INTERMITTENT HYPOXIA HAS ORGAN-SPECIFIC EFFECTS ON
OXIDATIVE STRESS

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Running Head: Intermittent Hypoxia and Oxidative Stress

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ABSTRACT

Obstructive sleep apnea (OSA) is characterized by upper airway collapse, leading to intermittent hypoxia (IH). It has been postulated that IH-induced oxidative stress may be a contributing factor to several chronic diseases associated with OSA. We hypothesize that IH induces systemic oxidative stress by up-regulating NADPH oxidase, a superoxide-generating enzyme. NADPH oxidase is regulated by a cytosolic p47phox subunit, which becomes phosphorylated during enzyme activation. Male C57BL/6J mice were exposed to IH with a FiO2 nadir 5% 60 times/hr during the 12 hr light phase (9am-9pm) for one or four weeks. In the aorta and heart, IH did not affect levels of lipid peroxidation (malondialdehyde, MDA), nitrotyrosine, or p47phox expression and phosphorylation. In contrast, in the liver, exposure to IH for 1 week resulted in a trend to an increase in MDA levels, whereas IH for 4 wks resulted in a 38% increase in MDA levels accompanied by up-regulation of p47phox expression and phosphorylation.

Administration of an NADPH oxidase inhibitor, apocynin during IH exposure attenuated IH-induced increases in hepatic MDA. In p47phox deficient mice, MDA levels were higher at baseline, and unexpectedly decreased during IH. In conclusion, oxidative stress levels and pathways under IH conditions are organ and duration specific.

Keywords: Intermittent hypoxia, oxidative stress, obstructive sleep apnea, NADPH oxidase
INTRODUCTION

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 oxygen desaturations in patients with OSA (24; 64; 85). A causal role of OSA in atherosclerosis is supported by evidence that CPAP treatment 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; 44-46; 65; 68; 88). CPAP therapy ameliorates aminotransferase elevations (46) and experimental IH causes inflammatory liver injury in mice on a high fat, high cholesterol diet (77). A mechanism has not yet been established for these observations.

Diseases associated with OSA are thought to be due to, at least in part, oxidative stress (51; 97). The hypothesis that IH causes oxidative stress stems from observations that ischemia-reperfusion (I/R) 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). In contrast to the above, other investigators have 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 pathologic 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. Cytosolic 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 ApoE−/− mice (7), demonstrating the relevance of this enzyme to cardiovascular disease. Inhibition of NADPH oxidase with apocynin also attenuates cholesterol-induced liver injury (61).

Our study set out to answer three questions. First, does IH per se induce circulatory or tissue oxidative stress? Secondly, does IH affect NADPH oxidase activity in tissues? Third, does NADPH oxidase activation contribute to IH-induced oxidative stress in the aorta, heart or liver?
METHODS

Animals

Sixty-four 8 week-old male, lean C57BL/6J mice, and sixteen 8-week-old male p47^phox^-/- mice were purchased from Jackson Laboratory (Bar Harbor, Maine). 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. As animals lacking functional NADPH oxidase are prone to infection, animals were housed two animals per cage, food and bedding was 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 hours prior to bleeding and sacrifice. For biochemical assays, the heart, descending aorta, and livers 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, nitrogen and oxygen into customized cages housing the mice as previously described (72). During each period of IH, the FIO_{2} was reduced from 20.9 to 4.9 ± 0.1 % over a 30 second period and then rapidly reoxygenated to room air levels in the subsequent 30 second
period. Animals were kept in a controlled environment (22-24 °C with a 12 hour : 12 hour light : dark cycle; lights on at 09.00) with free access to food and water. Control animals were exposed to intermittent air (IA), with a flow pattern similar to that of the IH group, but at a fixed 21% FIO₂. IH and IA states were induced during the 12 hour light phase alternating with 12 hours of constant room air during the dark phase.

Apocynin injections

Mice were given daily intraperitoneal injections of apocynin, 2 mg/kg/day dissolved in 0.9% saline according to the method used by Hart et al. (35) A matched control group (n=8) received i.p. 2 mg/kd/day 0.9% saline placebo.

Lipid peroxidation

Tissues were isolated and homogenized in 10 µL/mg ice-cold PBS containing 5 mM butylated hydroxytoluene to inhibit ex vivo oxidation. Serum and tissue thiobarbituric acid (TBARS) was determined using commercially available kits (Zeptometrix, Buffalo, NY). For determination of oxidized LDL (ox-LDL), 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 minutes according to method described by Comanici et al.(19). Oxidized-LDL was assessed by measurement of TBARS in the solubilized LDL pellet.

3-Nitrotyrosine(3-NT)
3-NT levels, a marker of peroxynitrite formation, were assessed with a commercially available ELISA assay (Oxis Research, Portland, OR). Tissues were first homogenized and diluted to a concentration of 1 mg/mL protein in supplied dilution buffer. 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. A tetramethylbenzidine substrate was added, yielding a yellow product which was measured at 450 nm. All unknowns were run in triplicate, and log 3-NT levels interpolated by nonlinear regression using Prism software (GraphPad software, San Diego, CA). 3-NT levels were normalized to mg protein as determined by Bradford assay. Nitration albumin was used as a competitive blocking control to verify specificity of observed interactions. Liver from mice treated with 4 weeks of daily 200 mg/kg i.p injections of acetaminophen was used as a positive control and demonstrated elevated hepatic and serum 3-NT levels (data not shown).

Glutathione (GSH) and Reduced:Oxidized glutathione ratios (GSH:GSSG)

Total glutathione and GSH:GSSG ratios were determined by use of commercially available colorimetric assays. (Oxis Research, Portland, OR) For GSSG measurement in erythrocytes, an aliquot of 100 µL freshly drawn whole blood was immediately mixed with 10 µL 1-Methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) to scavenge reduced glutathione prior to freeze-thaw lysis. Frozen tissues were placed in PBS containing 10% M2VP prior to homogenization. Samples were further diluted in
metaphosphoric acid and assayed according to manufacturer protocol. The GSH:GSSG ratio was calculated by the formula \((\text{[GSG]}-2\times\text{[GSSG]})/\text{[GSSG]}\).

**Superoxide Dismutase (SOD) Activity**

Liver or heart tissue was homogenized and diluted to a concentration of 5 mg/mL protein per sample. 40 µL homogenate was then used in commercially available kinetic assay (Oxis Research, Portland, OR). For SOD activity in red cells, whole blood was centrifuged to obtain the erythrocyte pellet which was diluted in 4 volumes ice-cold water for hypotonic lysis. The lysate was diluted another four-fold in supplied assay buffer prior to assay. The assay measures the SOD-mediated increase in the rate of auto-oxidation of 5,6,6a,11b-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 minutes, and the slope of the inflection point \((V_s)\) was divided by the slope of a blank auto-oxidation sample \((V_c)\). The \(V_s/V_c\) ratio was used to calculate the SOD units, where 1 unit of SOD activity is defined as the activity that doubles the auto-oxidation rate of the control blank \((V_s/V_c = 2)\)

**p47phox protein abundance and serine phosphorylation**

Descending aorta was homogenized in 150 mM NaCl 20 mM Tris, pH 7.2 1% Triton X-100 1 mM DTT with complete protease inhibitor (Roche Diagnostics, Mannheim, Germany). Homogenate was electrophoresed in 4–15% Tris-glycine sodium dodecyl sulfate polyacrylamide with a Bio-rad pre-cast system. Proteins were transferred onto polyvinyl diethyl fluoride membranes and probed with anti-p47phox (C20, Santa Cruz
Biotechnology, 1:100 dilution) followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:5000 dilution). Immune complexes were visualized with enhanced chemiluminescence (ECL) detection system. After immunoblotting with anti-p47^{phox}, serine phosphorylation was assessed by stripping the membrane and re-probing with phospho-Ser/Thr Akt substrate antibody (Cell Signaling Technologies, 1:1000 dilution) Actin was used as a loading control for all samples.

Statistical Analysis

All values are reported as mean ± standard error of the mean (SEM). Statistical comparisons between groups of mice were performed by a general linear model ANOVA across two independent variables, hypoxia and time course, followed by the Tukey’s post-hoc test. In experiments involving apocynin, ANOVA was performed independently for the 1 week and 4 week data set. (Standard error_{1H}/Mean MDA_{1H}) + (Standard error_{1A}/Mean MDA_{1A}) was used to estimate the error of the ratios.
RESULTS

Body Weight and Food Intake

In both the 1 and 4-week 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 first week, 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 weeks did not produce detectable changes in serum MDA or 3-NT, erythrocyte SOD activity or GSH:GSSG ratios (Table 2). There was a trend towards decreased erythrocyte GSH after one week ($P=0.17$), which was no longer the case after four weeks. To determine the effect of IH on oxidized LDL, 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. Surprisingly, there was a decrease in ox-LDL in the serum, after 1 week of IH (Table 2). At 4 weeks, there was no difference in ox-LDL 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 either time point. There was also no apparent change in p47phox expression or serine phosphorylation, suggesting unchanged NADPH oxidase activity (Fig. 1 and 2). Superoxide dismutase
(SOD) activity, GSH, and GSH:GSSG ratios in the heart were unchanged across all groups (Table 4). In aortic tissue, there was a trend towards increased MDA after 1 week of IH (Table 3) but not after 4 weeks. Otherwise, there were no IH-induced increases in MDA, 3-NT, or in 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 weeks, leading to a 38% increase in hepatic MDA levels. There was a concurrent increase in p47phox expression and serine phosphorylation after 4 weeks of IH exposure (Fig. 1 and 2). Total glutathione trended towards a decrease (P=.058) after 1 week but was restored at 4 weeks, and the ratio of GSH:GSSG was unaffected at either time point (Table 4). There was no effect of IH on liver 3-NT (not shown), or SOD activity (Table 4). IH did not induce any overt liver injury by serum aminotransferase levels or organ weight (Table 5). Serial sections of liver were also examined without evidence of tissue injury.

Effect of Altered NADPH oxidase function on Liver Lipid Peroxidation

Apocynin prevented a rise in MDA level at 1 week, and attenuated IH-induced increases in liver MDA after 4 weeks (Fig.3, 4). In p47phox-/- mice, baseline liver MDA levels were approximately 20% higher than in wild-type C57BL/6J animals. In contrast to wildtype mice, in p47phox-/- mice, IH resulted in a significant decrease of the hepatic MDA levels (Fig. 3, 4). Neither apocynin nor p47phox deletion had any detectable effect on mouse weight, liver phenotype, non-invasive blood pressure, 3-NT, GSH, GSH:GSSG, or SOD
activity (data not shown). In spite of stringent hygiene for all animals, several \textit{p47}^{\text{phox}}^{-/-} mice developed lung granulomata at time of sacrifice. After one week exposure, 2 of 8 control \textit{p47}^{\text{phox}}^{-/-} mice and 3 of 8 hypoxic \textit{p47}^{\text{phox}}^{-/-} mice had evidence of pulmonary infection; after the 4-week exposure, pulmonary infection was present in 5 of 8 control \textit{p47}^{\text{phox}}^{-/-} mice and 4 of 8 \textit{p47}^{\text{phox}}^{-/-} hypoxic animals. No evidence of infection was seen in any of the placebo or apocynin-injected animals.
DISCUSSION

We found that IH had variable effects, depending on both 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 knockout of p47phox. All of these effects became significant after 4 weeks. 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 length 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 up-regulation of ROS in the brain, carotid bodies, adrenal glands and the 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 this study, the liver was uniquely susceptible to IH. We found an increase in lipid peroxidation over time with up-regulation of liver NADPH oxidases. A trend towards decreased total glutathione stores was seen at 1 week with restored levels after four weeks. Because the GSH:GSSG ratios were unaffected, we infer that glutathione 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 glutathione stores (62; 89).

Two lines of evidence point to NADPH oxidase as a source of IH-induced ROS in our model. First, there was an increase in liver p47phox expression and phosphorylation in the liver coinciding with the increase in MDA. Second, wildtype mice treated with apocynin and p47phox−/− animals had blunted or even decreased MDA levels after IH. Our finding of increased MDA under IA conditions 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 that of decreased food intake and weight loss from IH may have become unmasked under p47phox−/− conditions. Thus, the inhibitory effects of apocynin and p47phox deletion demonstrate that functional NAPDH 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? First, 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. Second, responses to the hypoxic insult may differ between liver and heart. Liver I/R injury was attenuated in mice given apocynin (58) or lacking gp91phox (33). On the contrary, p47phox/- mice were not protected from myocardial ischemia-reperfusion injury (37). The liver is also host to phagocytic Kupffer cell NADPH oxidases that release superoxide during ischemia (90) and which mediate oxidative injury from toxins (21; 67). Lastly, 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 this 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 possibility for the discrepancy is that superoxide was generated during reoxygenation that 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 this study. Our investigation was designed to primarily examine oxidative stress rather than functional or anatomic tissue injury. Therefore, we conducted our experiments on relatively healthy C57BL/6J mice on regular diets, knowing that mice on 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 high-fat diet or acetaminophen (77; 79). Not surprisingly, in this “single hit” study of IH, we demonstrated modest oxidative stress without visibly affecting organ structure or function. It is therefore beyond the scope of the current 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 physiologic roles, or induce downstream pathologic changes 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.
While reasons for the weight loss and decreased food intake are not the focus of this study, caloric restriction is protective against oxidative stress (4; 11; 14; 31; 81), potentially negating the injury of IH. Indeed, dietary changes provide a logical explanation for several of our observations. Early decreases in liver and erythrocyte GSH, serum ox-LDL, and MDA levels in p47^{phox/-} 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 in the future.

We must also acknowledge limitations with the use of apocynin and p47^{phox/-} mice. Apocynin is 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 pharmacologic agents such as diphenylene iodonium (94). p47^{phox/-} mice are susceptible to infections, and several mice in our experiment had evidence of lung granulomas at time of sacrifice. It is difficult to know what impact this had on our results, even though a similar number of mice between IH and control groups was affected. We also did not see a significant difference in liver MDA levels between p47^{phox/-} mice with granulomas and those without. In spite of these limitations, we feel 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-week 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 oxidases. Our data show that querying 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.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

**Figure 1.** p47phox protein expression in the heart, aorta, and liver of mice exposed to 1 or 4 weeks of intermittent hypoxia (IH). Representative immunoblots are shown, arranged to show 2 bands out of n=4 per group. In the heart and aorta, IH did not induce any changes in p47phox expression (shown) or in serine phosphorylation (not shown) at either time point. In the liver, IH caused an increase in p47phox expression and serine phosphorylation after 4 weeks.

**Figure 2.** p47phox protein expression in the heart, aorta, and liver of mice exposed to 1 and 4 weeks of intermittent air (white) or intermittent hypoxia (black). Optical densitometry of immunoblots from Figure 1 shown, using p47phox/actin ratios. IH induced a twofold increase in hepatic p47phox protein expression and phosphorylation. *denotes p<0.05 for the difference between IA and IH.

**Figure 3.** Liver MDA after exposure to 1 or 4 weeks of intermittent air control (IA) or intermittent hypoxia (IH) in mice given placebo, apocynin, (2 mg/kg/day), or mice lacking functional p47phox. At 1 week, there was a trend toward increased MDA with IH (p= 0.18). At 4 weeks, IH induced a 38% increase in MDA. (*denotes p <0.005) Apocynin attenuated the rise in MDA. p47phox-/- mice exhibited increased baseline MDA levels which normalized under IH conditions. n=96, 8 animals per group.
Figure 4. Mice given placebo, apocynin (2 mg/kg/day), or mice lacking functional p47phox were exposed to 1 or 4 weeks of intermittent air control (IA) or intermittent hypoxia (IH). IH/IA MDA ratios are shown. At 4 weeks, the time point at which IH resulted in significant increases in MDA, apocynin significantly attenuated the rise in liver MDA (*p < 0.01 vs. placebo) p47phox−/− mice exhibited decreased MDA after IH as a result of increased oxidative stress in the IA condition (†p <0.005 vs. placebo). The decrease in MDA was greater in p47phox mice than apocynin-treated mice (p<0.05). Error bars for the ratios are the sum of the standard errors of both IA and IH groups as a percentage of the MDA total.
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Ref Type: Generic


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Table 1. Mouse weight at the start and end of intermittent air (IA) or intermittent hypoxia (IH) exposures, and average daily food consumption per animal.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight in grams (± SEM)</th>
<th>p</th>
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<tbody>
<tr>
<td></td>
<td>IA</td>
<td>IH</td>
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<tr>
<td>1 week</td>
<td>Start 23.6 ± 0.4</td>
<td>21.2 ± 0.8</td>
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<td></td>
<td>End 25.0 ± 0.7</td>
<td>20.1 ± 0.7</td>
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<td></td>
<td>Δ weight 1.4</td>
<td>-1.1</td>
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<td></td>
<td>Daily food 3.4 ± 0.2</td>
<td>2.9 ± 0.4</td>
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<tr>
<td>4 week</td>
<td>Start 26.5 ± 0.7</td>
<td>26.9 ± 0.9</td>
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<td></td>
<td>End 28.7 ± 0.5</td>
<td>25.4 ± 0.7</td>
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<tr>
<td></td>
<td>Δ weight 2.1</td>
<td>-1.4</td>
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<td></td>
<td>Daily food 3.8 ± 0.2</td>
<td>3.3 ± 0.1</td>
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<tr>
<td>Group</td>
<td>IA</td>
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<tr>
<td>Serum MDA (µM/mL)</td>
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<tr>
<td>1 week</td>
<td>0.36 ± 0.01</td>
<td>0.34 ± 0.01</td>
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<td>4 week</td>
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<td>Serum ox-LDL (µM/mL)</td>
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<tr>
<td>1 week</td>
<td>92.9 ± 5.2</td>
<td>66.9 ± 4.2*</td>
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<td>4 week</td>
<td>93.3 ± 6.7</td>
<td>88.1 ± 6.7</td>
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<td>Serum 3-NT (nM/mL)</td>
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<tr>
<td>1 week</td>
<td>4.1 ± 1.0</td>
<td>3.5 ± 0.9</td>
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<td>4 week</td>
<td>3.2 ± 0.6</td>
<td>3.4 ± 0.8</td>
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<td>RBC SOD (U/100 µL RBCs)</td>
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<tr>
<td>1 week</td>
<td>7.86 ± 1.24</td>
<td>7.38 ± 1.84</td>
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<tr>
<td>4 week</td>
<td>7.57 ± 0.98</td>
<td>7.64 ± 1.61</td>
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<td>RBC GSH (µM/100 uL RBCs)</td>
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<td>1 week</td>
<td>200.9 ± 4.36</td>
<td>185.2 ± 9.72†</td>
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<td>4 week</td>
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<td>RBC GSH/GSSG ratio</td>
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<td>1 week</td>
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<td>156 ± 56.8</td>
</tr>
<tr>
<td>4 week</td>
<td>138 ± 31.4</td>
<td>164 ± 38.4</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *P<0.001 and †P<0.017 for the difference between IH and IA.
Table 3. Biomarkers of oxidative stress in heart and aorta of mice exposed to intermittent air control (IA) or intermittent hypoxia (IH). n=32, 8 mice per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart</th>
<th></th>
<th></th>
<th>Aorta</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>IH</td>
<td>IA</td>
<td>IH</td>
<td></td>
</tr>
<tr>
<td>MDA(μM/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>3.56 ± 0.42</td>
<td>3.18 ± 0.24</td>
<td>4.47 ± 0.96</td>
<td>6.87 ± 1.08*</td>
<td></td>
</tr>
<tr>
<td>4 week</td>
<td>3.38 ± 0.30</td>
<td>2.76 ± 0.49</td>
<td>4.28 ± 1.04</td>
<td>4.13 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>3-NT (nM/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>1.63 ± 0.51</td>
<td>1.71 ± 0.65</td>
<td>11.01 ± 1.43</td>
<td>11.40 ± 2.26</td>
<td></td>
</tr>
<tr>
<td>4 week</td>
<td>1.34 ± 0.37</td>
<td>1.16 ± 0.23</td>
<td>16.07 ± 1.76</td>
<td>16.25 ± 1.38</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *P = 0.14 for the difference between IH and IA.
Table 4. Superoxide dismutase (SOD) activity, total glutathione (GSH) and reduced:oxidized glutathione ratio (GSH/GSSG) in heart and liver following exposure to intermittent air control (IA) or intermittent hypoxia (IH). n=32, 5 mice per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>IH</td>
</tr>
<tr>
<td>SOD (units/mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>11.6 ± 1.1</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td>4 week</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 week</td>
<td>17.3 ± 1.8</td>
<td>14.7 ± 1.4</td>
</tr>
<tr>
<td>4 week</td>
<td>18.5 ± 1.7</td>
<td>21.1 ± 2.0</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>176 ± 23.1</td>
<td>206 ± 36.2</td>
</tr>
<tr>
<td>4 week</td>
<td>191 ± 29.9</td>
<td>188 ± 35.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. P= 0.058 for the difference between IH and IA.
Table 5. Liver weight and function following exposure to intermittent air control (IA) or intermittent hypoxia (IH), n=32, 8 mice per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>IA</th>
<th>IH</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>1291 ± 70.3</td>
<td>1165 ± 43.4</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>1355 ± 32.6</td>
<td>1149 ± 45.2</td>
<td>NS</td>
</tr>
<tr>
<td>Liver/B. weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>0.052</td>
<td>0.058</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>0.047</td>
<td>0.045</td>
<td>NS</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>50.5 ± 2.63</td>
<td>48.0 ± 2.80</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>41.4 ± 3.0</td>
<td>47.3 ± 5.64</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>23.4 ± 1.15</td>
<td>18.7 ± 1.61</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>25.0 ± 1.0</td>
<td>24.8 ± 5.83</td>
<td>NS</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>4.6 ± 0.07</td>
<td>4.4 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>4.9 ± 0.05</td>
<td>5.0 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>3.5 ± 0.02</td>
<td>3.2 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>4 week</td>
<td>3.3 ± 0.05</td>
<td>3.5 ± 0.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.