Intermittent hypoxia and diet-induced obesity: effects on oxidative status, sympathetic tone, plasma glucose and insulin levels, and arterial pressure

Elena Olea, Maria Teresa Agapito, Teresa Gallego-Martín, Asunción Rocher, Angela Gomez-Niño, Ana Obeso, Constancio Gonzalez, and Sara Yubero

Department of Biochemistry and Molecular Biology and Physiology, University of Valladolid, School of Medicine, Valladolid, Spain; and Institute of Molecular Biology and Genetics, CIBER Enfermedades Respiratorias, CIBERES, Institute of Salud Carlos III, Valladolid, Spain

Submitted 28 May 2014; accepted in final form 31 July 2014

Intermittent hypoxia and diet-induced obesity: effects on oxidative status, sympathetic tone, plasma glucose and insulin levels, and arterial pressure.

J Appl Physiol 117: 706–719, 2014. First published August 7, 2014; doi:10.1152/japplphysiol.00454.2014.—Obstructive sleep apnea (OSA) consists of sleep-related repetitive obstructions of upper airways that generate episodes of recurrent or intermittent hypoxia (IH), OSA commonly generates cardiovascular and metabolic pathologies defining the obstructive sleep apnea syndrome (OSAS). Literature usually links OSA-associated pathologies to IH episodes that would cause an oxidative status and a carotid body-mediated sympathetic hyperactivity. Because cardiovascular and metabolic pathologies in obese patients and those with OSAS are analogous, we used models (24-wk-old Wistar rats) of IH (applied from weeks 22 to 24) and diet-induced obesity (O; animals fed a high-fat diet from weeks 12 to 24) to define the effect of each individual maneuver and their combination on the oxidative status and sympathetic tone of animals, and to quantify cardiovascular and metabolic parameters and their deviation from normality. We found that IH and O cause an oxidative status (increased lipid peroxides and diminished activities of superoxide dismutases), an inflammatory status (augmented C-reactive protein and nuclear factor kappa-B activation), and sympathetic hyperactivity (augmented plasma and renal artery catecholamine levels and synthesis rate); combined treatments worsened those alterations. IH and O augmented liver lipid content and plasma cholesterol, triglycerides, leptin, glycemia, insulin levels, and HOMA index, and caused hypertension; most of these parameters were aggravated when IH and O were combined. IH diminished ventilatory response to hypoxia, and hypercapnia and O created a restrictive ventilatory pattern; a combination of treatments led to restrictive hypoventilation. Data demonstrate that IH and O cause comparable metabolic and cardiovascular pathologies via misregulation of the redox status and sympathetic hyperactivity.

Intermittent hypoxia; oxidative stress; sympathetic activity; insulin resistance; hypertension

Obstructive sleep apnea (OSA) is defined as a repetitive complete (apnea) or partial (hypopnea) obstruction of upper airways (UA) occurring every night during sleep time and leading to recurrent episodes of hypoxia. The number of obstructions per hour, or apnea-hypopnea index, is the most common criterion used to define OSA severity, but classification criteria should also consider the degree of hemoglobin desaturation (1, 9). OSA is a primary central neurological disorder of unknown causes (5, 14), but secondary alterations of the sensory motor apparatus of UA are commonly present in OSA (14, 68). Additionally, there is a wide array of craniofacial conditions that while diminishing the diameters of the UA, constitute risk factors for OSA. In this regard, obesity outweighs all other craniofacial abnormalities in predisposing to OSA; the frequency and severity of OSA correlates with body mass index and weight loss, and bariatric surgery ameliorates the sleep disorder (63). However, aside from the mechanical factors (increased airflow resistances due to pharyngeal fat and a restrictive ventilatory pattern due to intrathoracic and intraabdominal fat), little is known about the mechanisms linking obesity to OSA (63, 71).

If the causes of OSA are not known, its consequences are epidemiologically well defined. Commonly, as time progresses, OSA generates the obstructive sleep apnea syndrome [OSAS; i.e., the combination of OSA and most notably cardio-circulatory (hypertension and acute vascular accidents)] and metabolic alterations [insulin resistance, glucose intolerance, nonalcoholic fatty liver disease (65)]. The number and severity of comorbidities vary among patients, but pathogenic mechanisms linking OSA to these pathologies remain unsettled.

Because intermittent hypoxia (IH) is the most direct consequence of OSA, we should inquire into the pathways linking IH to those alterations. Along these lines, it has been proposed that repetitive hypoxia-reoxygenation would mimic episodes of ischemia-reperfusion, generating oxygen reactive species (ROS) and vascular endothelium damage [see (42, 43)]; but see (38). ROS-regulated transcription factors would cause overexpression of inflammatory cytokines and vasoconstrictor factors that damage the endothelium further, decreasing vasodilator mediators and facilitating dyslipidemias and atherogenic processes, hypertension, and cardiovascular accidents. Confirming these notions, findings in laboratory animals indicate that IH, even of mild intensity (lowest SaO2 around 90%), causes oxidative stress and altered plasma lipid levels (59). On the other hand, the arterial P02 decrease occurring in each obstruction activates the carotid body (CB) chemoreceptors, whose increased drive to the brainstem generates an augmented output to the inspiratory and UA dilator muscles and a transient waking reaction, which together, overcome the obstruction and restore blood gases (14, 27). Soon after, another cycle of UA obstruction appears and the cycle repeats during sleep. This repetitive stimulation sensitizes the CB, leading to the sympathetic hyperactivity observed in patients with OSAS (38, 51) and IH animal models (44, 56). Apparently, recurrent-intermittent CB stimulation produces a bias in brainstem inte-
gration of the CB input, thereby strengthening the sympathetic activation (29, 37). Repetitive awakenings fragment sleep and lead to diurnal somnolence and anxiety that contribute to sympathetic hyperactivity (52). Thus sympathetic hyperactivity also seems a prime factor in the genesis of OSAS-associated cardiovascular and metabolic pathologies (38, 55, 62). It should be acknowledged, however, that two recent studies (11, 30) propose alternative, albeit noncoincident, pathways in the pathogenesis of cardiovascular alterations.

Besides that, it is well recognized that commonly, obese subjects have a cluster of cardiovascular and metabolic pathologies such as those encountered in OSAS [see (17)] and, as mentioned above, there is a high prevalence of OSAS among obese patients (63, 71). These two facts bring up new questions; namely: 1) Are obesity and OSA independent factors in producing metabolic and cardiovascular pathologies? 2) Do obesity and OSA share oxidative stress and sympathetic hyperactivity as pathogenic mechanisms? 2) Do obesity and OSA interact to worsen oxidative stress and sympathetic tone and to aggravate metabolic and cardiovascular pathologies? The aim of our study has been to answer those questions. To that end, we performed experiments on rats exposed to IH made obese by high-fat feeding, and with combined treatments. Our findings indicate that both treatments, independently applied, cause an oxidative status, augment sympathetic tone measured as plasma catecholamine levels, and generate a permanent status of inflammation; when treatments were combined by exposing obese animals to IH, the above parameters tend to deviate further from normality. IH and obesity caused in an independent manner liver steatosis and hyperlipidemia, glycemic alterations mimicking type 2 diabetes and hypertension, without evidence for baroreceptor reflex alteration. These pathologies were aggravated when obese animals were exposed to IH.

Finally, IH caused a hypventilatory response to hypoxia and hypercapnia, and obese animals exhibited a restrictive respiratory pattern; animals made obese and subjected to IH (OIH) exhibited a restrictive ventilatory pattern and hypoventilation, making them more prone to suffer hypoxic episodes. Mechanisms linking oxidative stress, sympathetic hypertone, and inflammatory status to the metabolic and cardiovascular pathologies are discussed. Mechanisms of aggravation of those pathologies when obesity and IH are associated are also considered.

MATERIALS AND METHODS

Animals and Anesthesia

We used a total of 160 male adult Wistar rats 24 wk of age. Rats were housed four per cage in the vivarium of the University of Valladolid, with free access to food and water, under controlled conditions of temperature and humidity, and in a stationary light-dark cycle. Animals were distributed to four different groups. The control group (C, 40 rats) was fed standard rat solid diet providing 3.8 kcal/g with 10% kcal as fat (D12450B; Open Source Diets) until they reached 24 wk of age. A second group of 40 animals was similarly fed and subjected to IH (control intermittent hypoxia group, CHI) from weeks 22 to 24. A third group of 40 rats received the same normal diet until they were 12 wk old, and then a fat-rich diet (5.2 kcal/g with 60% kcal as fat; D12492; Open Source Diets) from weeks 13 to 24 (obese group, O). The last group of 40 animals was given the same diet as the third group, and during weeks 22 to 24 was exposed to IH (obese intermittent hypoxia group, OIH). All experimental procedures to be described, except for IH exposure and plethysmography, were performed in animals anesthetized with sodium pentobarbital (ip; 60 mg/kg ody wt); in the experiments in which arterial blood pressure was measured the anesthesia was ketamine plus diazepam (100 and 5 mg/kg, respectively; ip). Experiments were performed in the morning (from 0830 to 1300) of the day after the last IH exposure with animals fasting overnight. In handling the animals, we followed the European Community Council Directive 24 of November 1986 (86/609/EEC) for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of Valladolid approved the protocols. Animals were killed by a cardiac overdose of sodium pentobarbital.

Exposure to Intermittent Hypoxia and Plethysmography

General design and functioning of our equipment to expose rats to IH have been described in previous publications (29, 59). The specific protocol of IH used in the present study consisted of cycles of exposure for 40 s to 5% O2, then exposure for 80 s to air for 8 h per day (from 0800 to 1600; i.e., during the animals’ time of inactivity) for 14 days. In recent experiments (59) we found that the lowest value of arterial PO2 measured was 37.2 ± 0.9 mmHg, with the percentage of HbO2 prior to hypoxic episodes being 96.5 ± 0.75% and lowest mean values being 73.5 ± 1.4%. The time that SaO2 was below 90% (<90%) represented 25% of the 8-h duration of the hypoxic exposure. There was no change in erythrocyte count or hemoglobin content after 14 days of IH.

Whole-body plethysmography in freely moving unrestrained animals was performed twice, the day before starting exposure to IH and the day before ending exposure to IH. The plethysmographic method has been described in detail elsewhere (29, 53).

Arterial Pressure Measurements

Ketamine-anesthetized rats were tracheostomized and pump-ventilated (CL Palmer, London, UK) with room air (60 cycles/min and a positive expiratory pressure of 2 cmH2O). To register the effect of hypoxic depressor responses, the inlet of the respirator was connected to a balloon filled with a mixture of 10% O2 + 90% N2. Arterial pressure was recorded from the right common carotid artery with a calibrated (with a mercury manometer) pressure transducer (Transpac IV; ICU Medical, San Clemente, CA) connected to an acquisition card (Power Lab 16SP; ADI Instruments, Castle Hill, Australia) and to the computer; pressure signals were monitored continuously and data were stored for off-line analysis. From these animals we collected epididymal and perirenal (retroperitoneal) adipose tissue.

Plasma and Tissue Catecholamine

Due to the duality of plasma catecholamine (CA) levels, special care was used in handling these animals. After anesthesia rats were tracheostomized and pump-ventilated with air. The chest was opened and blood was slowly withdrawn by direct puncture to the left ventricle. Citrated blood was centrifuged at 1,000 g for 5 min at room temperature. Supernatant was transferred to tubes containing 60 mg/ml of sodium metabisulfite and frozen at −80°C until HPLC analysis of CA. For analysis of endogenous CA in renal artery (RA), supernatants of glass-to-glass [0.1 N perchloric acid (PCA), 0.1 mM EDTA] homogenized tissues were directly injected into an HPLC apparatus. The chromatographic system and conditions have been described in detail elsewhere (29).

Measurement of the rate of CA synthesis in the RA. General procedures for the synthesis experiments have been described in previous publications (19, 69). In brief, RAs were incubated (37°C; 2 h) in Tyrode solution (in mM: NaCl, 140; KCl, 5; CaCl2, 2; MgCl2, 1.1; HEPES, 10; glucose, 5; pH 7.40) containing 30 μM 3,5-3H-tyrosine (the natural precursor of CA; 6 Ci/mmol; Perkin Elmer), 100 μM 6-methyl-tetrahydropterine, and 1 mM ascorbic acid [cofactors
for tyrosine hydroxylase and dopamine-ß-hydroxylase, respectively; (19, 69]). Tissues were washed in precursor-free Tyrode (4°C; 5 min), homogenized, and processed for HPLC as before. Identification and 3H-CA was performed against external standards and quantification by collecting the HPLC column effluents and scintillation counting.

**SDS-PAGE and Western Blotting**

The general SDS-PAGE procedure and immunoblotting have been previously described (6, 59). Cytoplasmic and mitochondrial fractions were obtained as described in (59) and used, respectively, for Cu-Zn-superoxide dismutase (SOD) and MnSOD assays. Samples of cytoplasmic and mitochondrial fractions (50 μg of protein) were electrophoretically fractionated on 12% SDS-polyacrylamide gels, and protein bands were transferred to PVDF membranes by electroblotting (Mini Trans-blot cell transfer; Bio-Rad, Hercules, CA). Membranes were blocked [5% nonfat dry milk in Tris-buffered saline (TBS)] and incubated overnight (4°C in TBS containing 0.1% Tween-20 and 0.5% BSA) with primary antibodies: anti-Cu-Zn SOD (rabbit antiserum, 1:5,000; Millipore, Billerica, MA), anti-Mn SOD (rabbit antiserum, 0.5 μg/ml; Millipore), and anti-β-actin (mouse antiserum, 1:5,000; Sigma). After washing, membranes were reincubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:20,000 for SODs; Millipore) and goat anti-mouse IgG (1:1,000, for β-actin; BD Pharmingen). Membrane signals were developed for enhanced chemiluminescence (ECL; Perkin Elmer) and visualized automatically (Versadoc; Quantity One 4.5.2.; Bio-Rad) or conventionally in a manual manner using ECL-sensitive film (Amer. sham Hyperfilm). Optical densities of the bands were determined with ImageJ software and normalized with correspondent β-actin signals to correct for minor deviations in protein loading. Normalized values were then averaged for all the replicated gels and used to calculate the relative change of enzyme expression in each group of animals.

**Measurement of SOD and Glutathione Peroxidase Activities in Liver Tissue; and Total Lipid Content, Lipid Peroxide Levels, and Nuclear Factor-Kappa B Activation in Liver**

All these measurements were made in liver segments removed from the animals used to assay plasma and renal artery CA. In every instance, except the total lipid content measurements, a small aliquot of the homogenates was used to determine proteins.

**Superoxide dismutases.** Liver segments were homogenized in ice-cold 0.25 M sucrose buffer (pH 7.4; w/vol 0.1) containing 10 mM Tris-HCl and 1 mM EDTA. The homogenates were centrifuged and the supernatant used to measure SOD. SOD activities were determined with an indirect method (SOD determination kit; Sigma, Madrid, Spain), which by inhibiting cytosolic SOD (3 mM potassium cyanide), allows us to differentially determine mitochondrial and cytosolic SOD activities. SOD is expressed as activity units, with one unit being the amount of enzyme capable of inhibiting by 50% cytochrome-c reduction in a coupled system with xanthine oxidase (pH 7.8, 25 °C) in a 3-ml reaction mixture.

**Glutathione peroxidase.** Liver was homogenized in 20 mM Phosphate Buffer (PB) (pH 7.4; 0.2 w/v) and separated in two aliquots: one to determine lipid peroxide (LPO) (see below) and the other to determine glutathione peroxidase (GPx) activity. GPx activity was measured as the disappearance of NAPDH in a coupled reaction: first, GPx oxidizes GSH to Glutathione disulfide (GSSG) in the presence of peroxide; and second, GSSG is back-reduced to glutathione (GSH) by glutathione reductase using NADPH as a reducing agent (molar extinction coefficient 6,220 UA mol⁻¹). One unit of activity is equal to 1 μmol of NAPDH oxidized per minute per milligram of protein.

**Lipid peroxide levels.** Homogenates were treated with 10 μl/ml of 0.5 M butylated hydroxytoluene in acetonitrile to prevent oxidation. LPO levels were determined immediately after homogenization using a commercial kit and following the supplier’s instructions (Bioxytech LPO-586 kit; Oxis Health Products, Portland, OR). In this assay, malonaldehyde and 4-hydroxynalkenals react with a chromogen reagent at low temperature, yielding a stable chromophore (peak absorbance at 586 nm). The assay provides a reliable index of lipid peroxidation (7).

**Nuclear Factor-κB activity.** Nuclear extracts were prepared using the Nuclear Extract Kit (40010; Active Motif, Rixensart, Belgium), and the nuclear protein content of the samples was determined by the Bradford method. Nuclear extracts were used in the nuclear factor-κB (NF-κB) p65 TransAM transcription factor assay kit (40096; Active Motif) according to the manufacturer’s instructions. Activation was detected by incubation with the primary antibody anti-NF-κB, which specifically recognizes an epitope (p65) accessible only when the factor is activated and bound to its DNA target. A secondary anti-IgG HRP conjugate allows spectrophotometric (450 nm) detection of the activated NF-κB. Data are presented as control fold increase.

**Total lipid content.** Total lipids in liver were obtained following the solvent extraction method described by Folch et al. and gravimetrically assessed (22). The extracted material was transferred to small Petri dishes and dried until two successive weights were identical. Results are expressed as % of total weight of the original piece of liver tissue.

**Measurement of Metabolic Parameters and Plasma C-Reactive Protein and Leptin Levels**

Measurement of all the parameters described under this heading occurred in a new group of animals. We withdrew a sample of blood to measure glucose, triglycerides, cholesterol, insulin, leptin, and C-reactive protein (CRP). Thereafter, we performed a glucose tolerance test. The consumption of glucose in isolated muscle was performed in tissues obtained from animals used to measure plasma CA. Glucose level in blood and glucose tolerance tests. Glucose in blood was measured with a glucose meter (Ascensia Breeze 2; Bayer). To perform the glucose tolerance tests, rats received 2 g (ip) of d-glucose/kg in saline, and glucose was measured at 0, 15, 30, 60, 90, and 120 min in tail blood.

Plasma triglyceride and total cholesterol levels in plasma were determined using enzymatic colorimetric assay kits (Chemex Labkit; Barcelona, Spain) following the manufacturer’s instructions.

Plasma insulin, leptin, and CRP levels were measured using commercial rat-specific ELISAs from DRG Diagnostics (Germany) (for insulin) and Cusabio Biotech; bioNova Cientifica, Madrid, Spain (for leptin and CRP).

**Glucose transport in skeletal muscle.** Glucose transport in muscle was measured in strips of gluteus superficialis freed of surrounding tissues. Muscle strips were placed in 20-ml glass vials kept at 37°C and containing 4 ml of Tyrode bicarbonate solution (composition as above, except for the substitution of 24 mM NaCl with 24 mM NaHCO₃) continuously bubbled with 20% O2/5% CO₂, balance N₂, saturated with water vapor. Basal transport was measured in tissues preincubated for 30 min in Tyrode solution, and insulin effects in tissues preincubated in Tyrode for 15 min + 15 additional min in Tyrode-containing insulin (20 μU/ml). Incubation proceeded for additional 30 min by addition of [14C(U)] 2-deoxy-D-glucose (310 mCi/mmol; Perkin Elmer) to the vials to yield a final radioactivity concentration of 1.55 μCi/ml. On completion of the incubation, tissues were washed for 5 min at 0–4°C in isoosmotic free Tyrode, weighed, homogenized (1 ml, 0.4 N PCA), and radioactivity was measured in 800 μl of supernatant in a Beckman scintillation counter. Glucose transport rates are expressed as nanomole/milligram of tissue.

**Statistics**

Data are expressed as means ± SE. When comparing two groups, statistical significance of differences was assessed using a two-tailed Student’s t-test for unpaired data and for comparisons of more than two groups, we used a one-way ANOVA followed by a Newman-Keuls multiple comparison test. Analysis of ventilatory parameters...
and positive interactions between treatments were assessed by the use of two-way ANOVA.

RESULTS

Body Weight Gain and Visceral Fat Deposits

Figure 1A shows the body weight evolution during the 12 wk the experiments lasted. Note that it took nearly 4 wk of high-fat intake for the weight of animals to deviate from controls. Figure 1B shows that weight gain throughout the 12 wk was greater in the animals fed a high-fat diet (HFD; 329.0 ± 10.9 g) than in control animals (C, 200.1 ± 4.1 g). Exposure to IH caused a nonsignificant (<10%) decrease in weight gain (C vs. CIH and O vs. OIH).

Weight of visceral fat pads was not different in C vs. CIH or in the two groups fed an HFD (O vs. OIH), yet epididymal and perirenal fat pads were 2–2.5 times greater in animals fed with high fat than in those fed a normal diet (Fig. 1, C and D).

IH and Obesity Alter Plasma Leptin and CRP Levels and Liver Redox Status and NF-κB Activation

Although literature data on the effects of IH on plasma leptin levels are controversial (see DISCUSSION), our data indicate (Fig. 2A) that both IH and obesity independently augment plasma leptin levels. Thus in C animals, plasma leptin was 8.9 ± 0.5 ng/ml and increased significantly in CIH and O animals, and combining HFD and IH (OIH animals) produced the highest plasma leptin levels (15.7 ± 0.2; $P < 0.001$ vs. C). Leptin levels were statistically higher in OIH than in O groups ($P < 0.05$).

CRP is an acute-phase reactant primarily synthesized in hepatocytes in response to inflammation mediators and released to plasma; in turn, CRP binding to cells may trigger generation of ROS, creating a vicious cycle that leads to development of chronic inflammation (72). According to this physiopathological schema and our data (Fig. 2B), IH and HFD cause an inflammatory status. Thus plasma CRP levels in C animals were 68.8 ± 6.5 μg/ml, nearly doubled in CIH and O animals, and increased further in OIH animals. In experiments in progress, we have observed that CRP continues to increase if the duration of IH is prolonged (data not shown).

In a recent study measuring up to 12 redox-related parameters in several tissues with two different levels of IH, we have found that liver is the most sensitive tissue to IH oxidative damage (59). Therefore, we measured only a few sensitive parameters in liver. Figure 2C shows that CIH caused a moderate 43% ($P < 0.05$) increase in liver LPO, whereas HFD augmented LPO by 225% ($P < 0.001$), and both treatments combined caused a higher increase (262%; $P < 0.001$). However, statistical analysis did not disclose a positive interaction between treatments. Liver activation of NF-κB (Fig. 2D) followed a comparable pattern: in CIH and O animals, NF-κB activation doubled control values ($P < 0.01$) and in OIH, it...
increased further by a factor of 2.5 ($P < 0.001$). Data for O and OIH animals were statistically different ($P < 0.05$).

Figure 3A shows activities of cytoplasmic and mitochondrial SOD. Note that both forms of SOD, cytoplasmic (CuZnSOD) and mitochondrial (MnSOD), were markedly diminished by the treatments given in isolation and combined. Control cytoplasmic and mitochondrial SOD activities were, respectively, 33.7 ± 2.7 and 2.7 ± 0.6 U/mg protein (i.e., the mitochondrial activity represents less than one-third of total liver SOD activity). Both activities decreased significantly in the three experimental groups, reaching a decrease of nearly 70% in OIH animals. However, at the protein level, both enzymes decreased only moderately, reaching a maximum decrease (30 – 35%) of the expression of MnSOD in OIH (Fig. 3B). The activity of liver GPx was not altered by any of the treatments (data not shown).

Markers of Sympathetic Activity: CA Levels in Plasma and Renal Artery CA Content and Rate of Synthesis

Sympathetic nerve endings are the main source (>80%) of plasma norepinephrine (NE), whereas epinephrine (E) comes entirely from the adrenal medulla. Therefore, levels of NE and E can represent a convenient index of the generalized sympathetic tone of mammalian organisms (26). In C animals, plasma NE was 19.6 ± 2.9 pmol/ml and increased significantly in the three experimental groups with a maximum in O animals (43.9 pmol/ml; $P < 0.001$) (Fig. 4A). Plasma E levels (30.5 ± 4.5 pmol/ml) in C animals evolved with the same pattern (Fig. 4B).

However, high CA plasma levels might also result from a decreased rate of their catabolism (26) with a normal level of sympathetic activity. Figure 4, C and D, excludes this possibility. NE content in the sympathetic endings of renal arteries increased in experimental groups as it did in plasma (Fig. 4C), and NE rate synthesis increased following the same pattern but in a higher percentage (Fig. 4D). Because in the steady-state conditions of our experiments NE synthesis rate represents the amount of NE released, data indicate that both obesity and CIH increase sympathetic tone.

Liver Lipid Content and Plasma Triglycerides and Cholesterol

These experiments explored whether IH alters lipid metabolism and interacts with HFD to deepen those potential alterations. Figure 5A shows that CIH produced a significant 16% increase in total liver lipid content, from 4.5 ± 0.07 to 5.2 ± 0.17% ($P < 0.05$). An HFD (O rats) more than doubled the liver lipid content, and association with IH (OIH rats) did not modify diet effects ($P < 0.001$ vs. C in both cases). Plasma total cholesterol and triglycerides rose by about a 30% in the three experimental conditions with no indications of interactions between IH and HFD (Fig. 5, B and C).
**Glycemic Metabolism**

Figure 6A shows basal levels of plasma glucose in our four groups of animals. Fasting glycemia in C animals was 75.9 ± 1.1 mg/dl, with minor but significant increases in the three experimental groups; data from O and OIH groups were statistically different \((P < 0.05)\), but there was not a statistically significant positive interaction between IH and HFD to promote an increase in glycemia. A similar trend was observed in plasma insulin levels (Fig. 6B). It rose from \(1.1 ± 0.04\ \mu g/liter\) in C animals to \(2.2 ± 0.07, 4.7 ± 0.11,\) and \(5.2 ± 0.23\ \mu g/liter\) in CIH, O, and OIH animals, respectively \((P < 0.001\ vs.\ C\ in\ all\ the\ cases)\). Again, insulin levels in O and OIH animals were different \((P < 0.01)\), but a statistical assessment of positive interaction between IH and HFD groups to augment plasma insulin was not significant. Evaluation of insulin resistance using the homeostasis model assessment \([HOMA;\ fast-ing\ insulin\ (\mu U/ml) \times \text{fasting glucose (mM)/22.5};\ see\ (48)]\) yielded values of 5.0, 10.4, 23.5, and 29.7 in C, CIH, O, and OIH animals, respectively \((P < 0.001)\). The HOMA in O and OIH animals was statistically different \((P < 0.001;\ Fig.\ 6C)\). Glucose tolerance tests indicate that CIH caused minor changes in the shape of the curve, with glycemia peaking at 30 min and nearly complete recovery at 120 min, but at any given time values, glucose tolerance in CIH animals was higher than in C animals (Fig. 6D). In O and OIH animals the glycemia curves depart from those of the animals fed a normal diet: basal and peak glycemia levels were higher and there was not a recovery of basal glycemia after 2 h (Fig. 6D); again, values in the curve of OIH animals were higher than in O animals. Estimation of the areas under the curve yielded values of 193, 202, 315, and 328 arbitrary units in C, CIH, O, and OIH, respectively.

**Arterial Blood Pressure and Baroreflex Response**

Figure 8A shows mean arterial blood pressure (AP) in the four groups of animals. In C animals, mean AP was \(88 ± 2\ mmHg\) and rose to \(120 ± 4\ mmHg\) in CIH animals, to \(135 ± 4\ mmHg\) in O animals, and to \(147 ± 4\ mmHg\) in OIH animals \((P < 0.001\ vs.\ C;\ P < 0.05\ O\ vs.\ OIH)\). Application of the hypoxic episode caused a significant decrease in AP in the four groups \((42%\ to\ 47%;\ P < 0.001)\), the effect being not different among groups (Fig. 8B). Basal heart rate before the 10% \(O_2\) acute hypoxic challenge was \(332 ± 10\ beats/min\) in C rats and slightly but significantly higher in all the experimental groups.
No significant differences in heart were observed among groups during the hypoxic challenge.

**Breathing Parameters**

Figure 9, A–C shows, respectively, breathing frequency (BF; breaths/min), tidal volume (TV; ml/kg), and minute ventilation (MV; ml·min⁻¹·kg⁻¹) in the four groups of animals while breathing 21% (air), 12%, 10%, and 7% O₂ and 5% CO₂. Data are presented according to the experimental protocol except for the elimination from the drawing of periods of resting air breathing (10 min) preceding the application of each stimulus (10 min); because there were no differences in all air-breathing periods, only the initial one is shown. In C animals, initial air BF was 71.8 ± 3.9 breaths/min and increased in the three hypoxic (12%, 10%, and 7% O₂) and in the hypercapnic (5% CO₂ in air) atmospheres. In CIH, BF followed the same pattern, but in any given atmosphere the BF tended to be slightly smaller. The same responses were observed in O animals, but in every condition BF was significantly higher than in C animals (*P < 0.05 to ***P < 0.001 vs. control (group C) (one-way ANOVA). Animals fed high fat and exposed to IH (OIH group) preserved the same pattern of BF, but IH lessened the BF, thereby increasing the effect observed in O animals; this decreasing effect was significant in hypercapnia (*P < 0.05 OIH vs. O animals). Initial TV in C animals while breathing air was 5.3 ± 0.07 ml/kg and reached a maximum of 8.7 ± 0.36 ml/kg in 7%
O2 (Fig. 9B). IH caused a tendency for TV to decrease in every condition but room air atmosphere; this decreasing effect of IH on TV was statistically significant under the most intense hypoxia tested (7% O2; P < 0.01 CIH vs. C). In the O group, TV was smaller than in the C group in every atmosphere (*P < 0.05 to ***P < 0.001 as drawn). In OIH animals the decrease in TV was even more intense than in O animals, particularly while animals were breathing in 7% O2 and 5% CO2 atmospheres (**P < 0.01 OIH vs. O animals); in other words, it would appear that HFD and IH led to a diminution in TV. Finally, MV in C animals oscillated between 395.0 ± 22.2 ml·kg⁻¹·min⁻¹ in air and 904.3 ± 29.6 ml·kg⁻¹·min⁻¹ in 7% O2 (Fig. 9C). In CIH animals, MV was statistically smaller in the three hypoxic and hypercapnic atmospheres (*P < 0.05 to ***P < 0.01 as drawn). O animals showed MV comparable to those observed in C, except for the most intense hypoxia in which MV was smaller (**P < 0.01). In the OIH group, MV was significantly smaller than in the C group in the three hypoxic and hypercapnic conditions. Further comparison of MV in O vs. OIH groups showed that MV was smaller in every condition in OIH (*P < 0.05, **P < 0.01 OIH vs. O); that is, IH treatment also diminished total ventilation in animals fed high fat. Penh (a unitless index) is the unique indicator of airway reactivity and airway resistance that can be obtained in conscious, unrestrained animals and therefore is freed from the bias imposed by anesthesia or restraining stress [see (53)]. We found that only in the O group did Penh show a tendency to increase in every condition, reaching statistical significance (P < 0.05) while breathing 7% O2 and 5% CO2 (i.e., at highest air flow; data not shown).

**DISCUSSION**

Our present findings show that high-fat feeding generates O because it increases total body weight and epididymal and perirenal fat, whereas IH does not. Individually, IH and O augment plasma leptin and CRP levels, evidencing an inflammatory status in both conditions; when combined, as in OIH animals, these two parameters increase further. The remaining findings can be grouped according to the following specific aims: 1) Individually, IH and O produce oxidative stress (increased LPO levels and decreased SOD activities in liver); when IH and O are combined (as in OIH rats), liver lipoxygenases increased and liver NF-κB activation followed the same pattern (Figs. 2 and 3). 2) Sympathetic tone (plasma NE and E and renal artery NE levels) augment in IH, O, and OIH.
animals; NE synthesis rate in the renal artery would indicate a preferential increase in renal sympathetic tone (Fig. 4). 3) Lipid and glucose metabolisms are altered by IH and O (Figs. 5–7). IH caused a moderate increase in the liver lipid content (CIH rats) and HFD caused a marked increase (O and OIH rats), with plasma cholesterol and triglyceride rising similarly in the three groups. All monitored glycemic metabolism indexes (basal glycemia, insulin levels, HOMA index, and glucose tolerance curve) exhibited prediabetic or diabetic deviations in CIH and O rats, with deviations being more pronounced in OIH animals. Insulin resistance was also manifested in skeletal muscle in vitro as a diminution of insulin-promoted increase in glucose uptake. 4) Individually, IH and O cause hypertension (Fig. 8; CIH and O rats) with a further increase in arterial pressure when treatments are combined (OIH animals); we found no evidence of baroreceptor reflex alteration. 5) Finally, Fig. 9 shows that CIH rats have a diminished MV, O animals maintained MV with a restrictive ventilatory pattern, and OIH rats exhibited lower MV and a restrictive ventilatory pattern. Overall, our findings demonstrate that IH and O interact to aggravate oxidative stress, sympathetic hypertony, and metabolic and cardiovascular alterations, yet the combined effect of IH and obesity is often less than additive, suggesting either an occlusive effect or the reaching of a saturation point. In the paragraphs that follow we will consider first the genesis of the oxidative status and sympathetic hypertony and, second, discuss the pathogenesis of metabolic and cardiovascular alterations.

The evolution of body weight gain and the final accumulation of epididymal and perirenal fat indicate the effectiveness of protocol feeding to promote obesity (54). Similarly, the 14 days of protocol of IH used has been shown to be adequate to promote systemic hypertension and metabolic disarrangements [see also (35, 57)]. Therefore, the experimental design meets the needs to study the interaction between O and IH. Additionally, our experimental protocol mimics the most common clinical situation in which for a long time, obesity precedes the appearance of OSA, or stated in a different way, obesity is the most important condition predisposing to OSA (63).

The mechanisms causing oxidative stress and alterations in the expression and specific activity of SODs in IH-exposed animals have recently been discussed on detail (59). Our observations showing an increase in the rate of ROS production in O animals agree with literature. Thus Furukawa et al. (24) showed that oxidative status correlated positively with body mass index in humans; these authors also showed that KKAy-obese mice had increased ROS levels (vs. C57BL/6 mice) and a diminished expression of SOD. Comparable findings have also been reported in ob/ob mice (41) and in diet-induced obesity (16, 54, 60). Although not addressed in our study, we might ask what an HFD does to generate the oxidative status in liver? Because mitochondria are responsible for fatty acid catabolism and major sources of ROS, it seems likely that they are playing a key role in the genesis of the oxidative status. In fact, as we have found in IH-exposed animals (59, 60), in the liver mitochondria of rats fed a high-fat diet, it is also observed that a marked decrease occurs in the quotient active/total aconitase, implying oxidative damage of the mitochondrial enzyme [see (10)]. It should be added that the changes we observe in MnSOD, both in IH-exposed animals and O animals, a decrease in activity $\geq$65% and a diminution of expression of $\sim$30% clearly indicate mitochondrial oxidative damage; oxidative mechanisms leading to the decreased expression and the deeper diminution in MnSOD activity have recently been discussed (59). Similarly, the diminution of CuZnSOD activity with a near normal level of protein expression would indicate an oxidative damage of the cytoplasmic enzyme (59, 70).

Activation of NF-κB by ROS is well established in many cells systems, and therefore it should be expected in our three experimental groups [see (59)]. When activated, p65/p50 NF-κB dimmers translocate to the nucleus and bind to the promoters of the regulated genes, particularly inflammatory cytokines (39), leading or contributing to the inflammatory status observed in experimental IH and in OSAS and in O animals and patients. Consistent with our observations and interpretation, it has been reported that quercetin, a known antioxidant, reduces activation of the NF-κB enzyme by an HFD as well as the oxidative and inflammatory status (54). Our statistical comparisons are evidence that the level of activation of NF-κB in OIH is higher than in O and CIH, but we cannot infer that O and CIH use unique or different mechanisms or pathways to activate NF-κB. IH causes plasma CRP levels paralleling the intensity of IH (59); because O generates inflammatory mediators in adipose tissue and liver, likely the dual origin of signals explains that in the OIH group, CRP levels were higher than in any of the two situations in isolation.

Data from the literature regarding levels of leptin in IH animals and in patients with OSAS are not consistent. Thus, although Messenger and Ciriello (50) found that short-term IH (80 s 6.5% O2/100 s 21% O2, 8 h) markedly augmented plasma leptin, Carreras et al. (8) found modest decreases in two models of IH of long duration (model A: cycles of 90 s 6.4% O2/90 s 21% O2, 12 h/day; model B: 8% O2, 12 h; 21% O2, 12 h; 5 wk). In patients with OSAS this has been reported as an increase in leptin levels (32), whereas other authors related plasma leptin levels to fat body mass and not to hypoxia (4, 64). Yet our

Fig. 7. Effect of insulin on 2-deoxy-D-glucose uptake and accumulation in segments of the gluteus superficialis. Data are means ± SE of 8–10 individual data. **$P < 0.01$, ***$P < 0.001$ vs. insulin-free in each group (two tailed unpaired t-test); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ insulin-treated tissues in all groups (one-way ANOVA).
findings unequivocally show that the IH paradigm used in the present study significantly augments plasma leptin. Because IH stabilizes and increases hypoxia-inducible factor 1-alpha (HIF-1α) levels (57), we propose that leptin increase is mediated by HIF-1α [see (67)]. In O animals leptin levels were about twice those observed in C rats, paralleling differences in fat pad weights, and in OIH, leptin increased further, appearing as if both fat tissue mass and hypoxia contribute to the genesis of the high leptin levels. However, IH is less effective in increasing leptin levels (and also CRP) in obese than in control animals. As just mentioned, leptin expression is under the control of HIF-1α. In obesity the density of white adipose tissue vascularization decreases (67), and therefore it should be expected that the applied IH causes a stronger hypoxia in the adipose tissue of obese animals. In this sense, it should be anticipated that IH would augment the production of leptin more in obese than in control animals. However, data indicate otherwise. This could imply that other unknown regulators are lessening the effects of hypoxia on the expression of leptin in obese animals.

Levels of CA in plasma increased in the three experimental conditions (CIH, O, and OIH) with maximum levels in the O group. Interestingly, in every instance, NE levels rose slightly more than E levels, implying that sympathetic activity did not increase uniformly; in other words, the spillover of NE from sympathetic endings (the main source of plasma NE) increases proportionally more than the rate of E release from the adrenal medulla. The rate of NE synthesis in renal artery increased proportionally more than NE content, particularly in O and OIH animals, indicating a marked increase in the spillover of NE from renal sympathetics in animals fed an HFD. Therefore, our data indicate that obesity causes a preferential activation of the renal sympathetics. Consistent with this idea, NE turnover time (NE content divided by NE rate of synthesis) in renal artery decreased from 33 h (C) and 29 h (CIH) to 18 and 20 h in O and OIH groups, respectively. Sympathetic activation in IH is mediated by the CB (20, 28, 58). In O animals, sympathetic activation with a preferential activation of the renal sympathetics could be mediated at least in part by leptin. In fact, a connection between leptin and sympathetic activity has been documented in humans (18) and other species [see (61) for references], being recently demonstrated that leptin activation of the renal sympathetics is mediated by the hypothalamic arcuate nucleus (33). The CB also appears to contribute to the augmented sympathetic tone in O animals (see below).

Mechanisms leading to fat accumulation in the liver and to increased plasma lipid levels could be related to a decrease in the activity of aconitase. Indeed both IH and fat feeding cause
mitochondrial oxidative damage in liver (Figs. 2 and 3) and decrease aconitase activity (59, 60). Diminished aconitase activity would lead to citrate accumulation in mitochondria and its export to cytoplasm, causing a stimulation of ATP citrate lyase and fatty-acid synthase, de novo lipogenesis, and hepatic fat accumulation (40). Additionally, IH augments the expression of stearoyl coenzyme A desaturase 1 via HIF-1α; an abundance of monounsaturated fatty acids increases the synthesis of cholesterol esters and triglycerides, accumulation of lipids in liver, and their incorporation into lipoproteins and secretion to blood stream (45). Decreased expression of carnitine palmitoyltransferase-1 in rats fed with high fat has also been described, being proposed that it would lead to a diminished oxidation of fatty acids and facilitation of their deposit in liver (54). Thus nonalcoholic liver steatosis appears multifactorial, being triggered by any situation causing oxidative stress and inflammation as IH and obesity do. A diminution of insulin-regulated clearance of triglyceride-rich lipoprotein would also contribute to observed hyperlipidemia (16, 40).

The alteration of glucose metabolism produced by CIH as we observe it is best defined as a prediabetic status of insulin resistance. Our findings agree with the clinical observations indicating that more than 80% of the OSAS in some series of patients exhibit obesity-independent type 2 diabetes or prediabetes (36). Our findings also agree with those reported by other authors in experimental models of IH. Thus acute IH, both in mice [9 h (46)] and humans [8 h (47)], and with lowest O2 saturation comparable to that used in the present work, causes a decrease in insulin sensitivity. In CIH models (15) it was found that CIH (nadir O2 6 –7%; 60 cycles/h; 12 h/day; 4-wk duration) in mice caused a significant increase in HOMA index and, in a more recent study with the same protocol, the

**Fig. 9. Ventilatory parameters in the four groups of animals. Breathing frequencies (A), tidal volumes (B), and minute ventilations (C) while animals breathed in the different atmospheres depicted. Data are means ± SE of 8 individual values. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control (group C) (two-way ANOVA); *P < 0.05, **P < 0.01 OIH group vs. O group (two tailed unpaired t-test).**
same laboratory reported that CIH mice were hyperglycemic (16). Thus the point to consider is the one or more mechanisms involved in the genesis of the alterations produced by IH on the glycemic metabolism. We believe that they are mediated by the CB: repetitive hypoxic stimulation → sensitization of the CB → biased integration in the brainstem of CIH input (29, 37) → increased sympathetic activity → insulin resistance. It has been known since the early 1980s (13) that CAs, particularly E, augment liver glucogenogenesis and glycogenolysis leading to hyperglycemia; at the same time, CAs impair sensitivity to insulin in both peripheral and hepatic tissue with the appearance of a picture of insulin resistance [see (55)] as that encountered in our experiments. The situation in obesity appears similar to the one just described for IH. Our group has recently demonstrated that activity of the CB is increased in animals fed an HFD, and that CIH resection shuts down diet-induced insulin resistance and hypertension, implying that the CB is implicated in the pathogenesis of metabolic and hemodynamic disturbances through sympatho-adrenal overactivation (62). Although statistically the interaction between CIH and O to alter glucose metabolism in OIH is less than additive, it is not less certain that every parameter is significantly more deviated from normality in OIH than in O and CIH animals; in other words, extrapolating findings to humans, we could assert that the appearance of OSA in obese subjects would worsen their glucose metabolic profile. Finally, although we inculcate the sympathetic system as the main mediator of the metabolic alterations, we do not exclude other factors (oxidative stress, inflammation, NF-κB activation) as important players in the genesis of metabolic alterations observed.

The magnitude of increase in mean arterial blood pressure observed in IH animals is comparable to that observed by other authors. For example, recording blood pressure in nonanesthetized animals exposed to a comparable IH protocol (20 cycles of hypoxia, 7 h/day for 14 days) it was found that mean arterial blood pressure increased from 109 ± 2 mmHg to 137 ± 4 mmHg [see (3)]. Similarly, Huang et al. (34), who recorded blood pressure in urethane-anesthetized rats, found that control animals had a mean arterial blood pressure of 98 mmHg and the IH animals had a pressure of 116 mmHg (7–7.5% O2, 30 cycles, 6 days/wk for 5 wk). Thus the use of different anesthetics does not preclude evidencing the hypertension produced by IH. Regarding the possible mechanisms involved in the genesis of hypertension, the discussion given for the metabolic alterations would apply for the AP and heart frequency; CB-mediated sympathetic activation would be the main player in the genesis of hypertension [see above; also see (38, 49, 55, 62)]. Additionally, we note that renal sympathetic activity in part mediates the control of renin secretion, and therefore the activity of the renin-angiotensin system could contribute to the genesis of hypertension and other cardiovascular pathologies in OSAS or IH (21). Similarly, a contribution of the endothelium in the genesis of hypertension seems very plausible: oxidative stress and inflammation present in our experimental groups would cause a progressive loss of the physiological antiadherent, vaso-relaxant, and antiproliferative phenotype of the vascular endothelium (2). Additionally, in patients with OSA, other variables associated to obstructions (such as swings in intrathoracic pressure, repetitive arousals, and hypcapnia) would be important factors in the genesis of hypertension and cardiovascular alterations (30); yet these factors do not appear to contribute to the hypertension encountered in IH animals because the swings in the thoracic pressure are absent and animals became hypocapnic in each hypoxic episode (59). Then whatever the array of mechanisms implicated, data indicate that IH, similar to that encountered in patients with OSA, aggravates the hypertension in obese animals. In fact, this observation matches the conclusion of another recent study (11): independent treatment of obesity (with diet) and OSA (with continuous positive airway pressure, or CPAP) ameliorate hypertension in patients with OSAS and the improvement is greater when both treatments are combined.

A last aspect to comment on is the potential role played by the baroreflex in the genesis of hypertension and other cardiovascular alterations in patients with OSA. In the present study with IH animals, we did not detect modifications in the baroreflex responses triggered by the hypotension elicited by hypoxia (magnitude of the hypotension itself and increase in heart frequency); therefore, our findings do not support a role for baroreceptor dysregulation as being responsible for the encountered hypertension. In patients with OSA, it is commonly accepted that baroreflex desensitizes (14, 23), becoming poorly sensitive to blood pressure alterations, but in rats it has been reported that IH impairs and increases the sensitivity of the reflex (12, 31). Therefore, further and more specific experiments are required to clarify these aspects in animal models.

The final data to be discussed relate to ventilation. CIH did not alter the ventilatory pattern but caused a moderate and yet significant decrease in MV. It should be recalled that this diminution contrasts with the aforementioned sensitization of the CB chemoreceptor activity: every author that has monitored activity in the output of the CB as chemoreceptor discharges in CIH animals has evidenced the sensitization, but when the ventilatory (or phrenic activity) reflex effects are monitored, both in IH animal models or patients with OSAS, many authors have found a decrease in MV [for references, see table 1 in (28, 29)], have interpreted these apparently opposing results as created by a bias in the integration of central nervous system activity at the brain stem level that would result in a diversion of the information arriving from the CB to the nuclei controlling sympathetic activity with the final outcome of decreased ventilation and an increased sympathetic tone [see also (37)]. In O animals, ventilation follows a typical restrictive pattern [increased BF and decreased TV; see (71)], although MV was maintained except during intense hypoxia. However, this ventilatory pattern would result in decreased alveolar ventilation, an expected finding because obese animals (including humans) consume less O2/kg body weight despite the exaggerated work of breathing (25). OIH animals retained the pattern of O animals and the diminution of MV of CIH, implying that the interaction between O and IH drives the animals to a situation in which the appearance of hypoxic episodes would be more severe.

In summary, our data demonstrate that IH in rats (which represent the experimental model of human OSA), as well as feeding a high-fat diet induced obesity and caused sympathetic hyperactivity and oxidative stress, indicating additionally that although IH augments general sympathetic activity, obesity activates renal sympathetics in a preferential manner. Data also show that IH and obesity cause, in independent manners, liver steatosis and plasma dyslipidemias, marked alterations in gly-
Intermittent Hypoxia and Diet-Induced Obesity • Olea E et al.

Cerebrovascular metabolism best defined as a marked insulin resistance, and arterial hypertension without noticeable alteration of the baroreflex. When IH and obesity were combined, all alterations tended to be aggravated so that they were significantly greater in OIH than in either O or CIH. Finally, we verify once again that CIH causes hypoventilation (in spite of CB sensitization) and that diet-induced obesity causes a clearly restrictive respiratory pattern while maintaining MV; combining high-fat feeding and IH in OIH animals creates a situation in which the efficacy of ventilation to ventilate at the alveolar level decreased, and therefore, potential genesis of hypoxic episodes were augmented. Finally, our findings should represent a signal of medical awareness in the sense that careful attention should be given to discover sleep disorders in obese subjects, because their appearance will aggravate all obesity-related pathologies.

ACKNOWLEDGMENTS
We thank Mª de los Llanos Bravo, E. Gonzalez, and A. Gordillo for technical assistance.

GRANTS
Support for this study was provided by Spanish Ministry of Economy and Competitiveness Grant BFU2012-37459 and Spanish Ministry of Health-Institute Carlos III Grant CIBER CB06/00050 to C.G.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


