Intermittent hypoxia has organ-specific effects on oxidative stress

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OBSTRUCTIVE SLEEP APNEA (OSA) results in repetitive cycles of hypoxia and reoxygenation, termed intermittent hypoxia (IH) (27). The potential role of OSA in the development of chronic diseases is becoming increasingly recognized. For example, OSA is associated with elevated risks of hypertension and atherosclerosis (24, 53, 64, 85). Endothelial dysfunction correlates with the severity of O2 desaturations in patients with OSA (24, 64, 85). A causal role of OSA in atherosclerosis is supported by evidence that continuous positive airway pressure (CPAP) improves early signs of atherosclerosis (23) and our animal data showing that chronic IH induced atherosclerosis in C57BL/6J mice fed a high-cholesterol diet (78). OSA is also associated with nonalcoholic steatohepatitis in obese individuals (1, 15, 44–65, 68, 88). CPAP therapy ameliorates aminotransferase elevations (46), and experimental IH causes inflammatory liver injury in mice fed a high-fat, high-cholesterol diet (77). A mechanism has not been established for these observations.

Diseases associated with OSA are thought to be due, at least in part, to oxidative stress (51, 97). The hypothesis that IH causes oxidative stress stems from observations that ischemia-reperfusion (IR) or anoxia-reoxygenation injury gave rise to reactive oxygen species (ROS) (3, 5, 20, 32, 48, 75, 99). Applicability of this concept to OSA has been shown in several studies, as exemplified by increased exhaled isoprostanes (9, 10), blood reactive oxygen metabolites (18), serum malondialdehyde (MDA) (6, 52), neutrophil superoxide production (80), and susceptibility of serum LDL to oxidation (80) in OSA.

Furthermore, CPAP treatment has been shown to significantly improve parameters of inflammation and oxidative stress (17, 22, 43) in patients with OSA. In rodent models of IH, oxidative stress has also been reported in association with left ventricular dysfunction (13), neurodegenerative changes (76, 96, 98), or dyslipidemia (54). However, other investigators found no increases in serum free nitrotyrosine (87), lipid peroxidation (69, 86), or LDL oxidation susceptibility (93) in OSA. Overall, there is lack of consensus regarding IH-induced oxidative stress and little investigation into a pathway by which pathological ROS might be generated.

A principal source of ROS in many tissues is NADPH oxidase (34, 63, 66). In neutrophils, two membrane-bound subunits, gp91phox and p22phox, comprise cytochrome b558. Cytochrome regulatory subunits include p47phox, p67phox, p40phox, and the small GTP-binding protein Rac (12, 25). Several isoforms of the enzyme have been described in various tissues. In most known isoforms, p47phox phosphorylation is the initial step in enzyme activation. Deletion of p47phox inhibits ROS generation in the aorta (7, 38, 47) and ameliorates atherosclerosis in apolipoprotein E-deficient mice (7), demonstrating the relevance of this enzyme to cardiovascular disease. Inhibition of NADPH oxidase with apocynin also attenuates cholesterol-induced liver injury (60).

Our study set out to answer three questions. 1) Does IH per se induce circulatory or tissue oxidative stress? 2) Does IH affect NADPH oxidase activity in tissues? 3) Does NADPH oxidase activation contribute to IH-induced oxidative stress in the aorta, heart, or liver?

METHODS

Animals. Sixty-four 8-wk-old male, lean C57BL/6J mice and sixteen 8-wk-old male p47phox-deficient (p47phox−/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME). The study was approved by the Johns Hopkins University Animal Care and Use Committee and complied with the American Physiological Society guidelines for animal studies. Inasmuch as animals lacking functional NADPH oxidase are prone to infection, animals were housed two per cage, food and bedding were autoclaved, and cages were thoroughly cleaned with detergent and ethanol every 3–4 days. The same protocol was used with C57BL/6J mice to equalize handling. For blood sample collection, surgical procedures, and tissue collection, anesthesia was
induced and maintained with 1–2% isoflurane administered through a facemask. All mice were fed a regular Purina chow diet (3.3 cal/g, 4% fat). Mice were fasted for 5 h before they were exsanguinated and killed. For biochemical assays, the heart, descending aorta, and liver were immediately frozen at −80°C for future analysis.

**Intermittent hypoxia.** A gas control delivery system was designed to regulate the flow of room air, N2, and O2 into customized cages housing the mice, as previously described (72). During each period of IH, the inspired O2 fraction was reduced from 20.9% to 4.9% over a 30-s period and then rapidly restored to room air levels in the subsequent 30-s period. Animals were kept in a controlled environment (22–24°C with a 12:12-h light-dark cycle; lights on at 0900) with free access to food and water. Control animals were exposed to 0.9% saline placebo (2 mg kg−1·day−1·ip) dissolved in 0.9% saline according to the method used by Hart et al. (35). A matched control group (n = 8) received 0.9% saline placebo (2 mg kg−1·day−1·ip).

**Apocynin injections.** Mice were given daily injections of apocynin (2 mg kg−1·day−1·ip) dissolved in 0.9% saline to inhibit ex vivo oxidation. Serum and tissue thiobarbituric acid-reactive substance was determined using commercially available kits (Zepptomixt, Buffalo, NY). For determination of oxidized LDL (oxLDL), lipids were first isolated from mouse serum by manganese citrate-heparin precipitation (29). As a positive oxidized control, a separate aliquot of serum was incubated with 50 μM CuSO4 for 300 min according to method described by Cominacini et al. (19). oxLDL was assessed by measurement of thiobarbituric acid-reactive substance in the solubilized LDL pellet.

**3-Nitrotyrosine.** 3-Nitrotyrosine (3-NT) levels, a marker of peroxynitrite formation, were assessed with a commercially available ELISA (Oxis Research, Portland, OR). Tissues were first homogenized and diluted to a concentration of 1 mg/ml protein in dilution buffer supplied by the manufacturer. Serum samples were diluted 1:10 in dilution buffer. Antigen captured by a solid-phase monoclonal antibody was detected with a biotin-labeled goat polyclonal anti-nitrotyrosine. Streptavidin peroxidase was allowed to bind to the biotinylated antibody. Addition of a tetramethoxybenzidine substrate yielded a yellow product, which was measured at 450 nm. All unknowns were run in triplicate, and log 3-NT levels were interpolated by nonlinear regression using Prism software (GraphPad, San Diego, CA). 3-NT levels were normalized to milligrams of protein as determined by Bradford assay. Nitrotyrosine was used as a competitive blocking control to verify specificity of observed interactions. Liver from mice treated for 4 wk with acetaminophen (200 mg·kg−1·day−1·ip) was used as a positive control and demonstrated elevated hepatic and serum 3-NT levels (data not shown).

**GSH and GSH-to-GSSG ratio.** GSH and GSH-to-GSSG ratio (GSH/GSSG) were determined by use of commercially available colorimetric assays (Oxis Research). For GSSG measurement in erythrocytes, an aliquot of 100 μl of freshly drawn whole blood was immediately mixed with 10 μl of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate to scavange reduced glutathione before freeze-thaw lysis. Frozen tissues were placed in PBS containing 10% 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate before homogenization. Samples were further diluted in metaphosphoric acid and assayed according to the manufacturer’s protocol. GSH/GSSG was calculated as follows: ([GSH] − (2[GSSG])/[GSSG]), where [GSH] and [GSSG] are GSH and GSSG concentrations.

**SOD activity.** Liver or heart tissue was homogenized and diluted to a concentration of 5 mg/ml protein per sample. Homogenate (40 μl) was then used in a commercially available kinetic assay (Oxis Research). For SOD activity in red blood cells, whole blood was centrifuged to obtain the erythrocyte pellet that was diluted in 4 vol of ice-cold water for hypotonic lysis. Before assay, the lysate was diluted another fourfold in assay buffer supplied by the manufacturer. The assay measures the SOD-mediated increase in the rate of autooxidation of 5,6,6-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Absorbance of the chromophore was measured over 3 min, and the slope of the inflection point (Vc) was divided by the slope of a blank autooxidation sample (Vb). The Vc/Vb ratio was used to calculate the SOD units, where 1 unit of SOD activity is defined as the activity that doubles the autooxidation rate of the control blank (Vc/Vb = 2).

**Abundance of p47phox protein and serine phosphorylation.** Descending aorta was homogenized in 150 mM NaCl, 20 mM Tris (pH 7.2), 1% Triton X-100, and 1 mM DTT with complete protease inhibitor (Roche Diagnostics, Mannheim, Germany). Homogenate was electrophoresed in 4–15% Tris-glycine SDS polyacrylamide with 80°C for future analysis. Immunoblot analysis was performed (22–24°C with a 12:12-h light-dark cycle; lights on at 0900) with the following primary antibodies: p47phox (1:1000), phospho-p47phox (Ser426, 1:1000). The abundance of p47phox protein and serine phosphorylation was quantified using a chemiluminescence detection system (AutoImager, Molecular Dynamics, Sunnyvale, CA).

### Table 1. Mouse weight at the start and end of IA or IH exposures and average daily food consumption

<table>
<thead>
<tr>
<th>Group</th>
<th>IA</th>
<th>IH</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>23.6±0.4</td>
<td>21.2±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>3.4±0.2</td>
<td>2.9±0.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 2. Biomarkers of oxidative stress and antioxidant status in serum and blood of mice exposed to IA or IH

<table>
<thead>
<tr>
<th>Group</th>
<th>IA</th>
<th>IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum MDA, μM/ml</td>
<td>0.36±0.01</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>Serum ox-LDL, μM/ml</td>
<td>92.9±5.2</td>
<td>66.9±4.2*</td>
</tr>
<tr>
<td>Serum 3-Nitrotyrosine, μM/ml</td>
<td>4.1±1.0</td>
<td>3.5±0.9</td>
</tr>
<tr>
<td>RBC SOD, U/μl</td>
<td>6.7±1.24</td>
<td>7.38±1.84</td>
</tr>
<tr>
<td>RBC GSH, μM/μl</td>
<td>200.9±4.36</td>
<td>185.2±9.72†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 32 (8 mice per group). MDA, malondialdehyde; oxLDL, oxidized LDL; 3-NT, 3-nitrotyrosine. *P < 0.001; †P = 0.17 vs. IA.

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diethyl fluoride membranes and probed with anti-p47phox (catalog no. C20, Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology; 1:5,000 dilution). Immune complexes were visualized with an enhanced chemiluminescence detection system. After immunoblotting with anti-p47phox, we assessed serine phosphorylation by stripping the membrane and reprobing with phosphorylated (Ser/Thr) Akt substrate antibody (Cell Signaling Technologies; 1:1,000 dilution). Actin was used as a loading control for all samples.

**RESULTS**

**Body weight and food intake.** In the 1- and 4-wk exposure groups, mice exposed to IA gained weight while mice exposed to IH lost weight. Most of the weight was lost by the end of the 1st wk, after which food intake increased (Table 1). Thus, chronic IH resulted in weight loss and decreased food intake, consistent with our previous observations (54).

**Serum and blood markers of oxidative stress and antioxidant status.** IH exposure for 1 or 4 wk did not produce detectable changes in serum MDA or 3-NT, erythrocyte SOD activity, or GSH/GSSG (Table 2). There was a trend toward decreased erythrocyte GSH after 1 wk ($P = 0.17$), but not after 4 wk. To determine the effect of IH on oxLDL, a recognized precursor to atheroma formation (16, 40, 83, 84, 95), we isolated LDL and VLDL fractions from serum and measured lipid peroxidation in the solubilized lipid pellet. We were surprised to find a decrease in oxLDL in the serum after 1 wk of IH (Table 2). At 4 wk, there was no difference in oxLDL between controls and experimental animals.

**Cardiovascular oxidative stress, NADPH oxidase activity, and antioxidant status.** Biomarkers of oxidative stress and antioxidant status in the heart and aorta are shown in Table 3. In the heart, IH did not lead to increases in MDA or 3-NT at 1 or 4 wk. There was also no apparent change in p47phox expression or serine phosphorylation, suggesting unchanged NADPH oxidase activity (Figs. 1 and 2). SOD activity, GSH, and GSH/GSSG in the heart were unchanged across all groups (Table 4). In aortic tissue, there was a trend toward increased MDA after 1 wk of IH (Table 3), but not after 4 wk. There were...
no IH-induced increases in MDA, 3-NT, or p47phox expression and serine phosphorylation.

Liver oxidative stress, NADPH oxidase activity, and antioxidant status. IH led to increases in lipid peroxidation in the liver (Fig. 3). The effect was significant after 4 wk, leading to a 38% increase in hepatic MDA levels. There was a concurrent increase in p47phox expression and serine phosphorylation after 4 wk of IH exposure (Figs. 1 and 2). GSH trended toward a decrease (P = 0.058) after 1 wk but was restored at 4 wk, and GSH/GSSG was unaffected at 1 and 4 wk (Table 4). There was no effect of IH on liver 3-NT (not shown) or SOD activity (Table 4). IH did not induce overt liver injury, as indicated by serum aminotransferase levels or organ weight (Table 5). Serial sections of liver showed no evidence of tissue injury.

Table 4. SOD activity, GSH, and GSH-to-GSSG ratio in heart and liver following exposure to IA or IH

<table>
<thead>
<tr>
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<th>Heart</th>
<th>Liver</th>
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<tr>
<td></td>
<td>IA</td>
<td>IH</td>
</tr>
<tr>
<td>SOD, U/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>11.6±1.1</td>
<td>10.9±1.4</td>
</tr>
<tr>
<td>4 wk</td>
<td>10.0±1.2</td>
<td>10.8±1.2</td>
</tr>
<tr>
<td>GSH, μM/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>17.3±1.8</td>
<td>14.7±1.4</td>
</tr>
<tr>
<td>4 wk</td>
<td>18.5±1.7</td>
<td>21.1±2.0</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>176±23.1</td>
<td>206±36.2</td>
</tr>
<tr>
<td>4 wk</td>
<td>191±29.9</td>
<td>188±35.0</td>
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</table>

Values are means ± SE; n = 32 (8 mice per group). *P = 0.058 vs. IA.
Neither apocynin nor p47phox deletion had a detectable effect on mouse weight, liver phenotype, noninvasively measured blood pressure, 3-NT, GSH, GSH/GSSG, or SOD activity (data not shown). Despite stringent hygiene for all animals, several p47phox−/− mice developed lung granulomas at the time of death. After 1 wk of exposure, two of eight control p47phox−/− mice and three of eight hypoxic p47phox−/− mice had evidence of pulmonary infection; after 4 wk of exposure, pulmonary infection was present in five of eight control p47phox−/− mice and four of eight p47phox−/− hypoxic animals. There was no evidence of infection in any of the placebo- or apocynin-treated animals.

**DISCUSSION**

We found that IH had variable effects, depending on exposure length and the organ examined. IH did not affect markers of oxidative stress in the circulation or cardiovascular system; NADPH oxidase expression in the heart and aorta did not change. In contrast, in the liver, IH increased lipid peroxidation, NADPH oxidase p47phox subunit protein levels, and phosphorylation. The IH-induced hepatic lipid peroxidation was attenuated by apocynin and abolished by knock out of p47phox. All these effects became significant after 4 wk. Taken together, our data demonstrate a spectrum of tissue susceptibilities to oxidative stress from IH, and this susceptibility appears related to activation of NADPH oxidases.

We did not detect significant oxidative stress in the circulation and cardiovascular organs during IH. It is clear from the literature that myocardial I/R induces an injurious cascade of ROS (2, 26, 36, 92). Our model of hypoxia-reoxygenation does not lead to a similar phenomenon and suggests that the nature of the IH insult does not closely parallel I/R physiology. This could be related to the short duration of hypoxia cycles, incomplete anoxia, or absence of other flow-related I/R events, such as altered ion gradients, capillary damage, and neutrophil influx. However, our group and other researchers employing similar, if not milder, IH exposures in mice have shown upregulation of ROS in the brain, carotid bodies, adrenal glands, and liver (50, 54, 71, 76, 79, 98). IH in the rat led to progressive cardiac lipid peroxidation (13). It is readily apparent that IH-induced oxidative stress is a phenomenon that is species, organ, and time dependent.

Among the organs considered in the present study, the liver was uniquely susceptible to IH. We found an increase in lipid peroxidation over time with upregulation of liver NADPH oxidases. A trend toward decreased GSH stores was seen in 1 wk, and levels were restored after 4 wk. GSH/GSSG was unaffected; we infer that GSH synthesis may have been impaired, but the pool of available GSH was not oxidized by IH. Decreased overall food intake with a relative cysteine deficiency can rapidly deplete hepatic GSH stores (62, 89).

Two lines of evidence point to NADPH oxidase as a source of IH-induced ROS in our model. 1) There was an increase in liver p47phox expression and phosphorylation in the liver coinciding with the increase in MDA. 2) MDA levels in wild-type mice treated with apocynin and in p47phox−/− animals were blunted or even decreased after IH. Our finding of increased MDA during IA in p47phox−/− mice is consistent with other reports showing paradoxically increased ROS in the absence of the p47phox subunit (56). It has been postulated that p47phox actually inhibits NADPH oxidase in unstimulated cells (57). In the absence of functional NADPH oxidase, other ROS-generating systems may also become activated in a compensatory manner, as has been described in the context of acute lung injury (49). The reason for the decrease in MDA to control levels during IH is less clear. An otherwise competitive antioxidant effect, such as decreased food intake and weight loss induced by IH, may have become unmasked under p47phox−/− conditions. Thus, the inhibitory effects of apocynin and p47phox deletion demonstrate that functional NADPH oxidase in the liver is necessary and sufficient for lipid peroxidation during IH.

What predisposed the liver, an organ with high antioxidant reserves, to IH-mediated oxidative stress? 1) The liver likely faced a greater hypoxic insult than the cardiovascular system. Under hypoxic and stressful conditions, blood flow and oxygen delivery are diverted to the cardiovascular and cerebral tissue beds at the expense of visceral organs. Lactate generated from anaerobic glycolysis is readily metabolized by the heart and skeletal muscle (30, 73) but must be salvaged for gluconeogenesis in the liver. The liver was thus effectively hypoxic for longer periods of time. 2) Responses to the hypoxic insult may differ between liver and heart. Liver I/R injury was attenuated in apocynin-treated (58) or gp91phox-deficient (33) mice. On the contrary, p47phox−/− mice were not protected from myocardial I/R injury (37). The liver is also host to phagocytic Kupffer cell NADPH oxidases, which release superoxide during ischemia (90) and mediate oxidative injury from toxins (21, 67). 3) Liver oxidative stress may have occurred via an indirect pathway. Chronic IH elevates serum and hepatic cholesterol levels (54, 55). In rats fed a high-cholesterol diet, liver injury was attenuated by apocynin with concurrent reversal of oxidative stress (60).

MDA, a marker of lipid peroxidation, and 3-NT, a marker of tyrosine oxidation and peroxynitrite formation, are widely used biomarkers of oxidative stress chosen in the present study for their documented relevance to atherosclerosis (16, 70, 82–84, 95) and hepatic oxidative stress (41, 42, 59, 91). In our study, MDA, but not 3-NT, changed with IH exposure. One possibil-
ity for the discrepancy is that superoxide was generated during reoxygenation and quickly reacted with regional lipids, rather than with nitric oxide (39). Furthermore, protein nitration may occur in subcellular compartments (8, 28) without detectable increases during assessment of total tissue homogenates.

We must acknowledge several limitations of the present study. Our investigation was designed primarily to examine oxidative stress, rather than functional or anatomic tissue injury. Therefore, we conducted our experiments on relatively healthy C57BL/6J mice fed regular diets, with the knowledge that mice of the same genetic background developed atherosclerosis only if exposed to IH and a high-cholesterol diet (78) and suffered liver injury only if exposed to IH and another insult such as a high-fat diet or acetaminophen (77, 79). In this “single-hit” study of IH, we were not surprised to find modest oxidative stress without a visible effect on organ structure or function. It is therefore beyond the scope of the present study to conclude that the observed oxidative stress causes a detrimental phenotype.

With regard to detection of oxidative stress, it is conceivable that our methods lacked sensitivity. Antioxidant defenses and tissue repair mechanisms may have obscured oxidative stress generated by IH. Lack of change in antioxidant levels might be interpreted as an absence of an oxidizing insult, or they could also reflect ample, unaltered antioxidant reserve in the face of a mild insult. We also should be careful to distinguish oxidative stress from ROS in general. Low, signal-level ROS can have physiological roles or induce downstream pathological responses without overt tissue modification (74). Another potential confounder was the effect of IH on diet and body weight. Mice exposed to IH lost weight and consumed less food than IA controls. Although reasons for the weight loss and decreased food intake are not the focus of the present study, IA controls. Although reasons for the weight loss and decreased food intake are not the focus of the present study, dietary changes provide a logical explanation for several of our observations. Early decreases in liver and erythrocyte GSH, serum oxLDL, and MDA levels in p47phox-deficient mice coincide with early reduced food intake, and some of these parameters normalized when food intake improved later in the time course. A weight-matched experiment or one with a food-restricted control group might enable us to answer this question.

We must also acknowledge limitations with the use of apocynin and p47phox−/− mice. Apocynin is a drug with nonspecific effects and antioxidant properties potentially unrelated to NADPH oxidase inhibition (94). Nevertheless, it has been widely used in vivo and with more specificity than other pharmacological agents such as diphenylene iodonium (94). p47phox−/− mice are susceptible to infections, and there was evidence of lung granulomas in several mice in our experiment at the time of death. It is difficult to know the impact of this finding on our results, even though a similar number of mice in IH and control groups was affected. We also did not see a significant difference in liver MDA levels between p47phox−/− mice with granulomas and those without. Despite these limitations, we believe that our collective data suggest a role for NADPH oxidase during IH.

Perspective and Significance

To our knowledge, this is the first investigation of oxidative stress induced by chronic IH in multiple organ systems. There were strikingly heterogeneous responses to IH across different organs over time. Over the 4-wk time course of IH, there was no evidence of oxidative stress in the circulation, heart, or aorta, whereas the liver appeared to be affected. We also capture the differential role of NADPH oxidase in different tissues during IH and provide evidence that IH-induced liver lipid peroxidation is mediated via NADPH oxidasas. Our data show that oxidative stress in the circulation during IH does not necessarily reflect end-organ effects and that serum markers may be of limited use in assessing oxidative stress in the OSA patient.

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GRANTS

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INTERMITTENT HYPOXIA AND OXIDATIVE STRESS