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Endothelin regulates intermittent hypoxia-induced lipolytic remodelling of adipose tissue and phosphorylation of hormone-sensitive lipase

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Key points

- Endothelin-1 (ET-1) is upregulated upon intermittent hypoxia and has lipolytic effects.
- Intermittent hypoxia induces adipose tissue lipolysis that may be mediated by ET-1.
- In the present study, we show that ET-1 is involved in adipose tissue remodelling induced by intermittent hypoxia and that phosphorylation of hormone-sensitive lipase could be one mechanism mediating this effect.
- We also show that ET-1 upregulates its own and its type A endothelin receptor expression, possibly leading to an autoactivatory loop.
- These results help us better understand the mechanisms of dyslipidaemia in disorders associated with intermittent hypoxia, such as obstructive sleep apnoea.

Abstract Obstructive sleep apnoea syndrome is characterized by repetitive episodes of upper airway collapse during sleep resulting in chronic intermittent hypoxia (IH). Obstructive sleep apnoea syndrome, through IH, promotes cardiovascular and metabolic disorders. Endothelin-1 (ET-1) secretion is upregulated by IH, and is able to modulate adipocyte metabolism. Therefore, the present study aimed to characterize the role of ET-1 in the metabolic consequences of IH on adipose tissue *in vivo* and *in vitro*. Wistar rats were submitted to 14 days of IH-cycles (30 s of 21% FiO_2 and 30 s of 5% FiO_2; 8 h day^{-1}) or normoxia (air–air cycles) and were treated or not with bosentan, a dual type A and B endothelin receptor (ETA-R and ETB-R) antagonist. Bosentan treatment decreased plasma free fatty acid and triglyceride levels, and inhibited IH-induced lipolysis in adipose tissue. Moreover, IH induced a 2-fold increase in ET-1 transcription and ETA-R expression in adipose tissue that was reversed by bosentan. In 3T3-L1 adipocytes, ET-1 upregulated its own and its ETA-R transcription and this effect was abolished by bosentan. Moreover, ET-1 induced glycerol release and inhibited insulin-induced glucose uptake. Bosentan and BQ123 inhibited these effects. Bosentan also reversed the ET-1-induced phosphorylation of hormone-sensitive lipase (HSL) on Ser^{660}. Finally, ET-1-induced lipolysis and HSL phosphorylation were also observed under hypoxia. Altogether, these data suggest that ET-1 is involved in IH-induced lipolysis in Wistar rats, and that upregulation of ET-1 production and ETA-R expression by ET-1 itself under IH could amplify its effects. Moreover, ET-1-induced lipolysis could be mediated through ETA-R and activation of HSL by Ser^{660} phosphorylation.

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Abbreviations ATGL, adipose triglyceride lipase; DMEM, Dulbecco's modified Eagle's medium; ET-1, endothelin-1; ETA-R, type A endothelin receptor; ETB-R, type B endothelin receptor; FBS, fetal bovine serum; FFA, free fatty acid; HIF-1, hypoxia-inducible factor 1; HSL, hormone-sensitive lipase; IH, intermittent hypoxia; OSA, obstructive sleep apnoea; PKA, protein kinase A; PS, penicillin–streptomycin; qPCR, quantitative PCR.

Introduction

Obstructive sleep apnoea (OSA) syndrome is a highly prevalent disease affecting 5–20% of the population and representing a growing health concern as a result of its association with cardiovascular mortality and morbidity (Levy *et al.* 2012). It is characterized by periodic upper airway collapse during sleep, leading to sleep fragmentation, respiratory efforts and chronic intermittent hypoxia, which appears to be the most deleterious consequence promoting cardiovascular disease through oxidative stress and an inflammatory cascade (Garvey *et al.* 2009; Levy *et al.* 2009).

Intermittent hypoxia (IH) is a hallmark of OSA and is associated with metabolic dysfunction (Drager *et al.* 2010). Experimental data suggest that IH leads to dyslipidaemia (Li *et al.* 2005; Trzepizur *et al.* 2013) through up-regulation of liver lipid biosynthesis (Savransky *et al.* 2008), increased lipolysis accompanied by free fatty acid (FFA) release and fat remodelling (Jun *et al.* 2010; Poulain *et al.* 2014) and decreased lipoprotein clearance as a result of inhibition of lipoprotein lipase (Drager *et al.* 2013). IH has also been shown to induce IL-6 release by epididymal fat and to decrease adiponectin secretion (Magalang *et al.* 2009; Poulain *et al.* 2014).

Finally, IH induces insulin resistance as a result of its pleiotropic effects through the sympathetic nervous system and the response of different tissues such as the muscle, liver and adipose tissue (Drager *et al.* 2010). Indeed, FFAs released by lipolysis of adipose tissue upon IH reduce insulin-mediated glucose uptake in muscles (Delarue & Magnan, 2007).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide mainly produced by vascular endothelial cells. It is upregulated upon hypoxia by hypoxia-inducible factor-1 (HIF-1) transcription factor (Yamashita *et al.* 2001). Moreover, we have shown that ET-1 plays a major role in the development of IH-induced cardiovascular alterations such as hypertension and increased myocardial infarction (Belaidi *et al.* 2009). Beside its cardiovascular consequences, endothelin also has a strong impact on adipose tissue, in particular on adipocyte glucose and lipid metabolism. Indeed, ET-1 induces lipolysis in 3T3-L1 adipocytes (Juan *et al.* 2005), rat adipocytes (Juan *et al.* 2006) and primary human adipocytes (Eriksson *et al.* 2009), an effect mediated by type A endothelin receptors (ETA-R). Finally, ET-1 decreases insulin sensitivity, possibly through inhibition of Glut4 translocation (Ishibashi *et al.* 2001), resulting in decreased fatty acid and glucose uptake (Chien *et al.* 2011). The experimental data therefore outline various similarities between the effects of IH and of ET-1 on the adipose tissue, suggesting that ET-1 might be one of the mediators of the metabolic consequences of IH.

In adipocytes, lipolysis is regulated by several key enzymes, including adipose triglyceride lipase (ATGL; also known as PNPLA2) and hormone-sensitive lipase (HSL; also known as LIPE)(Fruhbeck *et al.* 2014). ATGL, HSL and monoglyceride lipase sequentially hydrolyse triglycerides into glycerol and fatty acids. The activity of both ATGL and HSL is submitted to transcriptional and post-transcriptional regulation. HSL regulation requires serine phosphorylations with opposite effects: phosphorylation of Ser^{563} and $Ser^{659\text{-}660}$ by protein kinase A (PKA) increases its activity, whereas that of Ser^{565} by cyclic AMP-dependent protein kinase inhibits its activity by preventing Ser^{563} phosphorylation (Lampidonis *et al.* 2011).

The present study aimed to determine whether activation of the endothelin system by IH could contribute to its metabolic and lipolytic effects on adipose tissue. Using a combination of *in vivo* and *in vitro* approaches, we demonstrate that endothelin and its ETA-Rs participate in the lipolytic effects of intermittent hypoxia and that regulation of HSL activity could be one of the mechanisms involved.

Methods

Animals and ethical approval

Thirty-two male Wistar rats (8 weeks old, weight 300–350 g; Janvier Labs, Le Genest-Saint-Isle, France) were used in the present study. The animals were kept under control conditions (21 ± 1°C, 12:12 h light/dark cycle) and fed *ad libitum* with standard chow for 1 week. The animals were then divided into four experimental groups ($n = 8$ per group) exposed to intermittent hypoxia or normoxia and treated or not with bosentan (100 mg kg^{-1} day^{-1} admixed in chow). All experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other

Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986) and were approved by an Institutional Animal Care and Use Committee (Cometh, University Grenoble Alpes).

IH

IH was performed as described previously (Arnaud *et al.* 2011). Animals were exposed in their cages during their daytime sleeping period to 8 h of consecutive 1 min cycles (alternating 30 s of 21% and 30 s of 5% FiO_2, 60 cycles h^{-1}) for 14 consecutive days. FiO_2 was measured with a gas analyser (ML206; ADInstruments, Oxford, UK) throughout the experiment. Control normoxic rats were exposed to similar air–air cycles to reproduce the noise and air turbulences of the IH stimulus. Ambient air temperature was maintained at 20–22°C.

Blood and tissue collection

At the end of IH exposure, animals were fasted overnight and anaesthetized with sodium pentobarbital (50 mg kg^{-1}, I.P.). Blood was collected in heparinized and EDTA tubes and centrifuged at 1700 *g* for 10 min at 4°C. Plasma was frozen and stored at −80°C until analysis. Right and left epididymal fat pads were removed, weighted and either stored at −80°C for quantitative PCR (qPCR) analysis or fixed in 95% ethanol and paraffin-embedded for histological studies.

Plasma analysis

Heparinized plasma glucose and triglyceride concentrations were assayed on a modular analyser (Roche Diagnostics, Mannheim, Germany). Circulating FFA levels were measured in ETDA plasma by an enzymatic assay using the NEFA FS DiaSys® kit (Diasys Diagnostic Systems, Holzheim, Germany).

Immunohistochemistry

Paraffin-embedded epididymal fat was sectioned in 5 μm slices and stained with anti-ETA antibody (dilution 1:100; Alomone Labs, Jerusalem, Israel) and secondary anti-rabbit antibody (dilution 1:1000). Slices were viewed and photographed with a Nikon Eclipse 80i® microscope (Nikon, Tokyo, Japan). Quantifications were made using ImageJ (NIH, Bethesda, MD, USA) and NIS (Nikon) software.

Cell culture

Murine 3T3-L1 preadipocytes were cultured (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (PS) and 0.1% amphotericin B. Two days after confluence, the medium was replaced with differentiation medium, containing DMEM/HAM F-12 Glutamax (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with FBS, PS, amphotericin B and insulin (10 μg ml^{-1}), dexamethasone (250 nM) and 3-isobutyle-1-methylxanthine (0.1 mM). After 72 h, differentiation medium was replaced with adipocyte medium: DMEM/HAM F-12 Glutamax supplemented with FBS, PS, amphotericin B and insulin (5 μg ml^{-1}). Seven days after the end of differentiation, adipocytes were deprived with DMEM without glucose for 24 h. Cells were then pre-treated with or without BQ123 (a specific ETA-R, antagonist, 10 μM), BQ788 [a specific endothelin receptor B (ETB), antagonist, 10 μM] or bosentan (mixed ETA and ETB antagonist, 10 μM) for 1 h, followed by insulin (5 nM) or ET-1 (10 nM) for 4–24 h. For hypoxia experiments, differentiated cells were cultured with 3% O_2 and 5% CO_2 in a trigaz incubator (MCO-18 M; Sanyo, Moriguchi, Japan) for 4 or 24 h. At the end of the experiments, supernatants and cell pellets were stored at −80°C until further use.

Glycerol release and glucose uptake

3T3-L1 glycerol release was measured in the culture medium by colourimetric assay for triglyceride on a modular analyser (Roche Diagnostics) as described previously (Nagele, 1985). The glucose concentration in the culture medium was assayed using the hexokinase method on the same analyser (Schmidt, 1961).

Real-time qPCR

Total RNA extraction was performed using Trizol followed by RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) in accordance with the manufacturer's instructions. Total RNA was treated with RNase-free DNase I (Qiagen). cDNA was reverse transcribed from 1 μg of total RNA with the SuperScriptIII First-Strand Synthesis System (Life Technologies, Saint Aubin, France). RNase H treatment was added as recommended by the manufacturer.

Real-time PCR was conducted using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Mx3005P qPCR system (Stratagene, La Jolla, CA, USA).

Primers were chosen to include intron spanning and were synthesized by Life Technologies. Primer sequences and experimental qPCR conditions are reported in the Supporting information (Table S1).

Gene expression was quantified using the comparative threshold cycle (C_t) method (Giulietti *et al.* 2001). The amount of target gene, normalized to three endogenous reference genes (ACTB, CYCA and HPRT1) was expressed relative to that of control cells.

Table 1. Body weights before and after 14 days of exposure to normoxia or intermittent hypoxia with or without bosentan treatment

Group	Day 0	Day 14	Weight change
Normoxia	353.3 ± 4.6	383.9 ± 6.8	30.6 ± 5.5
Intermittent hypoxia	353.8 ± 6.7	346.7 ± 7.1	−7.1 ± 3.3*
Normoxia + bosentan	363.8 ± 4.4	402.5 ± 6.0	38.7 ± 4.0
Intermittent hypoxia + bosentan	350.4 ± 3.9	359.7 ± 8.1	9.3 ± 6.2*†

*$P < 0.05$ *vs.* corresponding normoxia group. †$P < 0.05$ *vs.* corresponding untreated group; two-way ANOVA.
Weights (g) are expressed as the mean ± SEM.

Western blot analysis

3T3-L1 adipocytes and epididymal adipose tissue were lysed in RIPA lysis buffer supplemented with protease and phosphatases inhibitors. Protein concentration was measured using the bicinchoninic acid method (Pierce BCA Protein Assay Kit®; Thermo Fisher Scientific). Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membrane were incubated overnight at 4°C with anti-ET-1, anti-ETA, anti-ETB, anti-pHSL(Ser^{565}), anti-pHSL(Ser^{660}) and anti-ATGL antibodies (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes were incubated for 1 h with secondary anti-rabbit HRP-linked antibody. Results were normalized with anti-actin antibody (dilution 1:1000; Sigma-Aldrich, St Louis, MO, USA). Signal density was analysed with ImageJ software.

Statistical analysis

Results are expressed as the mean ± SEM and were analysed using Prism (GraphPad Software, La Jolla, CA,

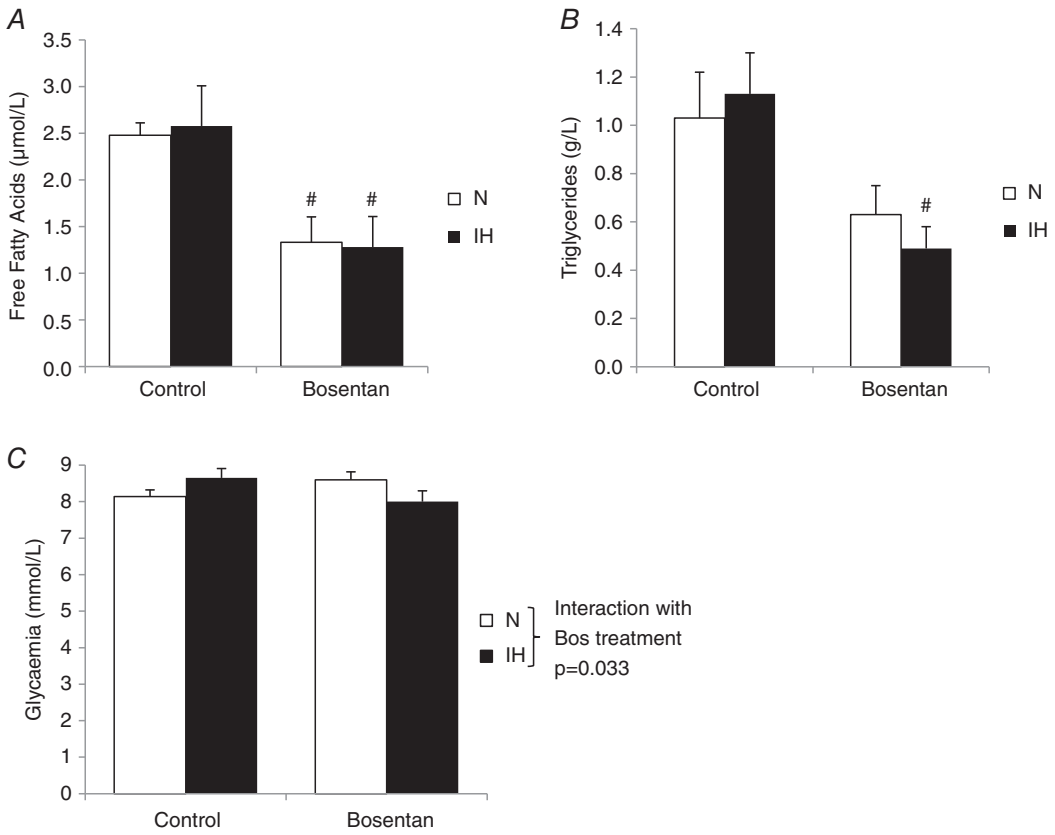

Figure 1. Plasma metabolic parameters in rats
Plasma levels of (*A*) FFAs (μmol L^{-1}), (*B*) triglycerides (g L^{-1}) and (*C*) glucose (mmol L^{-1}) in rats submitted to 14 days of normoxia (N) or intermittent hypoxia (IH) with or without oral bosentan treatment (100 mg kg^{-1} day^{-1}) ($n = 8$ rats per group). #$P < 0.05$ *vs.* control, two-way ANOVA. There was a significant interaction between IH exposure and bosentan treatment for glycaemia ($P = 0.033$).

USA). $P < 0.05$ was considered statistically significant. Comparison of data from the four groups of rats was performed by two-way ANOVA followed by Tukey's *post hoc* analysis. Comparison of data from cell culture experiments was performed by one- or two-way ANOVA depending on the number of factors, or by non-parametric ANOVA on ranks when a normal distribution of the values was not achieved.

Results

In vivo, ET-1 is involved in IH-induced metabolic perturbations and lipolysis

The body weights of Wistar rats submitted to 14 days of normoxia or IH with or without oral bosentan treatment are reported in Table 1. Rats submitted to IH did not gain weight compared to normoxic animals and this difference was significantly attenuated by bosentan treatment. Plasma FFAs were not modified by IH exposure but were significantly reduced by bosentan treatment in both normoxic and IH groups (Fig. 1*A*). Similarly, plasma triglycerides were not affected by IH exposure but were significantly reduced by bosentan treatment in the IH group (Fig. 1*B*). Finally, a significant interaction between IH exposure and bosentan treatment was found for glycaemia ($P = 0.033$) (Fig. 1*C*).

IH induced a significant loss of epididymal adipose tissue mass in untreated animals, which was significantly reversed by bosentan treatment (Fig. 2*A*). This was associated with an IH-induced decrease in mean adipose cell volume in untreated but not in bosentan-treated rats, as shown in haematoxylin-stained slices of epididymal fat (Fig. 2*B* and *C*).

ET-1 and ETA-R expression in adipose tissue in response to IH exposure

We observed that ET-1 mRNA levels were increased almost two-fold in the epididymal adipose tissue of IH rats compared to normoxic rats (Fig. 3*A*). The ET-1 protein

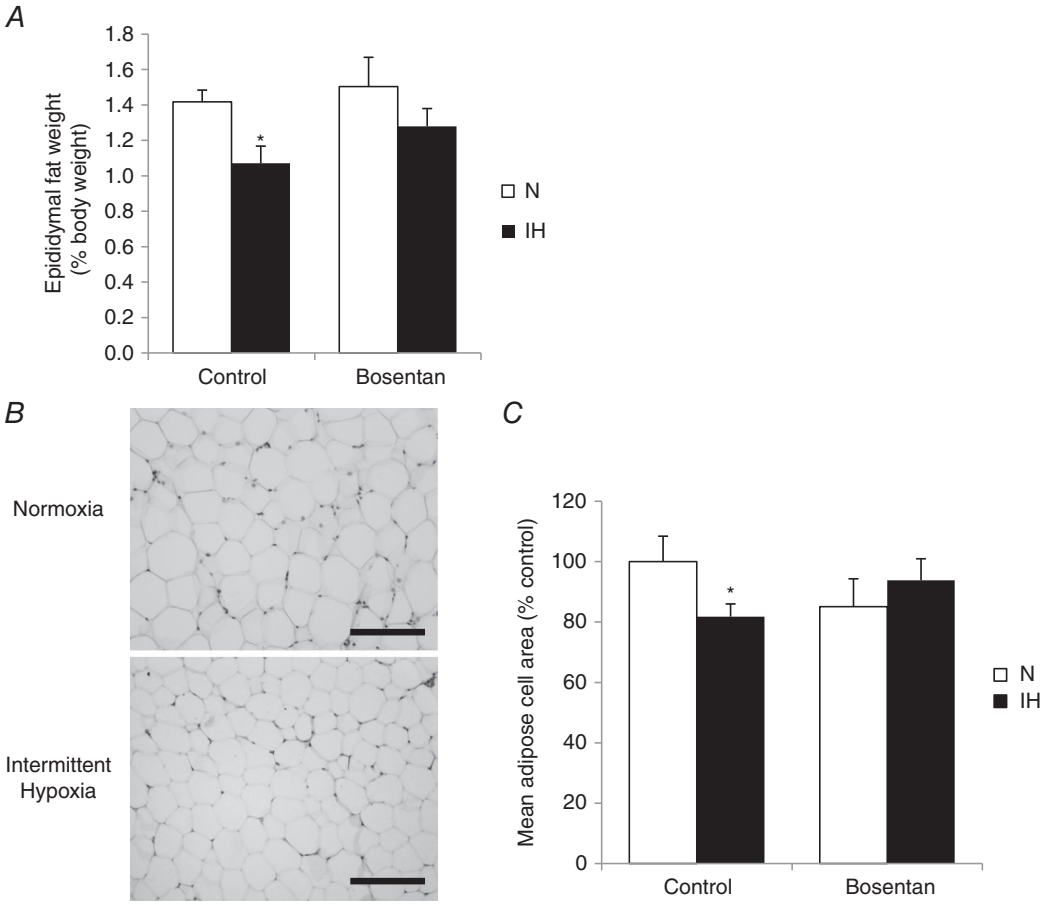

Figure 2. ET-1 contributes to intermittent hypoxia-induced adipose tissue lipolysis
A, ratio (%) of epididymal fat to total body weight after 14 days of exposure to normoxia (N) or intermittent hypoxia (IH) with or without oral bosentan treatment (100 mg kg^{-1} day^{-1}). *B*, hematoxylin staining of representative slices of adipose tissue from untreated N and IH rats. Scale bar = 40 μm. *C*, quantification of mean adipose cell area on at least three epididymal fat sections per rat ($n = 8$ rats per group) $^*P < 0.05$ *vs.* N; two-way ANOVA.

level in epididymal fat extracts was not significantly modified by IH but was significantly decreased by bosentan (Fig. 3*B*).

Adipose tissue ETA-R and ETB-R were not induced by intermittent hypoxia at the transcriptional level (data not shown). However, we observed that IH induced a marked increase in protein ETA-R expression (normalized to total cell area because of a decreased cell size after IH exposure) in immunostained epididymal fat sections, with this effect being abolished by bosentan treatment (Fig. 3*D*).

ET-1 regulates its own expression in 3T3-L1 cells

In differentiated 3T3-L1 adipocytes, ET-1 mRNA levels were induced 12-fold when cells were incubated for 24 h with recombinant ET-1 (10 nM) and this effect was abolished by co-incubation with a selective ETA-R antagonist (BQ123), a selective ETB-R antagonist (BQ788) or a non-selective ETA-R/ETB-R antagonist (bosentan) (Fig. 4*A*). Similarly, although ETB-R expression was low (data not shown), ETA-R gene expression was increased 1.4-fold in ET-1 stimulated adipocytes and this effect was also abolished by all three antagonists (Fig. 4*B*). At the protein level, we observed a 20% increase in ETA-R content in 3T3-L1 cells incubated with ET-1, which was abolished by bosentan co-treatment (Fig. 4*C*).

ET-1 induces lipolysis in 3T3-L1 cells

Because bosentan treatment abolished the decrease in adipocyte size induced by IH exposure, we wanted to further investigate the metabolic effects of ET-1 *in vitro*. We measured supernatant glycerol release and glucose concentration in culture medium as markers of lipolysis and glucose uptake, respectively. 3T3-L1 cells were cultured under hypoxia (3% O_2) for 4 or 24 h. The addition of ET-1 (10 nM) to the culture medium resulted in an increased glycerol release at both time points that was blocked by bosentan (Fig. 5A). We further evaluated the effects of ET-1 in normoxic conditions. As shown in Fig. 5*B*, the addition of ET-1 (10 nM) for 24 h induced a 50% increase in glycerol release that was

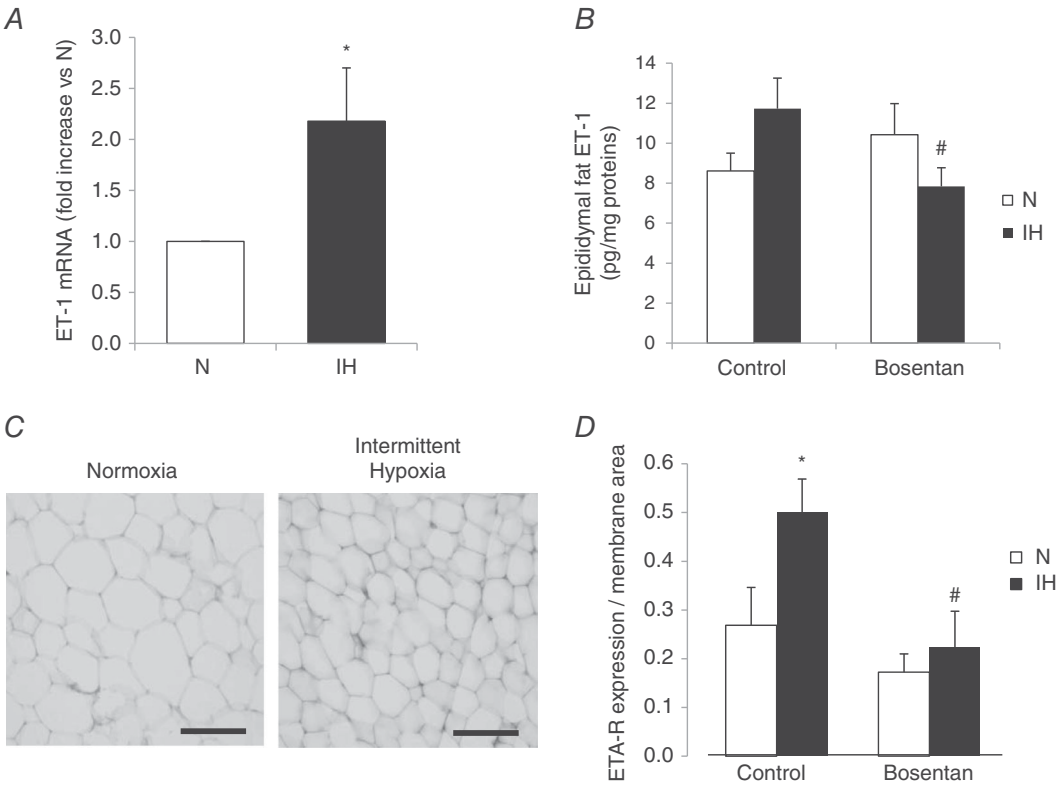

Figure 3. Intermittent hypoxia regulates adipose tissue ET-1 and ETA-R expression
A, qPCR quantification of ET-1 mRNA in epididymal fat of rats exposed to 14 days of normoxia (N) or intermittent hypoxia (IH). $^{*}P < 0.05$ *vs.* N; Mann–Whitney rank sum test. *B*, ET-1 protein level in epididymal fat extracts of rats exposed to 14 days of N or IH with or without oral bosentan treatment (100 mg kg^{-1} day^{-1}). $^{\#}P < 0.05$ *vs.* control; two-way ANOVA. *C*, immunohistochemistry of epididymal fat stained with anti-ETA-R antibody. Scale bar = 30 μm. *D*, quantification of ETA-R expression normalized to the area of cell membrane in the field, from at least three epididymal fat sections per rat ($n = 8$ rats per group). $^{*}P < 0.05$ *vs.* N and $^{\#}P < 0.05$ *vs.* control; two-way ANOVA.

totally abolished by ETA-R antagonist (BQ123) or mixed antagonist (bosentan) and partially blocked by ETB-R antagonist (BQ788). As expected, insulin (5 nM) inhibited glycerol release and the different endothelin receptor antagonists did not modify this effect. Co-incubation of adipocytes with insulin and ET-1 attenuated the endothelin-induced glycerol release (Fig. 5*B*). Insulin also significantly increased glucose uptake in 3T3-L1 cells (+72%) (Fig. 5*C*). ET-1 *per se* had no significant effect on glucose uptake but was able to reverse insulin-induced glucose uptake and this effect was inhibited by BQ123 and bosentan but not by BQ788 (Fig. 5*C*).

ET-1 regulates the phosphorylation of HSL

To better understand the mechanisms underlying the endothelin-induced lipolysis, we investigated its regulation of HSL and ATGL in 3T3-L1 adipocytes. We observed that total protein expression of HSL (Fig. 6*A*) and ATGL (Fig. 6*B* and *C*) did not change in the presence of ET-1 for 5 or 10 h. However, the activating phosphorylation of HSL on Ser^{660} was significantly increased by exposure to ET-1 for 5 and 10 h and this was abolished by bosentan (Fig. 6*C* and *D*). The inhibitory phosphorylation of HSL on Ser^{565} was not modified by ET-1 (Fig. 6*E*). Finally, HSL phosphorylation on Ser^{660} upon ET-1 treatment was also observed when the adipocytes were cultivated under a hypoxic environment with 3% oxygen (Fig. 7).

Discussion

By combining *in vivo* and *in vitro* approaches, the present study demonstrates that activation of the endothelin system in adipocytes is involved in the structural and functional adipose tissue remodelling induced by intermittent hypoxia exposure. We showed that endothelin promotes adipocyte lipolysis and also inhibits glucose

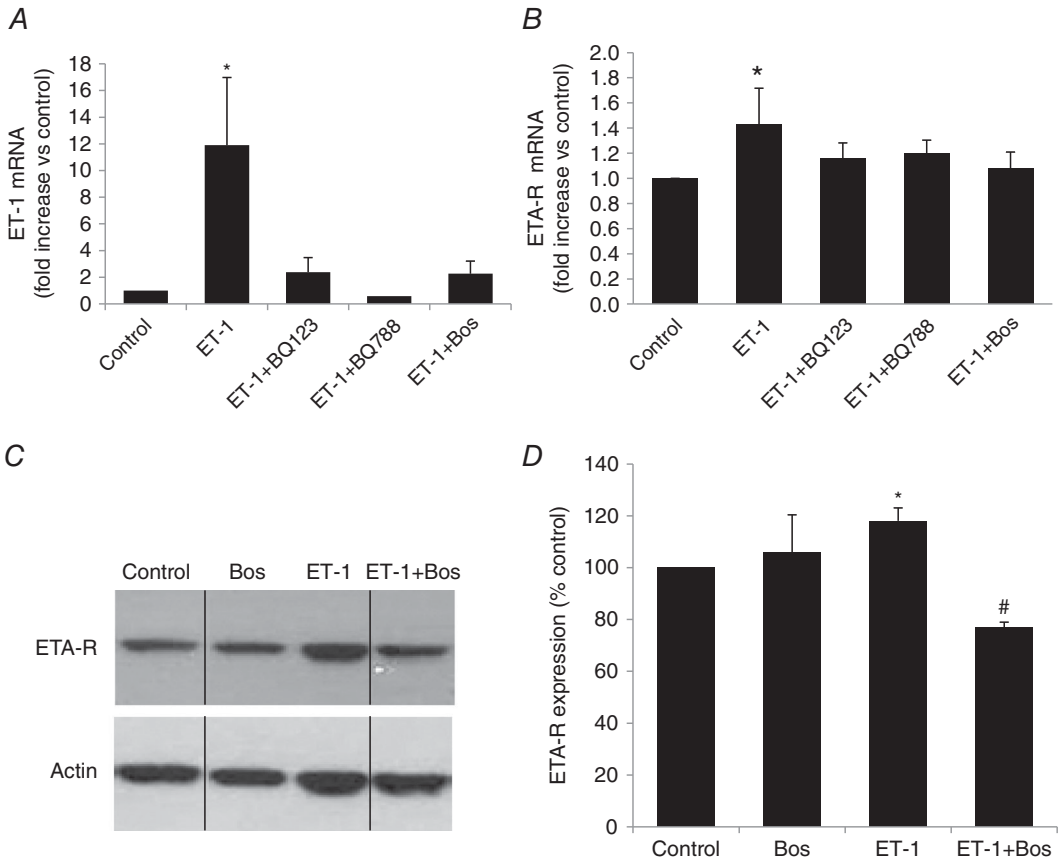

Figure 4. ET-1 regulates its expression and that of ETA-R in 3T3-L1 adipocytes
qPCR quantification of ET-1 (*A*) and ETA-R (*B*) mRNA in mature 3T3-L1 adipocytes exposed to ET-1 (10 nM) with or without BQ123, BQ788 or bosentan (Bos) (10 μM each) for 24 h. $^{*}P < 0.05$; one-way ANOVA on ranks. Western blot (*C*) and quantification (*D*) of ETA-R protein expression in mature 3T3-L1 adipocytes submitted to ET-1 (10 nM) with or without bosentan (10 μM) for 24 h. Quantification was performed on at least three independent experiments. $^{*}P < 0.05$ *vs.* control and $^{\#}P < 0.05$ *vs.* ET-1; two-way ANOVA. Vertical dividing lines indicate the components parts of a single image, where intercalating bands were suppressed.

uptake *in vitro*. We have also identified modulation of HSL activity as a potential mechanism behind the effects of endothelin on adipocyte metabolism (Fig. 8).

The decrease in adipose tissue mass and in adipocyte size induced by IH exposure is in agreement with recent data reported by our group (Poulain *et al.* 2014) and is indicative of increased lipolysis. We observed an epididymal fat loss of ∼1 g but it is probable that other fat compartments, such as subcutaneous fat, are also affected. Loss of body fat could be one of the mechanisms behind

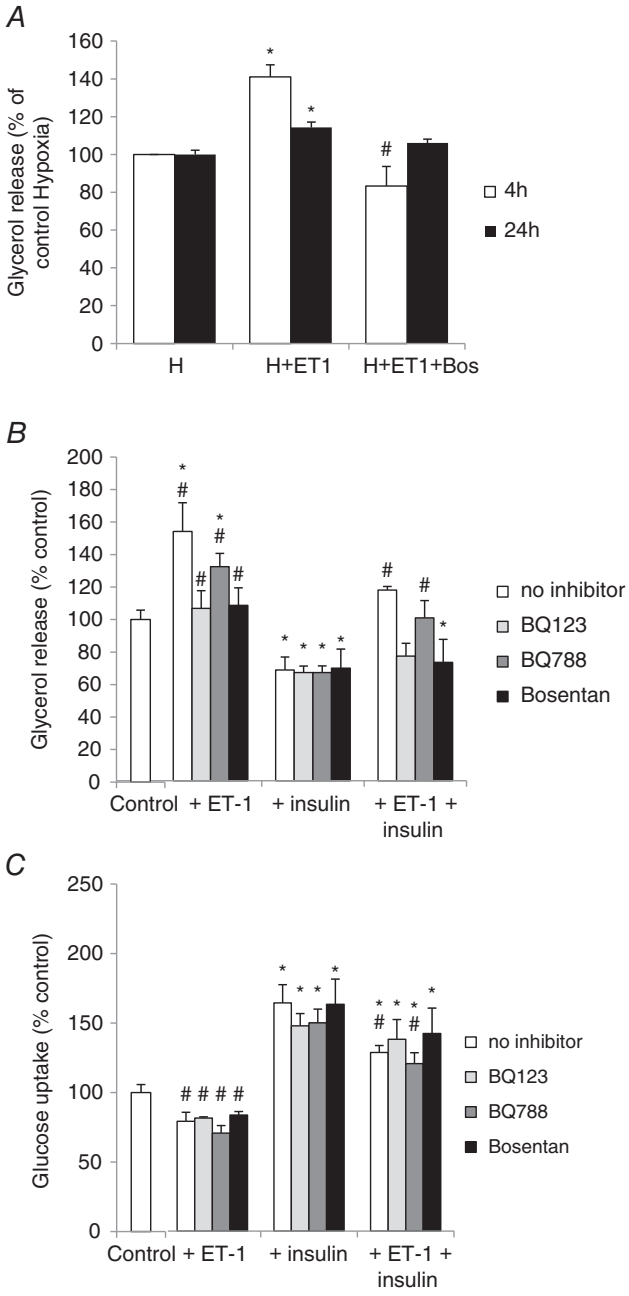

Figure 5. ET-1 induces lipolysis and reduces glucose uptake in 3T3-L1 adipocytes
A, lipolysis assessed by glycerol release (% of control hypoxic cells) in culture media of 3T3-L1 adipocytes cultivated under hypoxia (3% O_2) and treated or not with ET-1 (10 nM) and bosentan (Bos) (10 μM) for 4 or 24 h. $^{*}P < 0.05$ *vs.* control untreated cells. $^{\#}P < 0.05$ *vs.* the corresponding ET-1-treated cells; two-way ANOVA ($n = 4$ independent experiments). Lipolysis (*B*), assessed by glycerol release (% of control untreated cells) in culture media and glucose uptake (*C*) (% of control untreated cells) in 3T3-L1 adipocytes and treated or not for 24 h with ET-1 (10 nM), insulin (5 nM), BQ123, BQ788 or bosentan (10 μM each) under normoxia. $^{*}P < 0.05$ *vs.* control untreated cells. $^{\#}P < 0.05$ *vs.* the corresponding insulin-treated cells; one-way ANOVA on ranks ($n = 5$ independent experiments).

the lack of weight gain seen in IH-exposed rats compared to control rats. In agreement, a recent meta-analysis in OSA patients reported that continuous positive airway pressure treatment significantly increases the body mass index and body weight (Drager *et al.* 2015). Also relevant to the present study, there is clinical evidence for circulating ET-1 levels becoming elevated in OSA patients and being normalized by continuous positive airway pressure treatment (Jordan *et al.* 2005; Gjorup *et al.* 2007). In addition, triglyceride levels were improved in patients treated with atrasantan, an ET-1 receptor antagonist (Reriani *et al.* 2010). Similarly, in favour of a role of endothelin in the metabolic response to IH, we observed that bosentan treatment partially reversed fat loss and improved weight gain. Induction of the gene for ET-1 is under the control of the HIF-1 transcription factor in sustained hypoxia (Stow *et al.* 2011) and we have shown that the gene for ET-1 was upregulated by HIF-1 under intermittent hypoxia (Belaidi *et al.* 2009). In accordance, we observed that ET-1 transcription was increased in epididymal fat of rats exposed to IH. Nevertheless, other lipolytic factors, upregulated or not by HIF-1, might

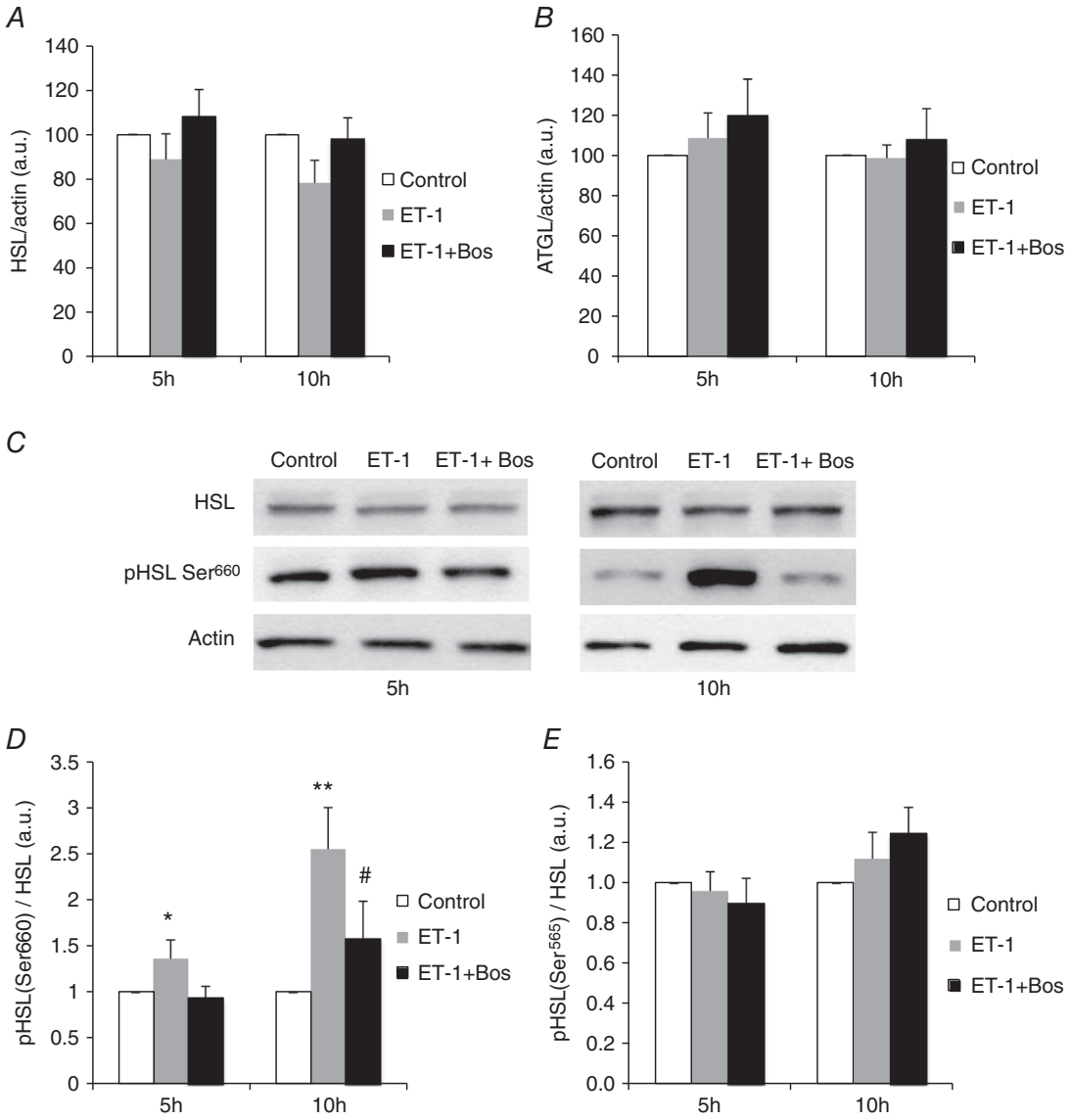

Figure 6. ET-1 regulates HSL phosphorylation but not HSL or ATGL expression
Western blot quantification (normalized to actin) of HSL (*A*) and ATGL (*B*) expression in 3T3-L1 adipocytes treated or not for 5 or 10 h with ET-1 (10 nM) and bosentan (Bos) (10 μM). *C*, western blots of HSL and phosphorylated (pHSL) on Ser^{660}. Quantification of pHSL (normalized to total HSL) on (*D*) activatory Ser^{660} and (*E*) inhibitory Ser^{565}. Western blots are representative of five independent experiments performed in duplicate except for (*B*) and (*E*) ($n = 3$ experiments performed in duplicate). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs*. untreated cells. $^{\#}P < 0.05$ *vs*. ET1-treated cells; two-way ANOVA.

be induced by intermittent hypoxia. Indeed, functional changes potentially leading to lipolysis were reported in adipocytes exposed to hypoxia, including a loss of insulin sensitivity, leptin upregulation and adiponectin downregulation (Trayhurn, 2014). Leptin, in particular, is associated with respiratory drive control (Campo *et al.* 2007) and has been shown to be upregulated in rodents exposed to CIH (Fu *et al.* 2015); moreover, leptin is involved in lipolysis in white adipocytes (Fruhbeck *et al.* 2001). Therefore, leptin may participate in the lipolytic remodelling of adipose tissue induced by IH exposure.

In accordance with the lipolytic effects of endothelin, we observed an ET-1-induced lipolysis *in vitro* in 3T3-L1 adipocytes along with inhibition of insulin-induced glucose uptake, and these findings were maintained in cells cultured under hypoxic conditions, supporting our hypothesis that endothelin may be one of the main mediators of the IH-induced reduction in adipocyte size. We also observed that 4 h, but not 24 h, of moderate hypoxia alone (3% O_2) induced lipolysis (124.7% *vs.* 100% in hypoxia *vs.* normoxia, respectively, $P = 0.028$). Thus, at 4 h of exposure, the effects of hypoxia and ET-1 were additive, leading to a global 75% increase in lipolysis compared to control normoxia. At 24 h, it appears that, in these cells and under moderate hypoxia (3% O_2), the effect of ET-1 is predominant over that of hypoxia alone. Lipolytic effects of hypoxia on 3T3-L1 adipocytes have also been reported in several other studies (Hashimoto *et al.* 2013; Regazzetti *et al.* 2009; Yin *et al.* 2009).

The effects of endothelin on lipolysis and glucose uptake in 3T3-L1 cells appear to be related to ETA, and not ETB, receptors, because they were blocked by BQ123 and bosentan but not by BQ788. It was also reported that ET-1-mediated lipolysis (Juan *et al.* 2005) and adiponectin secretion (Juan *et al.* 2007) were blocked by selective ETA-R but not by ETB-R antagonists. Similarly, a study on human adipocytes reported that ET-1 increases lipolysis via the activation of ETA-R (Eriksson *et al.* 2009). Therefore, endothelin appears to have anti-insulin effects on lipolysis and glucose uptake. In agreement, another study showed that ET-1 affects insulin signalling and counteracts insulin-induced inhibition of lipolysis by decreasing the expression of insulin receptor, IRS-1 and PDE-3B, although, by contrast with our results, in this study ETB-R through protein kinase C and calmodulin pathways, rather than ETA-Rs, appear to mediate ET-1 signalling (van Harmelen *et al.* 2008).

Although endothelin-induced lipolysis *in vitro* has already been reported, very little is known about the mechanisms involved. We therefore investigated whether

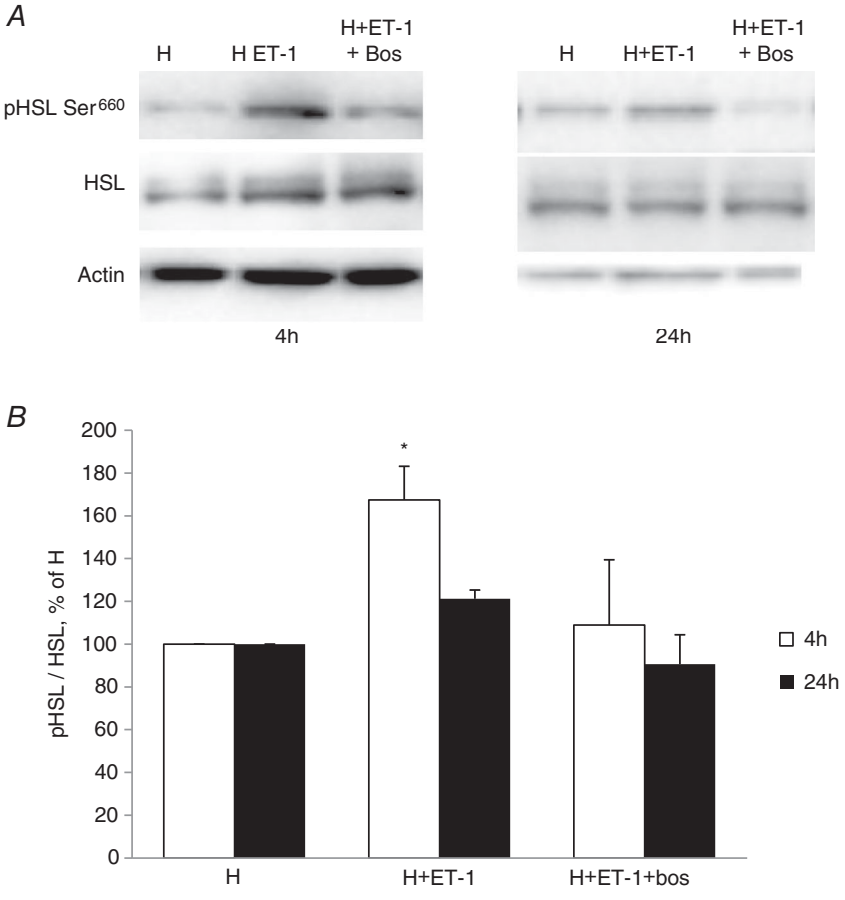

Figure 7. Endothelin-1 induces HSL phosphorylation in 3T3-L1 cultivated in hypoxia
A, HSL phosphorylation on Ser^{660} in 3T3-L1 adipocytes cultivated under hypoxia (H) (3% O_2) and treated or not with ET-1 (10 nM) and bosentan (Bos) (10 μM) for 4 or 24 h. *B*, quantification of the western blots. Western blots are representative of three independent experiments performed in triplicate.
$^*P < 0.05$ *vs.* control H; two-way ANOVA. $P = 0.054$ at 4 h for H + ET1 + Bos *vs.* H + ET1.

ET-1 could modify the expression and/or phosphorylation of the two main lipases involved in adipose tissue lipolysis, namely HSL and ATGL. Although the expression of both lipases was not modified, ET-1 induced the phosphorylation of HSL on Ser^{660} without affecting Ser^{565} phosphorylation. Because Ser^{660} phosphorylation is known to be activatory (Anthonsen *et al.* 1998), the ET-1-induced lipolysis could thus be explained by increased HSL activity, which promotes the second step of lipolysis. The classical pathway of Ser^{660}HSL phosphorylation and lipolysis activation in adipocytes is cAMP-dependent PKA activation. Although ET-1 signalling is not coupled to cAMP production, it has the ability to activate PKA in a cAMP-independent fashion (Dulin *et al.* 2001). Moreover, ET-1 receptors are coupled to various signalling pathways, including protein kinase C, extracellular signal regulated kinase 1/2 and p38 mitogen-activated protein kinase (Sugden, 2003), which can also modulate HSL and ATGL activity (Chaves *et al.* 2011) and be involved in its lipolytic effects.

In addition to the lipolytic effect of ET-1, it could be hypothesized that the adipose tissue remodelling induced by IH could also be a result of the inhibition of FFA intake and triglyceride lipogenesis. Indeed, ET-1 decreases adipogenesis during 3T3-L1 adipocyte differentiation (Bhattacharya & Ullrich, 2006) and increases the expression of resistin, an adipogenesis-inhibiting hormone (Tang *et al.* 2014). Although bosentan treatment decreased plasma triglycerides and FFAs in our rats, we did not observe any significant effect of intermittent hypoxia on these parameters. Most studies investigating the effects of IH on plasma lipid levels have been performed in mice and have shown that FFAs and/or triglycerides increase in response to short-term or long-term IH exposure (Li *et al.* 2005; Jun *et al.* 2010; Poulain *et al.* 2014). A possible explanation for our results could be that no lipoprotein lipase inhibitor was added to our samples. However, Perry *et al.* (2007) have shown that the duration of exposure was an important factor in determining the metabolic response to IH in rats because short-term exposure did not affect plasma lipids, whereas a 3-week exposure significantly increased triglyceride levels. Therefore, the lack of an effect observed in the present study could be the result of a species difference and/or the duration of IH exposure.

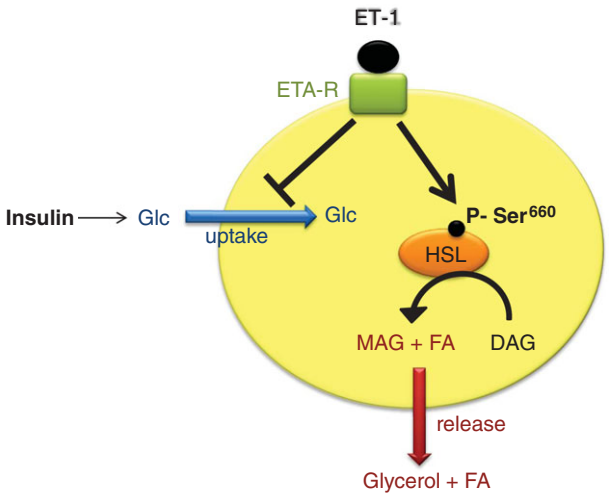

Figure 8. Proposed mechanisms explaining the dual action of endothelin-1 on glucose and lipid metabolism in adipocytes
ET-1 binding to its ETA receptor inhibits insulin-induced glucose uptake and activates HSL phosphorylation on Ser^{660}, thereby inducing diacylglycerol (DAG) lipolysis into monoacylglycerol (MAG) + fatty acid (FA). MAG conversion by monoglyceride lipase (not represented) results in glycerol and fatty acid release. Glc, glucose.

A novel finding of the present study is that intermittent hypoxia stimulates ET-1 and ETA receptor expression in adipose tissue *in vivo*. Although ETA-Rs were expressed by adipocytes, the origin of ET-1 mRNA in adipose tissue remains unclear. Indeed, endothelial cells are well known for synthesizing endothelin in response to hypoxia (Yamashita *et al.* 2001). Although ET-1 production by primary adipocyte has not been demonstrated, we observed a production of ET-1 mRNA in differentiated 3T3-L1 adipocytes suggesting, for the first time, that fat-like cells could also contribute to adipose tissue ET-1 secretion.

Another important finding is our observation that ET-1 *per se* stimulates its own expression, as well as its ETA-R expression, in cultured adipocytes. This could potentially induce a positive feedback loop contributing to enhanced endothelin system activation in response to IH. Our results, showing that bosentan treatment abolishes the adipose tissue increase in ET-1 and ET-A receptors, are in accordance with this hypothesis. However, the potential mechanisms involved remain to be determined. As already noted, the ET-1 gene is induced by the HIF-1 transcription factor and ET-1 is known to activate HIF-1 (Wilson *et al.* 2006; Spinella *et al.* 2007), possibly through its ability to promote NADPH expression and the generation of superoxide anions (Duerrschmidt *et al.* 2000; Li *et al.* 2003), which are powerful activators of HIF-1 (Semenza, 2009). Promoting HIF-1 activity could thereby provide a possible mechanism by which ET-1 might induce a positive retroactive effect on its own transcription.

To conclude, the present study shows that blockade of the effects of endothelin by bosentan is able to reverse the intermittent hypoxia-induced lipolysis *in vivo*. This could be explained by the ability of bosentan to inhibit the expression of both ET-1 and ETA-Rs. We confirmed these results in an adipocyte model *in vitro*, by showing that endothelin increases lipolysis in an ETA-R-dependent manner. Finally, we demonstrated that endothelin induces the phosphorylation of HSL on its activatory Ser^{660}, providing a possible mechanism to explain its lipolytic effect in adipocytes.

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Additional information

Competing interests

The authors declare that they have no competing interests.

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Author contributions

ABM, MH, DM, FHP and PF conceived, designed and performed the experiments, and analysed the data. DGR designed the study. ABM, DM, MH, PF, JLP and DGR were involved in manuscript preparation. All authors revised the manuscript for intellectual and form improvements. All authors have approved the final version of the manuscript and agree to be accountable for all

aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

The following supporting information is available in the online version of this article.

Table S1. Primer sequences and qPCR experimental conditions