hspa1* mRNA in subcutaneous white adipose tissue (B), skeletal muscle (C) and liver (D) of control, food-restricted (FR) and heat-stressed (HS) C57BL/6 mice. The rectal temperature of the animals was measured using a thermistor probe (Temp JKT; Oakton Instruments, Vernon Hills, IL, USA) during the daytime. Each mRNA level was determined using quantitative real-time PCR as described in the Materials and methods section. Values are normalised to mouse β -actin mRNA expression. Time zero was considered as baseline. Data points indicate the mean $\pm$ s.d., $n=6$. * $P<0.05$ indicates higher values in HS mice compared with their counterparts (C and FR mice) within day, ^{a,b,c} $P<0.05$ and $\dagger, \ddagger, \$P<0.01$ between treatments within day.

Figure 2 Plasma glucose (A) and non-esterified fatty acid (NEFA) concentrations (B) in control (C), food-restricted (FR) and heat-stressed (HS) C57BL/6 mice at days 2 and 5 of the experimental period. Plasma indices of metabolic status were determined by an automatic analyser (IL-300 Plus; Instrumentation Laboratory, Lexington, MA, USA). Plasma glucose (kit from Instrumentation Laboratory) and NEFA (NEFA-C kit; Wako Fine Chemical Industries, Dallas, TX, USA) were measured by enzymatic colorimetric methods. Data points indicate the mean $\pm$ S.D., $n=6$. * $P<0.05$ between days within treatment; ^{a,b,c} $P<0.05$ between treatments within day.

mice (Fig. 4B). FR mice had lower glucose ($P=0.0354$) than that observed in C but higher ($P=0.0371$) than that in HS mice. The decrease in the glucose AUC provided additional evidence that glucose clearance was affected by HS (Fig. 4C).

mRNA expression of specific adipokines, their receptors, IRS1 and glucose transporters

In Fig. 5, the specific mRNAs for leptin and adiponectin in the WAT are shown. Leptin mRNA expression in HS mice was up-regulated ($P=0.0002$) compared with C and FR mice (Fig. 5A). The differences in leptin mRNA expression remained significant until the end of the experimental period. Significant differences were observed in adiponectin mRNA expression (Fig. 5B) amongst the three groups. HS up-regulated ($P=0.0226$) adiponectin mRNA expression. Food restriction also up-regulated adiponectin compared with C both at day 2 ($P=0.0294$) and day 5 ($P=0.0305$).

Figure 6A1, A2 and A3 depict the changes in expression of *ObRb* in WAT, M and L respectively.

In WAT and M, the expression of *ObRb* was up-regulated in HS ($P=0.0172$) and FR ($P=0.0048$) compared with C, whereas in L, *ObRb* mRNA had the highest expression in HS mice after 2 days ($P=0.0016$) and 5 days of treatment ($P=0.0002$), and no differences were observed between C and FR mice.

The mRNA expression of adiponectin receptors (*Adipor1* in WAT and M and *Adipor2* in L) is shown in Fig. 6B1, B2 and B3. In WAT and M, *Adipor1* expression was up-regulated in HS ($P=0.0025$) and FR ($P=0.0018$) mice compared with control. In L, *Adipor2* mRNA expression was the highest ($P=0.0048$) in HS

Figure 3 Leptin (A), adiponectin (B) and insulin secretion (C) in control (C), food-restricted (FR) and heat-stressed (HS) C57BL/6 mice during the experimental period. Plasma leptin, adiponectin and insulin were measured using commercial ELISA kits. The Mouse Leptin ELISA kit (Millipore) was used for leptin (sensitivity: 0.05 ng/ml), and the Mouse Adiponectin ELISA kit (Millipore) was used for adiponectin (sensitivity: 0.5 ng/ml). Insulin was measured using the Mouse Insulin ELISA kit (Millipore), and its sensitivity was 0.2 ng/ml. Time zero was considered as baseline. Data points indicate the mean $\pm$ S.D., $n=6$. ^{a,b,c} $P<0.05$ between treatments within day.

Figure 4 Intraperitoneal glucose tolerance test (GTT) curves obtained in control (C), food-restricted (FR) and heat-stressed (HS) C57BL/6 mice at day 0 (A) and day 5 of the experimental period (B); GTT is expressed as the glucose area under the curve (AUC) calculated at day 5 (C). The animals ($n=6$ mice per group) had been fasted for 16 h and were injected with 1.5 g/kg body weight of glucose i.p. Blood glucose was measured 0, 30, 60, 90 and 120 min after injection using a glucometer (OneTouch Ultra Easy, Lifescan, Milano, Italy). Values are mean $\pm$ s.d., $n=6$. * $P<0.05$ between groups at 30 min after glucose injections; $a,b,cP<0.05$ between treatments.

mice, but food restriction also induced an increase in *Adipor2* mRNA expression, both at 2 days ($P=0.0023$) and 5 days ($P=0.0145$), compared with C.

The gene expression of *Irs1* in WAT, M and L is shown in Fig. 6C1, C2 and C3 respectively. In WAT and M, *Irs1* expression was up-regulated in HS ($P=0.0004$) and FR ($P=0.0034$) mice compared with C (Fig. 6C1 and C2). In L, *Irs1* mRNA was slightly ($P=0.0437$) up-regulated by food restriction and was markedly

increased ($P=0.0034$) by HS (Fig. 6C3). These differences in *Irs1* mRNA expression amongst the three groups were significant until the end of the experimental period.

The mRNA expression of *Glut4* and *Glut2* in WAT and in L, respectively, was higher in HS mice than FR ($P=0.0162$) and C ($P=0.0030$; Fig. 6D1 and D3), whereas in M, food restriction also increased *Glut4* mRNA until the end of the experimental period (Fig. 6D2).

Intestinal barrier integrity in HS mice

We performed electron microscopy on small intestine tissue samples to assess the effect of hyperthermia on epithelial morphology. Histological analysis by TEM revealed no significant changes in intestinal tissue in HS mice compared with mice kept under control conditions (Fig. 7). In particular, microvilli, mitochondria and tight junctions of HS mice were similar to those observed in the intestinal epithelium of C mice. Vacuolisation of epithelial cells was not visible in HS mice.

Figure 5 Leptin (A) and adiponectin mRNA expression (B) in subcutaneous white adipose tissue of C57BL/6 mice exposed to control (C), food restriction (FR) and heat stress (HS) conditions. Each mRNA level was determined using quantitative real-time PCR as described in the Materials and methods section. Values are normalised to mouse β -actin mRNA expression. Time zero was considered as baseline. Data points indicate the mean $\pm$ s.d., $n=6$. $a,b,cP<0.05$ and $A,BP<0.01$ between treatments within day.

Discussion

As expected, chronic HS ($35 \pm 1^\circ\text{C}$ for 5 days) resulted in decreased BW and food consumption and increased water intake and RT. The changes in those parameters, commonly used as indicators of the effects of HS on animal physiology, indicate that our mice were under conditions of moderate hyperthermia (Gordon 1993, Chung *et al.* 2008). The HS protocol used in this study was effective in inducing molecular responses in WAT, M and L, as shown by the increased expression of *Hspa1*. Interestingly, food restriction also induced an increase in *Hspa1* gene expression, which seems to confirm that those changes were independent of hyperthermia. The exposure to HS is associated with an increase in the synthesis of HSPs, which protect cells against hyperthermia (Horowitz 2002). Moreover, Aly *et al.* (1994) have

demonstrated an increase in HSP expression in tissues of animals maintained under caloric restriction. Low caloric intake is considered a mildly stressful condition that induces a survival response and therefore increases the organism's defences (Sinclair 2005).

The reduction of food intake commonly observed in HS animals is a response aimed to reduce digestion and metabolic heat production (Johnson 1987, Speakman & Król 2010). Considering that HS and food restriction generate stress conditions that induce a derangement of metabolic and behavioural homeostasis in animals, it was an important aim of our study to discriminate cellular/metabolic effects due to HS-induced reductions in food intake from those associated with food deprivation alone.

The up-regulation of leptin and adiponectin gene expression and secretion observed in this study agrees

Figure 6 mRNA expression of leptin receptor (*ObRb*) (A), adiponectin receptors (*Adipor1* and *Adipor2*) (B), insulin receptor substrate 1 (*Irs1*) (C) and glucose transporters (*Glut2* and *Glut4*) (D) in subcutaneous white adipose tissue (1), skeletal muscle (2) and liver (3) of C57BL/6 mice exposed to control (C), food restriction (FR) and heat stress (HS) conditions. Each mRNA level was determined using quantitative real-time PCR as described in the Materials and methods section. Values are normalised to mouse β -actin mRNA expression. Time zero was considered as baseline. Data points indicate the mean $\pm$ s.d., $n=6$. a, b, c $P < 0.05$ and A, B, C $P < 0.01$ between treatments within day.

Figure 7 Transmission electron micrographs of small intestine epithelial cells from mice exposed to control (C) or heat stress (HS) conditions. Control mice were maintained at an ambient temperature of $22 \pm 2^\circ\text{C}$ for 5 days. HS mice were continuously maintained at an ambient temperature of $35 \pm 1^\circ\text{C}$ for 5 days. Note: no damage to microvilli (M), tight junctions of cell membranes (TJ) or mitochondria (Mt) and no vacuolisation are present in HS mice. Microscopic magnification: (A and B), $10\,000\times$; (C and D), $25\,000\times$.

with our previous *in vitro* findings (Bernabucci *et al.* 2009), confirming a role of HS in modulating leptin and adiponectin gene and protein expression. In HS and FR mice, the circulating leptin and adiponectin levels paralleled the respective responses in WAT leptin and adiponectin gene expression. Our findings regarding leptin and adiponectin in FR mice agree with previous human and mouse studies, indicating that caloric restriction induces a reduction of leptin and an increase in adiponectin expression (del Mar Romero *et al.* 2009, Lee *et al.* 2010). The same result was not observed in HS mice, in which heat treatment significantly increased both leptin and adiponectin secretion and expression. Therefore, these data suggest that HS alters leptin and adiponectin levels independently of food restriction.

Hyperthermia increases intestinal permeability in a variety of mammalian species (Barthe *et al.* 1998, Lambert *et al.* 2002), facilitating the passive non-mediated diffusion of both small and large molecules (e.g. endotoxins) from the gastrointestinal lumen to the blood. Endotoxins entering the blood stream may induce the development of inflammation by stimulating the secretion and release of a variety of

pro-inflammatory factors (Lambert *et al.* 2002). Landman *et al.* (2003) reported that endotoxins stimulate the release of leptin into peripheral blood in rodents, humans and non-human primates. Lower levels of adiponectin are associated with low-grade chronic inflammation and increases in pro-inflammatory factors (Fantuzzi 2008). Under HS conditions in our study, the intestine maintained a normal epithelial morphology. This response might exclude the possibility of a heat-stressed inflammatory state mediated by altered intestinal permeability. Consequently, the observed changes in leptin and adiponectin gene expression and secretion in HS mice were likely an effect of HS on mechanisms that modulate the secretory function of WAT.

The molecular mechanisms by which HS regulates the expression and secretion of adipokines remain largely unknown. Park *et al.* (2005) suggested that changes in the fluidity of membrane lipids, induced by high temperature, may cause signal transduction that would induce a cellular heat-shock response, e.g. greater HSP expression, that could directly stimulate adipokine expression.

Chronic heat treatment, by up-regulating *ObRb* and *Adipor1* and *Adipor2* gene expression, seemed to elicit a significant increase in leptin and adiponectin sensitivity in WAT, M and L. Previous studies on HS animal models reported that despite marked reductions in nutrient intake, HS animals did not show an increase in plasma NEFA, had lower plasma glucose, increased basal insulin levels and stimulated glucose uptake (Torlinska *et al.* 1987, Ritchie *et al.* 1994, Ronchi *et al.* 1999, Gupta *et al.* 2009, Wheelock *et al.* 2010). All those responses and the up-regulation of *ObRb* and adiponectin receptors in this study suggest that adiponectin and leptin might play roles in modulating the post-absorptive energetic metabolism of HS subjects. Chronic HS and caloric restriction led to distinct patterns of expression of the leptin and adiponectin receptor genes in L, while these genes were similarly affected in WAT and M. The different responses of L, probably because of its major vulnerability and sensitivity to HS, emphasise the key role that L plays in orchestrating the main pathways of energy metabolism under HS conditions.

Adiponectin and leptin are important physiological insulin sensitisers (Rabe *et al.* 2008). These adipokines act locally or distally to modify insulin sensitivity in insulin target organs such as WAT, M and L. The suggested mechanism whereby adiponectin increases insulin sensitivity involves the suppression of hepatic glucose production and an increase in fatty acid oxidation by M and L, thereby reducing the levels of plasma NEFA (Berg *et al.* 2001). Adiponectin stimulates glucose uptake in adipocytes and M by activating 5'-AMP-activated protein kinase (Kadowaki *et al.* 2006, Klimcakova *et al.* 2006) and stimulates insulin secretion

by the pancreas. Leptin also activates 5'-AMP-activated protein kinase and enhances fatty acid oxidation in M and L (Klimcakova *et al.* 2006). It also modulates hepatic gluconeogenesis and pancreatic β -cell function (Rabe *et al.* 2008). Data from the current study suggest that similar mechanisms as those found previously may be activated by HS. Therefore, the results from this study provide initial evidence of the molecular mechanisms through which HS induces insulin sensitivity in peripheral tissues. In particular, HS seems to have a greater impact on insulin sensitivity in the L than in the WAT and M.

The present results lead to questions as to why chronic HS affected the gene expression and secretion of leptin and adiponectin, seemed to enhance leptin and adiponectin sensitivity in peripheral tissues and improved insulin sensitivity. On the basis of previous data, we suggest that these changes can be considered an acclimatisation of homeothermic animals to HS, i.e. a way to increase avenues of heat loss and reduce heat production in an attempt to remain euthermic. Those responses include decreased food intake, increased water intake and increased respiration and perspiration rates, all leading to increased maintenance costs.

The immediate response to heat load and the heat-induced changes in homeorhetic modifiers may alter post-absorptive energy, lipid and glucose metabolism. How these metabolic changes are initiated and regulated is not well known. The up-regulation of leptin and adiponectin expression in adipose cells exposed to heat shock might be one of the mechanisms involved in acclimation to a hot environment, i.e. a central action that leads to decreased food intake and limits body hyperthermia (Nogueiras *et al.* 2004, Hoyda *et al.* 2011). On the other hand, the up-regulation of adiponectin may be responsible for the increased pancreatic insulin secretion observed in HS animals. These adipokines might allow cells to better resist the damaging effects of HS. Indeed, our data show that HS animals experience a variety of post-absorptive metabolic changes that are not typical of animals under a lowered nutritional plane. With regard to lipid and carbohydrate metabolism, this is primarily characterised by the lack of WAT lipid mobilisation, increased basal and stimulated insulin release and increased glucose disposal.

An increase in glucose pool entry coupled with a decrease in blood glucose may suggest a higher rate of glucose leaving the circulating blood pool; thus, glucose appears to become a favoured fuel for HS animals. That suggestion is supported by the increase in respiratory quotient observed in hyperthermic humans (Febbraio 2001). The apparent switch in metabolism and the increase in insulin sensitivity are probably a mechanism by which animals decrease metabolic heat production. Despite having a much greater energy content, as a result of differences in the efficiencies of

capturing ATP, oxidising fatty acids generate more metabolic heat compared with glucose (O'Brien *et al.* 2010). Therefore, during HS preventing or blocking adipose mobilisation or breakdown and increasing glucose 'burning' is presumably a strategy to minimise metabolic heat production (Wheelock *et al.* 2010).

In summary, the current study provides some of the first evidence regarding the likely specific effects of chronic HS on leptin and adiponectin gene expression and secretion by WAT. HS appeared to improve leptin and adiponectin signalling in WAT, M and L. Changes in the levels and sensitivity of these adipokines may be considered an adaptive response to HS conditions. Moreover, our findings suggest that chronic heat treatment independent of reduced food intake was responsible for improving insulin sensitivity and glucose uptake in peripheral tissues, probably mediated by the adipokines. Collectively, these data indicate that moderate chronic heat treatment could be a potential therapeutic treatment for disorders associated with insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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