ORIGINAL RESEARCH

Inhibition of Lipolysis Ameliorates Diabetic Phenotype in a Mouse Model of Obstructive Sleep Apnea

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Abstract

Obstructive sleep apnea (OSA) is associated with insulin resistance, glucose intolerance, and type 2 diabetes. Causal mechanisms mediating this association are not well defined; however, augmented lipolysis in adipose might be involved. Here, we investigated the effect of acipimox treatment (lipolysis inhibitor) on glucose tolerance and insulin sensitivity in mice exposed to intermittent hypoxia (IH). C57BL6/J mice were exposed for 14 days to IH or control conditions. IH was created by decreasing the fraction of inspired oxygen from 20.9 to 6.5%, 60 times/h. Control exposure was air (fraction of inspired oxygen, 20.9%) delivered at an identical flow rate. Acipimox was provided in drinking water (0.5 g/ml) during exposures. After exposures, intraperitoneal insulin (0.5 IU/kg) and glucose (1 g/kg) tolerance tests were performed, and primary adipocytes were isolated for lipolysis experiments. IH elevated fasting glucose by 51% and worsened glucose tolerance and insulin sensitivity by 33 and 102%, respectively. In parallel, IH increased spontaneous lipolysis by 264%, and reduced epididymal fat mass by 15% and adipocyte size by 8%. Acipimox treatment prevented IH-induced lipolysis and increased epididymal fat mass and adipocyte size by 19 and 10%, respectively. Acipimox fully prevented IH-induced impairments in fasting glycemia, glucose tolerance, and insulin sensitivity. For all reported results, P less than 0.05 was considered significant. Augmented lipolysis contributes to insulin resistance and glucose intolerance observed in mice exposed to IH. Acipimox treatment ameliorated the metabolic consequences of IH and might represent a novel treatment option for patients with obstructive sleep apnea.

Keywords: intermittent hypoxia; diabetes; insulin resistance; obstructive sleep apnea; lipolysis

Clinical Relevance

This study suggests that adipose tissue lipolysis represents a mechanism linking obstructive sleep apnea syndrome with glucose intolerance, insulin resistance, and type 2 diabetes in a mouse model. Furthermore, the study shows that pharmacological inhibition of lipolysis ameliorated detrimental metabolic effects induced by intermittent hypoxic exposure.

Obstructive sleep apnea (OSA), with a prevalence of 5–15%, represents a common condition in the adult population (1). Repetitive partial or complete collapse of the upper airway during sleep results in periodic drops in blood oxygen levels (hypoxemia) and sleep fragmentation. Previous research has identified OSA as an independent risk factor for hypertension, cardiovascular diseases, stroke, and all-cause mortality (2, 3). More recently, studies indicate that OSA is also associated with glucose intolerance, insulin resistance, and type 2 diabetes mellitus (T2DM), independently of other confounding

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factors, including age, obesity, and central adiposity (4, 5).

Although epidemiological associations between OSA and impaired glucose metabolism have been repeatedly reported, mechanisms linking OSA with impaired glucose homeostasis are not well understood. Several animal studies have suggested that exposure to intermittent hypoxia (IH) compromised whole-body glucose homeostasis, reducing insulin sensitivity, glucose tolerance, and muscle glucose uptake; at the same time, it elevated fasting blood glucose, augmenting hepatic glucose release, and impairing secretion from pancreatic β cells (6–11). Similarly, exposure of healthy human volunteers to IH for 5 hours induced insulin resistance and impaired pancreatic β cell function (12). Although molecular mediators are only partially understood, increased sympathetic activity, elevated corticosteroid levels, oxidative stress, elevated plasma endothelin-1 levels, and activation of inflammatory pathways represent frequently suggested candidate mechanisms (4).

Impaired adipose tissue function has been shown to causally contribute to the pathogenesis of T2DM (13, 14) through changes in adipokine production and through the release of free fatty acids (FFAs) into the systemic circulation. Chronically elevated plasma FFA levels induce insulin resistance in muscle and liver (15) and impair pancreatic β cell function, resulting in decreased insulin secretion (16), which represents characteristic impairment defining T2DM. Importantly, endocrine and metabolic functions of adipose tissue are profoundly modified by tissue oxygen levels. Tissue O_2 levels as low as 15 mm Hg have been seen to develop in adipose tissue as adipocyte size increases in obese humans and rodents (17). Lowered tissue O_2 levels have been reported to increase lipolysis, decrease adipogenesis, alter the profile of secreted adipokines, and induce insulin resistance in adipose tissue (18). In contrast to the sustained adipose tissue hypoxia found in obesity, OSA is characterized by repetitive drops in arterial O_2 levels that induce tissue-specific changes in oxygenation in various organs (19). It is currently unknown whether adipose tissue metabolism is involved in IH-induced changes in glucose metabolism.

The purpose of this study was to test the hypothesis that increased adipose tissue lipolysis plays a central role in the pathogenesis of impaired glucose metabolism associated with IH exposure. We employed a mouse model of OSA to: (*1*) elucidate the impact of IH on basal and stimulated lipolysis in adipocytes; and (*2*) determine whether pharmacological suppression of lipolysis prevents development of hyperglycemia, glucose intolerance, insulin resistance, and β cell dysfunction.

Materials and Methods

A comprehensive description is provided in the online supplement.

Protocol for IH

Male mice (C57BL6/J, $n = 10$ per group) were exposed for 14 days to IH or control conditions. Oxygen levels inside cages were altered by administration of N_2 so that oxygen levels decreased from 21 to 6–6.5% during 30 seconds and subsequently returned to 21% in 30 seconds. Control experiments were performed by administration of compressed air. Acipimox (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) was administered in drinking water. The protocol was approved by the Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD; protocol no. MO11M263) and the National Institute of Public Health in Prague (Czech Republic; protocol no. 72,015).

Intraperitoneal Glucose and Insulin Tolerance Tests

Blood glucose was determined using a glucometer (Accu-Check Aviva; Roche, Indianapolis, IN) at 0, 10, 20, 30, 60, 90, and 120 minutes after intraperitoneal injection of 1 g/kg glucose ($n = 10$ per group) or at 0, 10, 20, 30, 40, 50, 60, 90, and 120 minutes after intraperitoneal injection of 0.5 IU/kg insulin (ITT; $n = 10$ per group).

Lipolysis Determination in Isolated Adipocytes

Epididymal fat pads were cut and digested using 1.25 mg/ml collagenase (Sigma-Aldrich) in Krebs Ringer bicarbonate buffer containing 10 mmol/L Hepes, 2% BSA, 500 nM adenosine, and 6 mmol/L glucose at pH 7.4. Catecholamine-stimulated lipolysis was investigated by adding isoprenaline (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, or 10^{-9} mol/L) to the Krebs Ringer bicarbonate buffer supplemented with 1 U/ml adenosine deaminase and 100 nM (−)-N^6-(2-phenylisopropyl)-adenosine and incubated for 120 minutes (20). Glycerol released into the buffer was determined and used as a marker of lipolysis. Total lipid content in each well was measured and adipocyte number was calculated. Lipolytic rate was expressed as glycerol released during 120 minutes per 10,000 cells.

Biochemical Measurements

Plasma insulin and leptin levels were determined using ELISA kits (Mouse Ultrasensitive EIA Kit; Alpco, Salem, NH; and Leptin Mouse ELISA Kit; Abcam, Cambridge, UK). Plasma glucose (Glucose Oxidase Assay Kit; Abcam) and FFA levels were quantified (NEFA-HR2; Wako Chemicals Inc., Richmond, VA).

Western Blotting and Gene Expression

Samples of femoral muscle and epididymal adipose tissues were homogenized and proteins separated using PAGE. Membranes were blocked with 5% albumin and incubated overnight with antibodies against total and phosphorylated Akt (product nos. 4691 and 4056; Cell Signaling, Danvers, MA); α-tubulin served as a loading control (prod no. ab7291; Abcam, Cambridge, MA). Gene expression of adenosine, leptin, and leptin receptors were assessed in epididymal adipose tissue using quantitative PCR. Total RNA was extracted using Tripure reagent and total RNA was reverse transcribed using the SuperScript III system (Invitrogen, Carlsbad, CA). Taqman probes Mm00545720, Mm00434759, and Mm00440181 were used. Glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene.

Statistical Analysis

Interaction between acipimox treatment and IH exposure was assessed using two-way ANOVA. All analyses were performed and figures were produced using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Values of P less than 0.05 were considered statistically significant. Data are presented as mean (±SEM).

Results

Parameters of Adiposity

Total body weight decreased with exposure to IH by 7.9 (±0.5%; 24.8 ± 0.5 g versus

22.6 ± 0.4 g; $P < 0.05$), whereas acipimox-treated mice lost only 2.2 (±1.0%; 23.8 ± 0.3 g versus 23.3 ± 0.3 g; $P < 0.05$) during IH exposure (ANOVA, $P < 0.05$ for interaction). Accordingly, exposure to IH reduced epididymal adipose tissue mass by 15% (583.3 ± 28.9 versus 494.7 ± 20.6 mg; $P < 0.05$) and decreased the proportion of epididymal fat to total body weight compared with weight-matched mice exposed to control conditions (2.5 ± 0.1 versus 2.2 ± 0.1%; $P < 0.05$) (Figures 1A and 1B). Furthermore, average adipocyte size was reduced by 8% (78.7 ± 1.8 versus 72.4 ± 1.4 μm; $P < 0.05$) and adipocyte size distribution was shifted to the left with IH exposure (Figure 1C). In contrast, acipimox treatment during IH exposure prevented changes induced by IH (i.e., increasing epididymal fat mass, 520.3 ± 30.4 versus 623.2 ± 38.9 mg, $P < 0.05$; preserving adipocyte size, 74.6 ± 3.4 versus 80.2 ± 1.7 μm, $P > 0.05$; and shifting adipocyte size distribution to the right; Figures 1A–1D). In a separate group of mice that were exposed to neither IH nor control conditions, acipimox administration for 14 days did not affect body weight compared with vehicle-treated mice (25.7 ± 0.6 g versus 26.2 ± 0.6 g and 25.6 ± 0.4 g versus 26.8 ± 0.5 g, respectively; $P > 0.05$).

The Effect of Acipimox on IH-Induced Impairments in Glucose Metabolism

Fasting glucose levels increased by 76% (86.6 ± 6 versus 152.2 ± 5.4 mg/dl; $P < 0.05$) and glucose tolerance worsened by 33% after IH exposure (area under curve$_{glucose}$ [AUC$_{glucose}$] = 18.2 ± 0.7 versus 24.2 ± 0.5 × 10^3 mg/dl/120 min × 10^3; $P < 0.05$). Acipimox administration prevented IH-induced changes in fasting glucose (137.0 ± 6 versus 148.9 ± 4.9 mg/dl; $P < 0.05$) and glucose tolerance (26.9 ± 0.9 versus 24.8 ± 0.7 × 10^3 mg/dl/120 min × 10^3; $P > 0.05$; two-way ANOVA, $P < 0.05$). Fasting plasma insulin levels as well as insulin sensitivity evaluated using the Homeostasis Model Assessment–insulin resistance index, reflected mainly hepatic insulin sensitivity, which was decreased by 46% and 36%, respectively, after IH exposure. Acipimox treatment did not prevent this effect (decline by 38% and 37%, respectively; all $P < 0.05$). In contrast, insulin sensitivity in a postprandial state (ITT test), reflecting muscle insulin sensitivity, worsened with exposure to IH by 34% (5.2 ± 0.5 versus 6.9 ± 0.3 × 10^3 mg/dl/120 min; $P < 0.05$), but this effect was completely ameliorated with acipimox administration during IH (10.0 ± 0.9 versus 8.2 ± 0.7 × 10^3 mg/dl/120 min; $P > 0.05$). Furthermore, IH exposure impaired pancreatic β cell function (Homeostasis Model Assessment–β cell function), which only partially improved ($P = 0.09$) with acipimox treatment. Data are summarized in Table 1 and Figure 2. Importantly, acipimox administration under physiological conditions had no detectable effect on glucose tolerance or insulin sensitivity, as demonstrated in a separate group of mice not exposed to insulin (mice were not exposed to either IH or control conditions; Figures 2E and 2F). Phosphorylation of protein kinase B (AKT) (threonine 308) in skeletal muscle was decreased by 30% during IH exposure (fold change = 1.00 ± 0.10 versus 0.70 ± 0.08 for control and IH-exposed groups, respectively; $P = 0.06$); however, the negative effect of IH was not present in the acipimox-treated group (fold change = 0.67 ± 0.11 versus 0.66 ± 0.04; $P > 0.05$). Opposite results were observed in adipose tissue, where phosphorylated AKT (pAKT) increased by 96% with IH exposure in the vehicle-treated group (fold change = 1.0 ± 0.12 versus 1.96 ± 0.09 for control and IH-exposed groups, respectively; $P < 0.05$) as well as in the acipimox-treated groups (fold change = 1.42 ± 0.14 versus 2.45 ± 0.18 for control and IH-exposed groups, respectively; $P < 0.05$). Acipimox administration decreased pAKT in muscle (fold change = 1.00 ± 0.10 versus 0.67 ± 0.11 for vehicle-treated and acipimox-treated control groups, respectively; $P = 0.07$), but had no effect on pAKT in adipose tissue (fold change = 1.0 ± 0.12 versus 1.42 ± 0.14 for vehicle-treated and acipimox-treated control groups, respectively; $P > 0.05$). Data are summarized in Figures 3A and 3B. Plasma leptin levels remained unchanged during IH exposure or acipimox

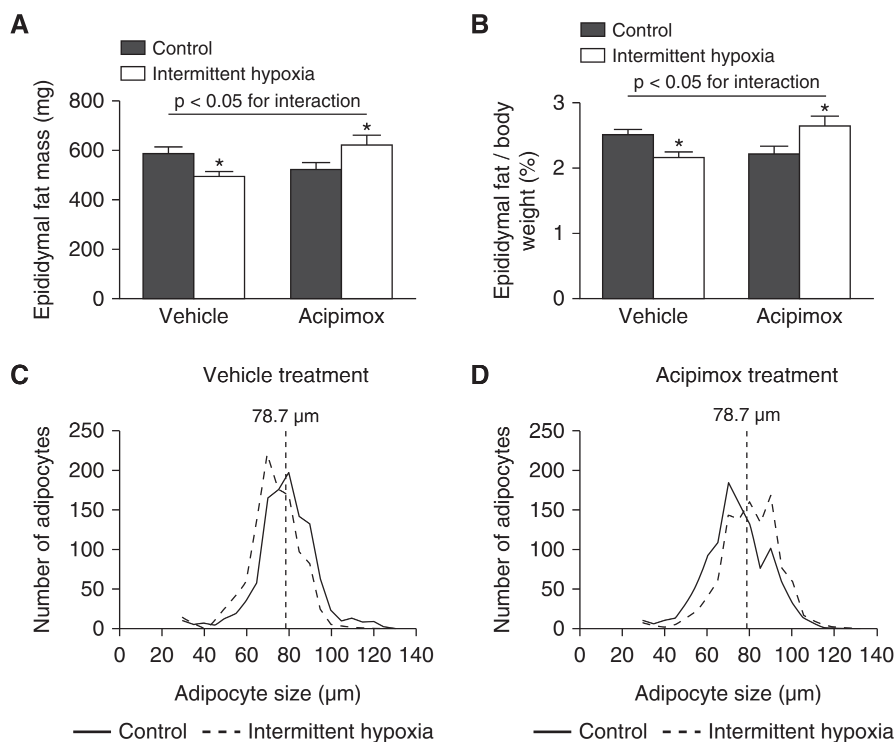

Figure 1. Parameters of adiposity and adipocyte size distribution. (*A*) Epididymal fat mass (mg) in mice exposed to control conditions or intermittent hypoxia (IH) for 14 days (d) while treated with acipimox or vehicle. (*B*) Proportion of epididymal fat mass to total body weight (%) in mice exposed to control conditions or IH for 14 d while treated with acipimox or vehicle. (*C*) Histogram of adipocyte size distribution in mice treated with vehicle. (*D*) Histogram of adipocyte size distribution in mice treated with acipimox. The *dashed lines* represent the average adipocyte size of the vehicle-treated control group. *$P < 0.05$ for the effect of exposure to IH ($n = 15$ for each group). Data are presented as mean (±SEM).

Table 1. Biochemical Parameters in All Treatment Groups

	Vehicle Treatment		Acipimox Treatment	
	Control	Intermittent Hypoxia	Control	Intermittent Hypoxia
Glucose, mg/dl	87 ± 6	152 ± 5.0*	137 ± 6.0	149 ± 5.0†
Insulin, ng/ml	0.38 ± 0.05	0.15 ± 0.02*	0.56 0.14	0.36 ± 0.11*
Leptin, pg/ml	352.6 ± 18.7	297.6 ± 23.1	295.5 ± 31.7	257.0 ± 33.7
GTT $AUC_{glucose}$, mg/dl/120 min × 10^3	18.2 ± 0.7	24.2 ± 0.5*	26.9 ± 0.9	24.8 ± 0.7†
ITT $AUC_{glucose}$, mg/dl/120 min × 10^3	5.2 ± 0.5	6.9 ± 0.3*	10.0 ± 0.9	8.2 ± 0.7*,†
HOMA-IR	2.5 ± 0.5	1.6 ± 0.3*	6.0 ± 1.5	3.8 ± 1.1*
HOMA-β	53.5 ± 9.8	18.5 ± 2.3*	68.0 ± 16.4	43.2 ± 12.7*
FFA, μmol/L	276.1 ± 22.3	420.9 ± 25.0*	323.3 ± 41.0	306 ± 28.1†

Definition of abbreviations: $AUC_{glucose}$, area under $curve_{glucose}$; FFA, free fatty acids; GTT, intraperitoneal glucose tolerance test; HOMA-β, Homeostasis Model Assessment–β cell function; HOMA-IR, Homeostasis Model Assessment–insulin resistance; ITT, intraperitoneal insulin tolerance test.
Values represent mean ± SEM (n = 10 for all groups).
*$P < 0.05$ for the effect of exposure to intermittent hypoxia.
†$P < 0.05$ for the interaction between exposure and acipimox treatment (two-way ANOVA).

administration ($P > 0.05$, two-way ANOVA), as summarized in Table 1.

The Effect of Acipimox on Adipocyte Lipolysis and Plasma FFA Levels

Lipolytic rate increased by 184% (19.0 ± 4.7 versus 53.8 ± 7.7 nmol/120 min/10,000 cells; $P < 0.05$) in adipocytes from mice exposed to IH. Treatment with acipimox prevented IH-induced stimulation of lipolysis (36.1 ± 4.8 versus 39.8 ± 6.8 nmol/120 min/10,000 cells for control and IH-exposed groups, respectively; $P > 0.05$), as shown in Figure 4A. In parallel, plasma FFA levels increased with exposure to IH by 54% (274.1 ± 22.3 versus 420.9 ± 25.9 μmol/L; $P < 0.05$), whereas this effect was ameliorated by acipimox treatment (324.3 ± 41.1 versus 306.5 ± 28.1 μmol/L for control and IH-exposed group, respectively; $P > 0.05$) (Table 1).

Administration of isoprenaline stimulated lipolysis in a dose–response manner under all experimental conditions (Figure 4). Sensitivity of the lipolytic pathway to isoprenaline stimulation, assessed as half-maximal effective concentration ($EC_{50[iso]}$), was unaffected by either IH exposure or acipimox treatment ($EC_{50[iso]}$ = 1.1 ± 0.4, 0.6 ± 0.4, 1.3 ± 0.5, and 1.5 ± 0.5 nmol/L for control, IH-exposed, acipimox-treated control, and acipimox-treated IH-exposed groups, respectively; two-way ANOVA, $P > 0.05$). However, responsiveness to isoprenaline, manifested as the maximal lipolytic rate achieved with administration of the highest isoprenaline concentration, was suppressed by exposure to IH in both vehicle- and acipimox-treated groups (144.3 ± 38.3 versus 31.2 ± 8.7 and 70.5 ± 12.0 versus 44.3 ± 9.0 nmol/120 min/10,000 cells, respectively; two-way ANOVA, $P < 0.05$), as shown in Figures 4B and 4C.

Gene expression of adenosine deaminase, representing a key enzyme inactivating adenosine, was not affected by IH in vehicle-treated (fold change = 1.0 ± 0.1 versus 1.0 ± 0.1 for control and IH-exposed groups, respectively) or acipimox-treated groups (fold change = 0.9 ± 0.1 and 0.9 ± 0.1 for control and IH-exposed groups, respectively; two-way ANOVA, $P > 0.05$). Similarly, gene expression of leptin and leptin receptors in adipose tissue was not modified by either IH exposure or acipimox administration ($P > 0.05$, two-way ANOVA; Figures 5A and 5B).

Discussion

This study investigated whether enhanced adipose tissue lipolysis represents a mechanism linking IH exposure with hyperglycemia, glucose intolerance, insulin resistance, and β cell dysfunction. We demonstrated that IH exposure significantly increased spontaneous adipocyte lipolysis, elevated plasma FFA levels, and impaired glucose homeostasis, whereas pharmacological inhibition of lipolysis with acipimox prevented IH-induced increases in lipolysis, elevation of plasma FFA levels, and ameliorated detrimental effects of IH exposure on glucose metabolism.

Stimulation of lipolysis under IH exposure reduced epididymal fat mass and adipocyte size, and resulted in total body weight reduction, which has also been reported in previously published studies (6, 7, 21); however, acipimox administration prevented IH-induced weight loss and preserved fat mass as well as adipocyte size. Consequently, acipimox-treated mice presented with higher body weight and larger adipocyte size compared with vehicle-treated groups at the end of exposures. Because metabolic parameters in mice are strongly determined by body weight (22) and adipocyte size (23), anthropometric differences between acipimox- and vehicle-treated groups might explain higher spontaneous lipolytic rates and higher fasting glucose levels in acipimox-treated versus vehicle-treated control groups. To address the possibility that acipimox administration itself elevated body weight and fasting glucose (and adversely affected metabolic profile), separate groups of mice were treated with acipimox or vehicle, but were not subject to any exposures (i.e., IH or control conditions). Results of this experiment showed that acipimox administration *per se* had no effect on body weight, fasting glucose, glucose tolerance, or insulin sensitivity, thus supporting the notion that acipimox-mediated suppression of lipolysis in mice exposed to IH is the actual mechanism mediating improvements in glucose metabolism.

Causal association between OSA and development of T2DM has been suggested, based on epidemiological data (1) supported by human (5, 12) and rodent (6, 7, 24, 25) experiments that documented the potential of IH exposure to induce

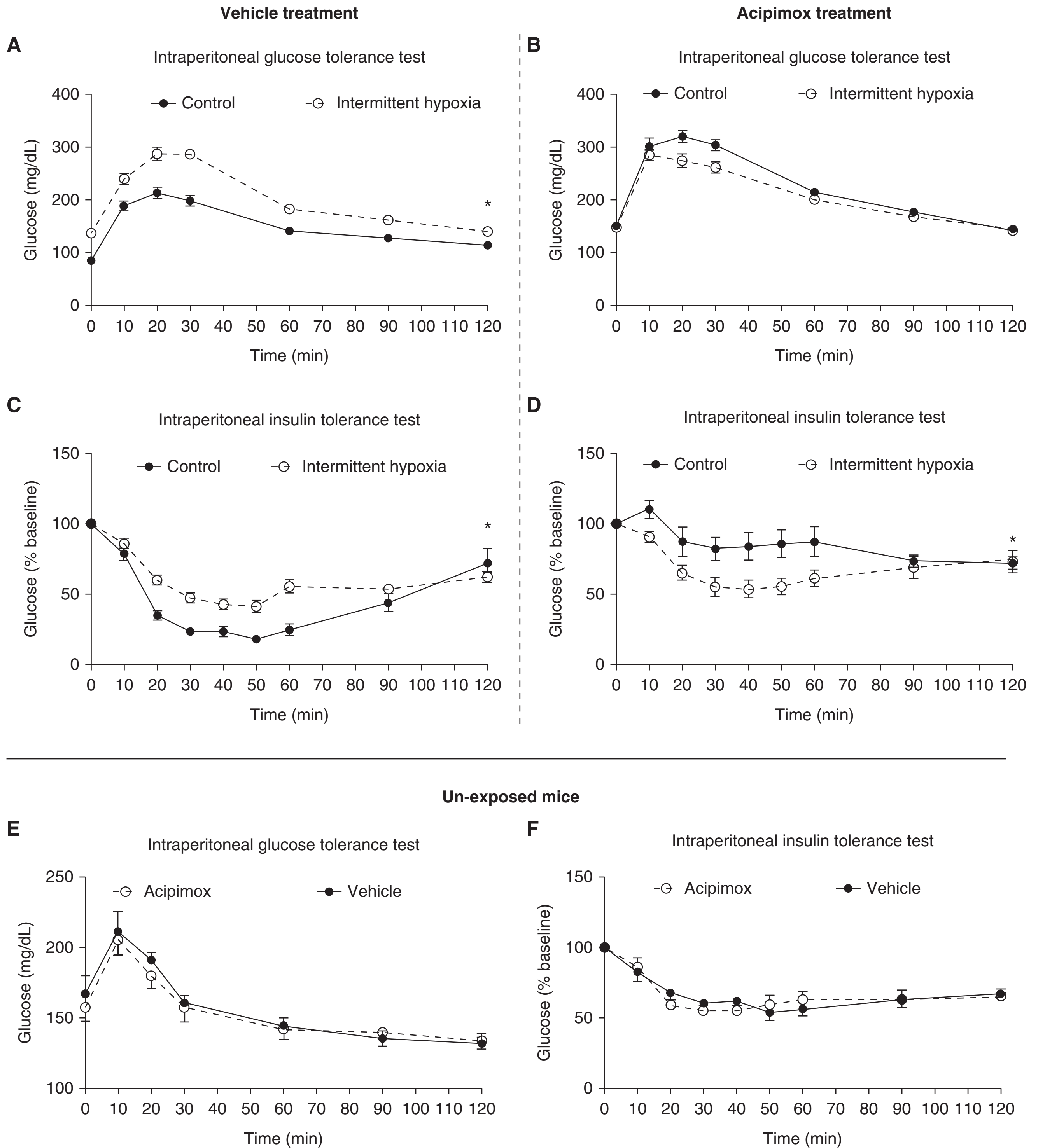

Figure 2. Intraperitoneal glucose and insulin tolerance tests. Individual values of plasma glucose after intraperitoneal administration of 1 g/kg glucose in mice exposed to control conditions and IH for 14 days while treated with vehicle (*A*) or acipimox (*B*). Individual values of plasma glucose after intraperitoneal administration of 0.5 IU/kg insulin in mice exposed to control conditions and IH for 14 days while treated with vehicle (*C*) or acipimox (*D*). Intraperitoneal glucose (*E*) and insulin tolerance (*F*) tests in unexposed animals (not exposed to IH or control conditions) treated with acipimox. $^{*}P < 0.05$ for the effect of exposure to IH ($n = 10$ for groups exposed to IH and control conditions; $n = 5$ for unexposed groups). Data are presented as mean (±SEM).

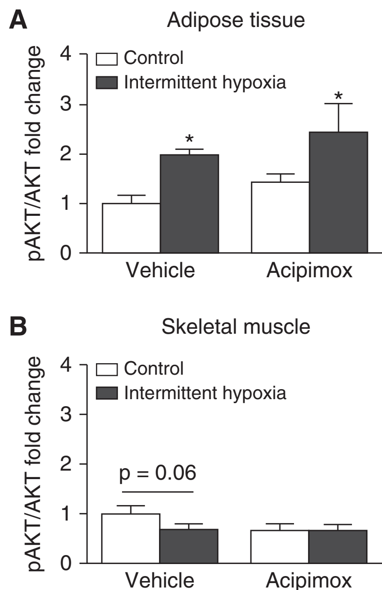

Figure 3. Insulin signaling pathway activation in muscle and adipose tissue. Phosphorylation of AKT (protein kinase B) was assessed in femoral muscle (*A*) and epididymal adipose tissue (*B*) of mice exposed to control conditions or IH for 14 days while treated with acipimox or vehicle. Data are expressed as fold changes over vehicle-treated mice exposed to control conditions. $^{*}P < 0.05$ for the effect of exposure to IH ($n = 8$ for skeletal muscle; $n = 5$ for adipose tissue). Data are presented as mean (±SEM). AKT, protein kinase B; pAKT, phosphorylated protein kinase B.

hyperglycemia, glucose intolerance, and insulin resistance, and to impair insulin secretion. However, molecular and endocrine mechanisms underlying this link remain only partially elucidated (4). We have previously demonstrated that cessation of IH exposure alone was not sufficient to reverse IH-induced impairments in glucose tolerance, insulin sensitivity, and β cell dysfunction (7), which warrants a search for novel treatments, particularly when we consider that a significant proportion of patients with OSA don't tolerate continuous positive airway pressure therapy (26). In view of this, adipose tissue lipolysis represents an attractive target and one that motivated this study. First, chronically elevated circulating FFAs, as a product of lipolysis, were found to be causally linked to the development of liver and muscle insulin resistance, glucose intolerance, and β cell dysfunction (15). Second, lipolysis can be effectively pharmacologically modulated (27) (e.g., by acipimox). Finally, sustained adipose tissue hypoxia or activation of hypoxia-regulated signaling pathways can stimulate lipolysis (18, 28, 29); however, it is worth noting that IH exposure is characterized by distinct cellular and molecular features, which are different from exposure to sustained hypoxia (30, 31). Using glycerol release and adipocyte size distribution as functional and morphological indices of adipocyte lipolysis, this study showed that IH exposure stimulated basal lipolysis, thus providing a functional explanation for results seen in previous studies that reported increased plasma FFA levels in patients with OSA and rodents exposed to IH (7, 25, 32, 33). These results demonstrate that lipolysis represents a process regulated by IH exposure *in vivo*, together with secretion of adipokines or gene expression regulation (4).

Plasma FFA concentration was determined by a balance between FFA release into circulation (lipolysis) and its uptake in peripheral tissues (34). Because low tissue O_2 levels can rapidly diminish FFA oxidation in muscle (35), it can be hypothesized that reduced FFA uptake, rather than augmented lipolysis, was responsible for elevated plasma FFA levels. However, based on our observation that acipimox administration prevented an IH-induced rise in plasma FFA, we conclude that adipocyte lipolysis represents the primary source of elevated FFA after IH exposure and a driving force for the associated diabetic phenotype. Analysis of downstream factors in the insulin signaling cascade supports such a notion by demonstrating reduced activation of AKT in skeletal muscle after exposure to IH, suggesting development of insulin resistance in muscle. Interestingly, a drop in pAKT was prevented by administration of acipimox; however, the overall pAKT quantity was also reduced with acipimox, which warrants caution in interpretation and suggests that regulation of AKT phosphorylation is a complex process with multiple signaling pathways (including apoptosis, proliferation, and glucose metabolism) that converge on this molecule (36). Similarly, the increase in pAKT in adipose tissue with IH exposure, needs to be evaluated in a context of the severe tissue hypoxia that develops during IH exposure (19), which is a proven strong stimulus

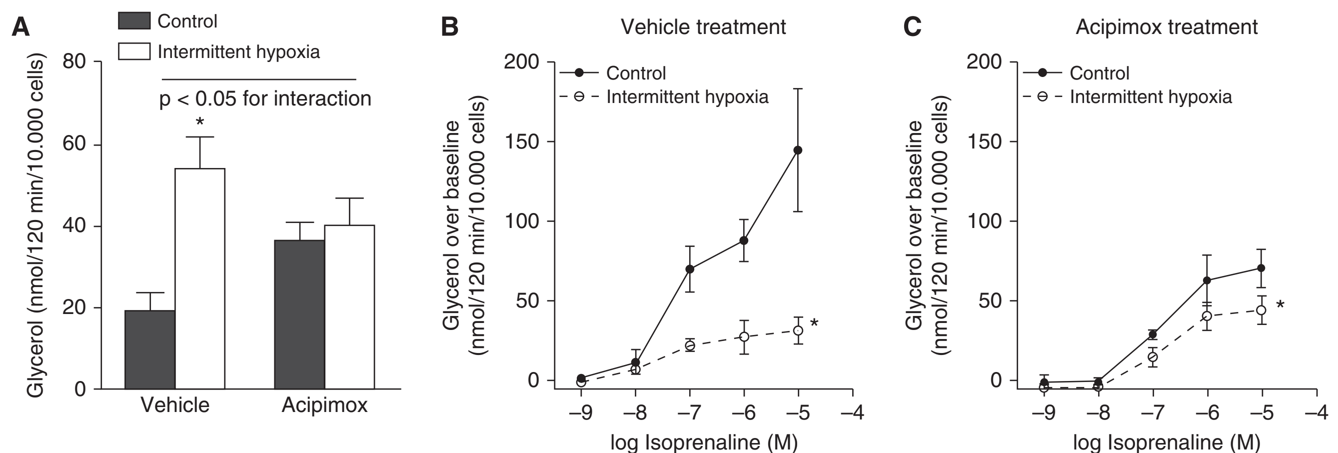

Figure 4. Basal and stimulated lipolysis. (*A*) Lipolytic rate in adipocytes isolated from mice exposed to control conditions or IH for 14 days treated with acipimox or vehicle. Isoprenaline stimulated lipolytic rate in mice exposed to control conditions and IH for 14 days while treated with vehicle (*B*) or acipimox (*C*). $^{*}P < 0.05$ for the effect of exposure to IH ($n = 5$ for all groups except stimulated lipolysis in the acipimox-treated group, where $n = 4$). Data are presented as mean (±SEM).

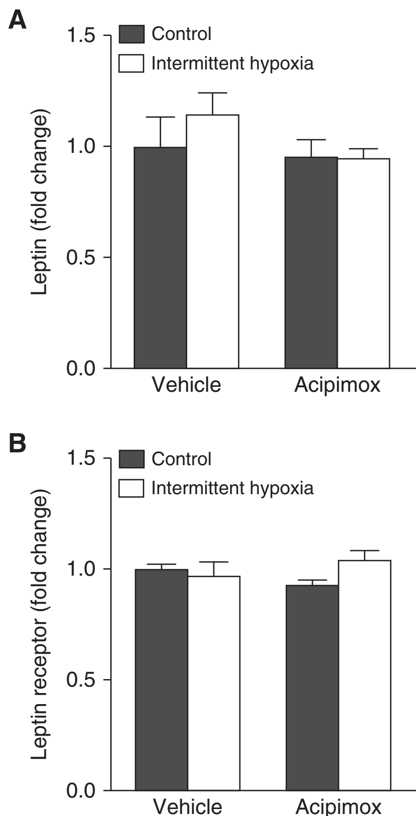

Figure 5. Adipose tissue gene expression of leptin and leptin receptor. Epididymal adipose tissue gene expression of leptin (*A*) and leptin receptor (*B*) in mice exposed to control conditions or IH for 14 days while treated with acipimox or vehicle. Data are expressed as fold changes over vehicle-treated mice exposed to control conditions ($n = 5$ for all groups). Data are presented as mean (±SEM).

for pAKT activation (37, 38), independently of insulin signaling or acipimox pharmacological effects. Further experiments employing acute exogenous insulin administration, as a stimulus for AKT phosphorylation, are warranted, as they could help to directly asses the insulin signaling pathway and insulin sensitivity under various experimental conditions. Despite conflicting results on insulin sensitivity, acipimox appears to be a promising agent, because its binding to G-protein coupled receptor 109A membrane receptor (39) prevents activation of a key lipase that regulates basal lipolytic rate; adipose triglyceride lipase (40) activity was increased in poorly vascularized and hypoxic tissues (41). Furthermore, hypoxia can up-regulate upstream adipose triglyceride lipase activators, such as cyclic adenosine monophosphate–dependent protein kinase A (42) and cyclic adenosine monophosphate levels in various cell types (43–45).

Besides the direct effect of lowered O_2 availability in adipose tissue, changes in endocrine regulators of lipolysis, including catecholamines, insulin, corticosteroids, and adipokines, might influence adipose tissue lipolysis during IH exposure. Enhanced sympathetic activity has been repeatedly reported in rodent and human models of IH as well as in patients with OSA (46); adrenergic stimulation has been suggested as one of mechanisms linking IH exposure with metabolic impairments. Indeed, pharmacological or surgical inhibition of the sympathetic system also ameliorated IH-induced impairments in glucose metabolism (25, 32). Our study partially supports this hypothesis, because we showed that adipocytes respond to catecholamine stimulation even after prolonged IH exposure. However, it should also be noted that the maximal lipolytic response (responsiveness) to a β-adrenergic agonist was clearly decreased in IH-exposed animals, probably due to the desensitization of the adrenoreceptor signaling cascade (47). Importantly, sensitivity to catecholamine stimulation ($EC_{50[iso]}$) was not affected by either IH exposure or acipimox treatment, suggesting the involvement of postreceptor signaling pathways localized downstream to membrane receptors, as observed in adrenoreceptor desensitization (47). It remains to be clarified whether further desensitization would diminish the effect of sympathetic drive after months or years of exposure. Leptin was previously shown to stimulate lipolysis and oppose the antilipolytic action of adenosine (48). Furthermore, sustained hypoxia and IH increased plasma leptin levels and gene expression (6, 49), even though contradictory findings have also been reported (50, 51). However, it seems unlikely that leptin is the causal mechanism linking IH exposure with increased lipolysis and whole-body metabolic impairments; this is because plasma leptin levels, as well as leptin and leptin receptor gene expression, in adipose tissue remained unaffected in our study, whereas lipolysis was strongly augmented. Furthermore, IH also induced insulin resistance in leptin-deficient *ob/ob* mice (6), suggesting that other mechanisms, including adipose tissue inflammation (51), could be involved.

Interpretation of the presented results should respect the limitations of the study. First, acipimox treatment in humans has been associated with significant improvements in insulin sensitivity and glucose metabolism (52); however, contradictory findings have been reported after prolonged administration, when a rebound in lipolysis was observed with increased plasma FFA levels (53). Similarly, we observed increased lipolysis after acipimox administration compared with a nontreated control group (not exposed to IH). The increased lipolysis was associated with improved, not worsened, insulin sensitivity and secretion. In addition, adipose tissue lipolysis is effectively regulated by tissue levels of a potent lipolysis inhibitor, adenosine. Although we observed no changes in gene expression of a key enzyme involved in adenosine degradation, adenosine deaminase, it is still possible that the activity of this enzyme was decreased, or other parameters of adenosine metabolism changed, with exposure to IH. In fact, adipose tissue concentrations of adenosine, representing a potent lipolysis-inhibiting compound in adipose tissue (54), are determined by a balance between its production by 5′-nucleotidase and its degradation mediated by adenosine deaminase and adenosine kinase. Future studies are needed to determine whether any part of adenosine metabolism is affected by IH and whether it represents a mechanism for the enhanced spontaneous lipolysis observed in OSA. Second, glucose and ITT tests provided no information about the function of the incretin system, which is responsible for approximately 50% of the postprandial insulin secretion (55). Third, a rather severe model of OSA, consisting of 60 hypoxic episodes per hour, was employed in this study. It should be noted that direct measurements of tissue O_2 levels during IH exposure in anesthetized mice suggested that, with increasing frequency of hypoxic periods, adipose tissue might become progressively hypoxic, and could start to resemble a state of sustained hypoxia (19). Therefore, it is possible that IH exposure mimics the hypoxic environment typical for obesity and leads to adipose tissue dysfunction.

In summary, this study showed that IH-induced impairments in insulin sensitivity, glucose tolerance, and β cell function can be prevented when IH-induced surges in basal lipolysis and plasma

FFA levels are pharmacologically prevented using acipimox. In addition to elucidating novel insights into mechanisms linking IH with impaired glucose metabolism, we hope that this study motivates exploration of the effectiveness of lipolysis inhibitors in treating metabolic impairments in subjects with OSA, especially those who are intolerant of continuous positive airway pressure therapy. ■

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References

1. Punjabi NM. The epidemiology of adult obstructive sleep apnea. *Proc Am Thorac Soc* 2008;5:136–143.
2. Sánchez-de-la-Torre M, Campos-Rodriguez F, Barbé F. Obstructive sleep apnoea and cardiovascular disease. *Lancet Respir Med* 2013; 1:61–72.
3. Punjabi NM, Caffo BS, Goodwin JL, Gottlieb DJ, Newman AB, O'Connor GT, Rapoport DM, Redline S, Resnick HE, Robbins JA, *et al*. Sleep-disordered breathing and mortality: a prospective cohort study. *PLoS Med* 2009;6: e1000132.
4. Briançon-Marjollet A, Weiszenstein M, Henri M, Thomas A, Godin-Ribuot D, Polak J. The impact of sleep disorders on glucose metabolism: endocrine and molecular mechanisms. *Diabetol Metab Syndr* 2015; 7:25.
5. Tasali E, Mokhlesi B, Van Cauter E. Obstructive sleep apnea and type 2 diabetes: interacting epidemics. *Chest* 2008;133:496–506.
6. Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP. Intermittent hypoxia increases insulin resistance in genetically obese mice. *J Physiol* 2003;552:253–264.
7. Polak J, Shimoda LA, Drager LF, Undem C, McHugh H, Polotsky VY, Punjabi NM. Intermittent hypoxia impairs glucose homeostasis in C57BL6/J mice: partial improvement with cessation of the exposure. *Sleep* 2013;36:1483–1490, 1490A–1490B.
8. Yokoe T, Alonso LC, Romano LC, Rosa TC, O'Doherty RM, Garcia-Ocana A, Minoguchi K, O'Donnell CP. Intermittent hypoxia reverses the diurnal glucose rhythm and causes pancreatic β-cell replication in mice. *J Physiol* 2008;586:899–911.
9. Xu J, Long YS, Gozal D, Epstein PN. β-cell death and proliferation after intermittent hypoxia: role of oxidative stress. *Free Radic Biol Med* 2009;46:783–790.
10. Fenik VB, Singletary T, Branconi JL, Davies RO, Kubin L. Glucoregulatory consequences and cardiorespiratory parameters in rats exposed to chronic-intermittent hypoxia: effects of the duration of exposure and losartan. *Front Neurol* 2012;3:51.
11. Lee EJ, Alonso LC, Stefanovski D, Strollo HC, Romano LC, Zou B, Singamsetty S, Yester KA, McGaffin KR, Garcia-Ocana A, *et al*. Time-dependent changes in glucose and insulin regulation during intermittent hypoxia and continuous hypoxia. *Eur J Appl Physiol* 2013;113:467–478.
12. Louis M, Punjabi NM. Effects of acute intermittent hypoxia on glucose metabolism in awake healthy volunteers. *J Appl Physiol (1985)* 2009; 106:1538–1544.
13. Boden G. Obesity and free fatty acids. *Endocrinol Metab Clin North Am* 2008;37:635–646, viii–ix.
14. Baldeweg SE, Golay A, Natali A, Balkau B, Del Prato S, Coppack SW; European Group for the Study of Insulin Resistance (EGIR). Insulin resistance, lipid and fatty acid concentrations in 867 healthy Europeans. *Eur J Clin Invest* 2000;30:45–52.
15. Frayn KN, Tan GD, Karpe F. Adipose tissue: a key target for diabetes pathophysiology and treatment? *Horm Metab Res* 2007;39:739–742.
16. Yaney GC, Korchak HM, Corkey BE. Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal β-cells. *Endocrinology* 2000;141: 1989–1998.
17. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of *ob/ob* and dietary obese mice. *Am J Physiol Endocrinol Metab* 2007;293: E1118–E1128.
18. Trayhurn P, Alomar SY. Oxygen deprivation and the cellular response to hypoxia in adipocytes—perspectives on white and brown adipose tissues in obesity. *Front Endocrinol (Lausanne)* 2015;6:19.
19. Reinke C, Bevans-Fonti S, Drager LF, Shin MK, Polotsky VY. Effects of different acute hypoxic regimens on tissue oxygen profiles and metabolic outcomes. *J Appl Physiol (1985)* 2011;111:881–890.
20. Viswanadha S, Londos C. Determination of lipolysis in isolated primary adipocytes. *Methods Mol Biol* 2008;456:299–306.
21. Iiyori N, Alonso LC, Li J, Sanders MH, Garcia-Ocana A, O'Doherty RM, Polotsky VY, O'Donnell CP. Intermittent hypoxia causes insulin resistance in lean mice independent of autonomic activity. *Am J Respir Crit Care Med* 2007;175:851–857.
22. Wang CY, Liao JK. A mouse model of diet-induced obesity and insulin resistance. *Methods Mol Biol* 2012;821:421–433.
23. Chen HC, Farese RV Jr. Turning WAT into BAT gets rid of fat. *Nat Med* 2001;7:1102–1103.
24. Iiyori N, Alonso LC, Li J, Sanders MH, Garcia-Ocana A, O'Doherty RM, Polotsky VY, O'Donnell CP. Intermittent hypoxia causes insulin resistance in lean mice independent of autonomic activity. *Am J Respir Crit Care Med* 2007;175:851–857.
25. Jun JC, Shin MK, Devera R, Yao Q, Mesarwi O, Bevans-Fonti S, Polotsky VY. Intermittent hypoxia–induced glucose intolerance is abolished by α-adrenergic blockade or adrenal medullectomy. *Am J Physiol Endocrinol Metab* 2014;307:E1073–E1083.
26. Weaver TE, Grunstein RR. Adherence to continuous positive airway pressure therapy: the challenge to effective treatment. *Proc Am Thorac Soc* 2008;5:173–178.
27. Langin D. Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. *Pharmacol Res* 2006;53:482–491.
28. Hashimoto T, Yokokawa T, Endo Y, Iwanaka N, Higashida K, Taguchi S. Modest hypoxia significantly reduces triglyceride content and lipid droplet size in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2013;440:43–49.
29. Famulla S, Schlich R, Sell H, Eckel J. Differentiation of human adipocytes at physiological oxygen levels results in increased adiponectin secretion and isoproterenol-stimulated lipolysis. *Adipocyte* 2012;1:132–181.
30. Ryan S, Taylor CT, McNicholas WT. Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. *Circulation* 2005;112:2660–2667.
31. Ryan S, McNicholas WT, Taylor CT. A critical role for p38 map kinase in NF-κB signaling during intermittent hypoxia/reoxygenation. *Biochem Biophys Res Commun* 2007;355:728–733.
32. Shin MK, Han W, Bevans-Fonti S, Jun JC, Punjabi NM, Polotsky VY. The effect of adrenal medullectomy on metabolic responses to chronic intermittent hypoxia. *Respir Physiol Neurobiol* 2014;203: 60–67.
33. Barceló A, Piérola J, de la Peña M, Esquinas C, Fuster A, Sanchez-de-la-Torre M, Carrera M, Alonso-Fernandez A, Ladaria A, Bosch M, *et al*. Free fatty acids and the metabolic syndrome in patients with obstructive sleep apnoea. *Eur Respir J* 2011;37:1418–1423.
34. Mensink M, Blaak EE, van Baak MA, Wagenmakers AJ, Saris WH. Plasma free fatty acid uptake and oxidation are already diminished in subjects at high risk for developing type 2 diabetes. *Diabetes* 2001; 50:2548–2554.
35. Horscroft JA, Murray AJ. Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. *Extrem Physiol Med* 2014;3:19.
36. Liao Y, Hung MC. Physiological regulation of Akt activity and stability. *Am J Transl Res* 2010;2:19–42.
37. Stegeman H, Kaanders JH, Wheeler DL, van der Kogel AJ, Verheijen MM, Waaijer SJ, Iida M, Grénman R, Span PN, Bussink J. Activation of AKT by hypoxia: a potential target for hypoxic tumors of the head and neck. *BMC Cancer* 2012;12:463.

38. Deguchi JO, Yamazaki H, Aikawa E, Aikawa M. Chronic hypoxia activates the Akt and β-catenin pathways in human macrophages. *Arterioscler Thromb Vasc Biol* 2009;29:1664–1670.
39. Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, Ohishi T, Hiyama H, Matsuo A, Matsushime H, Furuichi K. Molecular identification of nicotinic acid receptor. *Biochem Biophys Res Commun* 2003;303:364–369.
40. Miyoshi H, Perfield JW II, Obin MS, Greenberg AS. Adipose triglyceride lipase regulates basal lipolysis and lipid droplet size in adipocytes. *J Cell Biochem* 2008;105:1430–1436.
41. Gracey AY, Lee TH, Higashi RM, Fan T. Hypoxia-induced mobilization of stored triglycerides in the euryoxic goby *Gillichthys mirabilis*. *J Exp Biol* 2011;214:3005–3012.
42. Pagnon J, Matzaris M, Stark R, Meex RC, Macaulay SL, Brown W, O'Brien PE, Tiganis T, Watt MJ. Identification and functional characterization of protein kinase A phosphorylation sites in the major lipolytic protein, adipose triglyceride lipase. *Endocrinology* 2012;153:4278–4289.
43. Xiong Y, Qu Z, Chen N, Gong H, Song M, Chen X, Du J, Xu C. The local corticotropin-releasing hormone receptor 2 signalling pathway partly mediates hypoxia-induced increases in lipolysis via the cAMP protein kinase A signalling pathway in white adipose tissue. *Mol Cell Endocrinol* 2014;392:106–114.
44. Shaikh D, Zhou Q, Chen T, Ibe JC, Raj JU, Zhou G. cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial mesenchymal transition, migration, and invasion in lung cancer cells. *Cell Signal* 2012;24:2396–2406.
45. Torii S, Okamura N, Suzuki Y, Ishizawa T, Yasumoto K, Sogawa K. Cyclic AMP represses the hypoxic induction of hypoxia-inducible factors in PC12 cells. *J Biochem* 2009;146:839–844.
46. Mansukhani MP, Kara T, Caples SM, Somers VK. Chemoreflexes, sleep apnea, and sympathetic dysregulation. *Curr Hypertens Rep* 2014;16:476.
47. Lohse MJ, Engelhardt S, Danner S, Böhm M. Mechanisms of β-adrenergic receptor desensitization: from molecular biology to heart failure. *Basic Res Cardiol* 1996;91:29–34.
48. Frühbeck G, Gómez-Ambrosi J, Salvador J. Leptin-induced lipolysis opposes the tonic inhibition of endogenous adenosine in white adipocytes. *FASEB J* 2001;15:333–340.
49. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: differential effects of hypoxia on adipokine expression by preadipocytes. *J Endocrinol* 2008;198:127–134.
50. Yasumasu T, Takahara K, Nakashima Y. Hypoxia inhibits leptin production by cultured rat adipocytes. *Obes Res* 2002;10:128.
51. Carreras A, Zhang SX, Almendros I, Wang Y, Peris E, Qiao Z, Gozal D. Resveratrol attenuates intermittent hypoxia-induced macrophage migration to visceral white adipose tissue and insulin resistance in male mice. *Endocrinology* 2015;156:437–443.
52. Bajaj M, Suraamornkul S, Romanelli A, Cline GW, Mandarino LJ, Shulman GI, DeFronzo RA. Effect of a sustained reduction in plasma free fatty acid concentration on intramuscular long-chain fatty Acyl-CoAs and insulin action in type 2 diabetic patients. *Diabetes* 2005;54:3148–3153.
53. Vaag AA, Beck-Nielsen H. Effects of prolonged acipimox treatment on glucose and lipid metabolism and on *in vivo* insulin sensitivity in patients with non-insulin dependent diabetes mellitus. *Acta Endocrinol (Copenh)* 1992;127:344–350.
54. Johansson SM, Lindgren E, Yang JN, Herling AW, Fredholm BB. Adenosine A1 receptors regulate lipolysis and lipogenesis in mouse adipose tissue-interactions with insulin. *Eur J Pharmacol* 2008;597:92–101.
55. Nauck MA. Unraveling the science of incretin biology. *Am J Med* 2009;122(6 suppl):S3–S10.