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Dichotomous effects of chronic intermittent hypoxia on focal cerebral ischemic injury

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Abstract

Background and purpose—Obstructive sleep apnea (OSA), a condition associated with chronic intermittent hypoxia (CIH), carries an increased risk of stroke. However, CIH has been reported to either increase or decrease brain injury in models of focal cerebral ischemia. The factors determining the differential effects of CIH on ischemic injury and their mechanisms remain unclear. Here, we tested the hypothesis that the intensity of the hypoxic challenge determines the protective or destructive nature of CIH by modulating mitochondrial resistance to injury.

Methods—Male C57Bl/6J mice were exposed to CIH with 10% or 6% O₂ for up to 35 days and subjected to transient middle cerebral artery occlusion (MCAO). Motor deficits and infarct volume were assessed 3 days later. Intra-ischemic CBF was measured by laser-Doppler flowmetry and resting CBF by arterial spin labeling MRI. Ca²⁺-induced mitochondrial depolarization and reactive oxygen species (ROS) production were evaluated in isolated brain mitochondria.

Results—We found that 10% CIH is neuroprotective, while 6% CIH exacerbates tissue damage. No differences in resting or intra-ischemic CBF were observed between 6% and 10% CIH. However, 10% CIH reduced, while 6% CIH increases mitochondrial ROS production and susceptibility to Ca²⁺-induced depolarizations.

Conclusions—The influence of CIH on the ischemic brain is dichotomous and can be attributed in part to changes in the mitochondrial susceptibility to injury. The findings highlight a previously unappreciated complexity in the effect of CIH on the brain, which needs to be considered in evaluating the neurological impact of conditions associated with cyclic hypoxia.

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Conflict of interest/disclosures

The authors have no conflicts of interest or disclosures to declare.

Keywords

sleep apnea; cerebral blood flow; free radicals; mitochondria

Introduction

Obstructive sleep apnea (OSA) is a common form of sleep disordered breathing, affecting approximately 4% of men and 2% of women¹. It is characterized by cyclical periods of airflow cessation (apneas), increased intrathoracic pressure, intermittent hypoxia, hypercapnia and sleep deprivation². OSA is well established as an independent risk factor for stroke, increasing stroke risk by 2-3 fold³⁻⁵, an effect that seems to be related to the number of apnea-hypopnea episodes/hour of sleep⁵. Yet, the mechanisms by which OSA increases susceptibility to cerebral ischemia remain to be defined.

Chronic intermittent hypoxia (CIH) reproduces selected features of OSA and is commonly used as model of the human disease⁶. In CIH, as in OSA, there is an impairment in cerebrovascular regulation, consisting of attenuation of endothelial dependent responses and of the increases in cerebral blood flow (CBF) induced by neural activity⁷⁻¹⁰. While these deleterious cerebrovascular effects may reduce cerebrovascular reserves and contribute to the increase in the risk of stroke in OSA patients^{4,5}, there is experimental evidence that CIH can also reduce cerebral ischemic brain injury¹¹. Furthermore, mortality is reduced in older patients with OSA¹², possibly owing to a “preconditioning” effect of CIH¹³. This raises the intriguing possibility that the influence of CIH on the ischemic brain may in fact be dichotomous and that the fate of the ischemic tissue may depend on factors other than cerebrovascular reserves.

The intensity of the hypoxic episodes and the period of CIH exposure are likely to influence reaction of the brain tissue to ischemic injury. Indeed, studies in the heart have indicated that the duration of CIH exposure can determine the outcome of myocardial ischemia¹⁴⁻¹⁶, an effect in part related to changes in the susceptibility of mitochondria to damage^{17,18}. However, it is unclear how the duration of CIH and the intensity of the episodic hypoxia influence the outcome of cerebral ischemia and whether mitochondria may play a role in determining the fate of the ischemic tissue.

The key aim of this study was to test the hypothesis that intensity and duration of CIH are critical factors determining the impact of CIH on the post-ischemic brain. We found that CIH with cyclic hypoxia induced by 10% O₂ for 35 days is neuroprotective in a mouse model of middle cerebral artery occlusion (MCAO), while CIH with 6% O₂ exacerbates the injury. These effects could not be attributed to differences in cerebrovascular reserves, but to changes in mitochondrial function modulating mitochondrial depolarization and production of reactive oxygen species (ROS). Our findings support the hypothesis that the effects of CIH on the ischemic brain can be either protective or destructive, and that CIH-induced changes in mitochondrial resistance to injury have a key role in determining the fate of the ischemic tissue.

Methods

Mice

All procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Experiments were performed in male C57Bl/6J mice (aged 7-13 weeks) (Jackson Laboratory).

Chronic intermittent hypoxia (CIH)

CIH was used to reproduce the cyclic hypoxia experienced by patients with OSA. Although all OSA models have limitations¹⁹, CIH is frequently used to investigate the cerebral and extracerebral effect of cyclic hypoxia⁶. CIH was induced using a custom system designed by Dr George J. Delagrammatikas and described previously^{7,20}. Briefly, mice were randomly assigned to CIH (10% or 6% O₂) or sham (room air) groups. In CIH groups, oxygen levels within the animals' cage were changed from normal (21±1%) to low (10±1% or 6±1%) O₂ for 90 seconds at 90-second intervals, resulting in 20 hypoxic episodes per hour. The cages of sham treated mice were infused at the same rate with room air. Hypoxia/sham cycling was induced throughout the light (sleep) phase (8:00 am to 4:00 pm; 8 hrs)^{20,21}. During the remaining 16 hours of the day (4:00 pm to 8:00am), both CIH and sham cages were infused with room air. CIH and sham protocols were repeated for 14, 21 or 35 days, during which time mice had free access to food and water. The length of the CIH period was determined based on previous studies demonstrating that CIH for 35 days was sufficient to induce alterations in NMDA receptor trafficking and function²⁰, or cerebrovascular regulation⁷. Systolic blood pressure (SBP) was measured using a non-invasive tail cuff system (Model MC4000, Hatteras Instruments), as previously described²⁰.

Transient focal cerebral ischemia

Transient middle cerebral artery occlusion (MCAO) was induced using an intraluminal filament, as described²²⁻²⁵. Briefly, mice were anesthetized with isoflurane (1.5 - 2%) and rectal temperature maintained at 37° C. A heat-blunted suture was inserted into the right external carotid artery and advanced along the internal carotid artery until it obstructed the MCA. The common carotid artery was simultaneously ligated for the duration of the ischemic period (30 - 35 min). In all studies, cerebral blood flow (CBF) was monitored using laser Doppler flowmetry (Periflux System 5010, Perimed) in the ischemic territory (2mm posterior, 5mm lateral to bregma), as determined in preliminary studies²⁶. Three days after MCAO functional impairment was assessed using the hanging-wire test²⁷, and infarct volume was quantified in cresyl violet stained sections and corrected for swelling as previously described²⁴. Blood glucose and hematocrit were determined at the time of euthanasia.

Real-Time PCR

Total RNA was isolated from a 1 mm thick coronal slice collected at the center of the middle cerebral artery territory in non-ischemic mice^{23,28}. Quantitative determination of gene expression was performed on a Chromo 4 detector (Peltier Thermal Cycler, MJ Research) using a two-step cycling protocol, as described. Primers have been previously

described^{23,28}. Relative expression levels were calculated according to Livak and Schmittgen²⁹. Quantities of all targets in test samples were normalized to the mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene and values were correlated to sham-treated samples²⁸.

Mitochondria isolation and measurements of Ca²⁺ induced mitochondrial depolarization

Brain mitochondria were isolated and purified from the forebrain of CIH and sham naïve mice that were not subjected to MCAO, as described previously³⁰. Ca²⁺ induced mitochondrial depolarization was assessed using the fluorescent mitochondrial membrane potential indicator Safranin O (Molecular Probes)^{30,31}. Briefly, 100 µg of purified mitochondria were suspended in KCl buffer containing glutamate (5mM), malate (5mM) and ADP (0.2 mM). Safranin O (5 µM) was added and fluorescence intensity was measured spectrophotometrically. Boluses of 50 nmoles CaCl₂ were added sequentially until mitochondrial Ca²⁺ uptake declined, as indicated by the mitochondrial depolarization resulting from the mitochondrial permeability transition induced by Ca²⁺ overload. The amount of Ca²⁺ needed to cause mitochondrial depolarization was estimated from standard curves.

Measurement of mitochondrial reactive oxygen species

In isolated and purified brain mitochondria from CIH and sham mice (see above), ROS emission was measured by Amplex Red (Invitrogen) fluorescence, as described³². Briefly, 100 µg mitochondria were added to 1 mL incubation buffer. Standard curves were used to calculate H₂O₂ emission rates after sequential addition of substrate (5 mM glutamate, 2 mM malate), the complex I inhibitor rotenone (1 µM), and the complex III inhibitor antimycin A (1.8 µM).

Statistical Analysis

Mice were randomly assigned to the experimental groups, and analyses were performed by an investigator blinded to the treatment protocol. Data are expressed as mean±SE. Intergroup differences were analyzed using a Student's unpaired t-test or one-way ANOVA with Bonferroni's post-hoc analysis, as appropriate. Differences were considered statistically significant for p<0.05. Group sizes were calculated based on power analysis. We used a power of 0.8 and significance levels of 0.05.

Results

Influence of CIH on physiological parameters and HIF-1 α target genes

Exposure to CIH with 10% O₂ (10% CIH) for 14 – 35 days had no effect on body weight, plasma glucose or hematocrit (Table I). Exposure to 6% CIH transiently increased hematocrit and reduced plasma glucose at 21 days, and decreased body weight both at 21 and 35 days (Table I). SBP tended to increase, but the effect did not reach statistical significance (Table I). Next, we assessed messenger RNA expression of the HIF-1α target genes EPO, VEGF-A and Glut-1, which are induced by CIH³³. Both 10% and 6% CIH increased forebrain EPO mRNA expression at 35 days (Figure I). The induction was more pronounced with 6% than with 10% CIH, in accordance with the intensity of the hypoxic

challenge. Increases (<2 fold) in VEGF-A at 10% CIH and Glut-1 at 6% CIH were also observed (Figure I).

Opposing effects of CIH on ischemic brain injury

Exposure to 6% or 10% CIH for 14 days had no effect on the size of the infarct (Fig 1A,B). Exposure to 6% CIH for 21 days exacerbated ischemic brain injury, increasing infarct size by $\approx 50\%$, while 10% CIH was without effect (Fig 2A,B). Surprisingly, exposure to 10% CIH for 35 days reduced infarct volume by $\approx 50\%$, whereas 6% CIH increased infarct volume and functional impairment at the hanging wire test (Fig. 3A,B). The reduction in infarct volume with 10% CIH was not associated with motor improvement at the hanging wire test, perhaps reflecting the ability of this test to detect more readily worsening than improvement in this model.

CIH does not affect resting or inraischemic CBF

Since CIH alters cerebrovascular reactivity and may reduce cerebrovascular reserves^{7,10}, we examined whether the differences in tissue outcome between 6 and 10% CIH could be related to changes in inraischemic CBF. However, no differences in the reduction in CBF produced by MCA occlusion were observed among the groups (Figure IIA; $p>0.05$). Since LDF cannot measure resting CBF quantitatively, we used ASL-MRI to examine if the worsening of injury with 6% CIH could be related to reductions in resting CBF, which could result in more severe ischemia after MCAO. However, resting CBF did not differ between mice exposed to sham treatment or CIH (CIH 10%: Sham 146 ± 2 , CIH: 144 ± 10 ; CIH 6%: Sham 170 ± 6 , CIH 181 ± 5 ml/100g/min) at 35 days (Figure IIB; $p>0.05$).

Effects of CIH on pro-inflammatory gene expression in brain

Next, we examined if CIH could influence the outcome of cerebral ischemia by modulating inflammatory gene expression. Exposure to 6% CIH for 35 days increased expression of regulated and normal T cell expressed and secreted/CCL5 (RANTES), monocyte chemotactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) (Figure 4). There was a trend for pro-inflammatory gene expression to be downregulated in mice exposed to 10% CIH, particularly iNOS, which was significantly reduced (Figure 4). These changes in inflammatory gene expression were not associated with evidence of tissue injury both with 6% or 10% CIH, as assessed by fluoro jade-B (Figure IIIA,B). As a positive control, fluoro jade-B was able to detect brain damage after MCAO (Figure IIIC).

Opposing effects of CIH on mitochondrial susceptibility to Ca^{2+} induced depolarization

Mitochondria are critical modulators of ischemic brain injury and are influenced by intermittent hypoxia^{17,18,34,35}. Therefore, we tested the hypothesis that CIH-induced changes in mitochondrial resistance to injury play a role in the opposing effects on post-ischemic brain damage. Mitochondria were isolated from non-ischemic mice subjected to sham treatment or 10% or 6% CIH for 35 days, and their susceptibility to Ca^{2+} -induced loss of membrane potential (Ψ) was tested. Sham treated mice maintained Ψ in response to up to 200 nmoles CaCl_2 . Beyond this point, administration of an additional aliquot of CaCl_2

(40 nmol) resulted in a complete loss of Ψ , consistent with mitochondrial permeability transition pore (mPTP) opening (Figure 5A & B). In contrast, mitochondria isolated from mice exposed to 10% O₂ CIH had an increased Ca²⁺ capacity, maintaining Ψ in response to up to 240 nmoles CaCl₂ (Figure 5A), whereas mitochondrial Ca²⁺ capacity of mice exposed to 6% O₂ CIH was reduced, with loss of Ψ occurring in response to 120 nmoles CaCl₂ (Figure 5B).

Effect of CIH on mitochondrial ROS production

ROS promote mPTP opening and subsequent loss of membrane potential³⁴. In order to gain an insight into the mechanism by which CIH alters susceptibility of mitochondria to Ca²⁺ induced depolarization, we evaluated mitochondrial ROS production with substrate (malate, glutamate), rotenone (complex I inhibitor) and antimycin A (complex III inhibitor), in mice exposed to 10% and 6% CIH for 35 days. Consistent with a reduced susceptibility to Ca²⁺ induced membrane depolarization and mPTP transition, exposure to 10% CIH attenuated mitochondrial ROS production, as compared to sham-treated mice (Figure 6A). In contrast, ROS production was similar in mice exposed to either sham treatment or 6% CIH (Figure 6B). While there was a tendency for ROS production in response to substrate to increase in 6% CIH mice relative to control, this effect did not reach statistical significance ($p>0.05$).

Discussion

We have demonstrated that CIH can either ameliorate or worsen ischemic brain injury depending on the intensity of the hypoxic challenge. Thus, 10% CIH induces neuroprotection while 6% CIH exacerbates ischemic brain injury. Differences in the effects of 10% and 6% CIH on outcome were independent of alterations in CBF, as both basal cerebral perfusion, measured using ASL-MRI, and the degree of blood flow reduction in response to MCAO were similar in 10% and 6% CIH mice. However, the expression of key pro-inflammatory mediators was altered by CIH, increasing with 6% CIH and showing a tendency to decrease with 10% CIH, in agreement with their effects on outcome. Furthermore, the ability of cerebral mitochondria to maintain membrane potential in response to Ca²⁺ overload was enhanced by 10% CIH and reduced by 6% CIH, an effect associated with differences in mitochondrial ROS production. Collectively, our findings unveil a dichotomous effect of CIH on the post-ischemic brain that is dependent on the severity of cyclic hypoxic episodes.

CIH exposure exerts opposing effects on the ischemic brain

Decreasing the intensity from 10% to 6% CIH resulted in a switch from beneficial to detrimental effects of CIH on the ischemic brain. These opposing effects of 10% and 6% CIH were first observed after 21 days exposure, when 6% CIH increased susceptibility to ischemic brain injury. By 35 days, however, the dichotomy was fully developed, with 10% CIH decreasing and 6% CIH exacerbating the injury. These effects could not be attributed to changes in SBP, blood glucose, hematocrit or CBF, because changes in these parameters did not correlate with the post-ischemic outcome. Interestingly, while the pathogenic effects of CIH developed more rapidly, neuroprotective effects of CIH on the ischemic brain took more than 21 days to develop. This observation is consistent with the idea that exposure to

CIH induces delayed cerebral ischemic tolerance³³. Indeed, others have shown that hypoxic episodes can induce short-term³⁶⁻³⁹ or long lasting¹¹ preconditioning in the brain. However, our results indicate that the effects of CIH are not uniformly beneficial, and that their protective or destructive character depends on the intensity and duration of the recurring hypoxic challenge. Clinical data suggest that older patients with moderate OSA may have reduced mortality compared to the general population¹², attesting to a potential preconditioning effect of mild CIH¹³. Therefore, it would be important to test if the dichotomous effect of CIH also occurs in older animals.

CIH induced vascular dysfunction does not influence the outcome of cerebral ischemia

Post-ischemic cerebral perfusion has profound effects on the outcome of MCAO⁴⁰. Thus, alterations in CBF regulation reduce intra-ischemic CBF and exacerbate focal ischemic brain injury⁴¹⁻⁴³, whereas counteracting post-ischemic cerebrovascular dysregulation ameliorates the damage²⁶. Since 10% CIH alters critical vasomotor responses of the cerebral circulation⁷, we anticipated that intra-ischemic CBF would be reduced, owing to the impaired vasodilatory capacity of cerebral blood vessels and failure of collateral flow. Contrary to this prediction, however, we found that 10% CIH does not exacerbate the CBF reduction induced by MCAO, and results in less damage, due to its beneficial parenchymal effects. Collectively, these observations indicate that the “preconditioning” effect of CIH may protect the post-ischemic brain from the deleterious effects of cerebrovascular dysfunction.

CIH influences inflammation and the tolerance of mitochondria to Ca²⁺-induced membrane depolarization

Pro-inflammatory markers were slightly upregulated in the brain of mice exposed to 6% CIH in the absence of changes in tissue injury, and tended to be reduced in mice exposed to 10% CIH. This supports the idea that inflammation contributes to the CIH-induced changes in susceptibility to ischemic brain injury and is in agreement with previous studies reporting alterations in mediators and markers of inflammation in the brain following CIH^{11,44} and in the blood of OSA patients^{45,46}. However, the significance of these relatively minor changes in inflammatory gene expression remain to be established and a more detailed analysis of the cellular and molecular features of post-ischemic inflammation would be needed to assess the impact of CIH-induced immunomodulation on the outcome of cerebral ischemia.

A more striking and novel finding of the present study is that CIH exerts a profound influence on cerebral mitochondria. Mitochondria play a central role in cell death and survival⁴⁷ and are critical for buffering excessive post-ischemic intracellular Ca²⁺ levels³⁴. Here, we found that cerebral mitochondria isolated from mice exposed to 10% CIH had a significant reduction in susceptibility to Ca²⁺ induced membrane depolarization, consistent with a protection from injury, while 6% CIH increased susceptibility to Ca²⁺ induced depolarization. Although changes in mitochondrial function have been reported following CIH³³, we found, for the first time, that the effects of CIH on mitochondrial Ca²⁺ buffering capacity can be either protective or harmful depending on the intensity of the cyclic hypoxia.

Induction of mPTP and loss of mitochondrial membrane potential occurs due to excessive accumulation of Ca^{2+} and results in impaired ATP production and cell death. This loss of mitochondrial membrane potential can be promoted by oxidative stress³⁴. Indeed, we observed a reduction in mitochondrial ROS production in mice exposed to 10% CIH, in which susceptibility to Ca^{2+} induced membrane depolarization is attenuated. This is consistent with changes in mitochondrial ROS production in response to CIH, altering the stability of the mPTP and the susceptibility of the brain to ischemic injury. In agreement with this hypothesis, 6% CIH did not increase mitochondrial ROS production relative to control, whereas mitochondrial ROS levels were higher in 6% than in 10% CIH. This supports the idea that mitochondrial ROS play an important role in the effects of CIH on cerebral mitochondria and subsequent injury development. Interestingly, we previously reported that cerebrovascular ROS production is increased by 10% CIH, an effect mediated by a NOX2-containing NADPH oxidase⁷. In contrast, in the present study we observed that mitochondrial ROS are reduced by the same CIH regimen. While cell-type specific effects cannot be ruled out, these observations raise the intriguing possibility that CIH has differential effects on ROS production depending on the enzymatic system and/or cellular compartment generating the radicals. Further studies targeting specifically mitochondrial and extramitochondrial sources of ROS would be required to address this issue.

Conclusions

This study demonstrates that CIH can have both beneficial and deleterious effects on the ischemic brain. Neuroprotection was observed following exposure to 10% CIH, while injury exacerbation was seen with 6% CIH, suggesting that the intensity of the hypoxic insult is critical in determining the effect of CIH on tissue outcome. At the mechanistic level, this study provides novel evidence for a key role of mitochondria in the effects of CIH on the ischemic brain, with alterations in the susceptibility to Ca^{2+} -induced membrane depolarization and mitochondrial ROS production observed. While further studies are required to define the mechanisms of these mitochondrial effects, the dichotomous nature of the impact of CIH on the post-ischemic brain unveils a novel and intriguing aspect of the pathobiology of CIH. This may be of great relevance to clinical conditions associated with cyclic changes in oxygenation levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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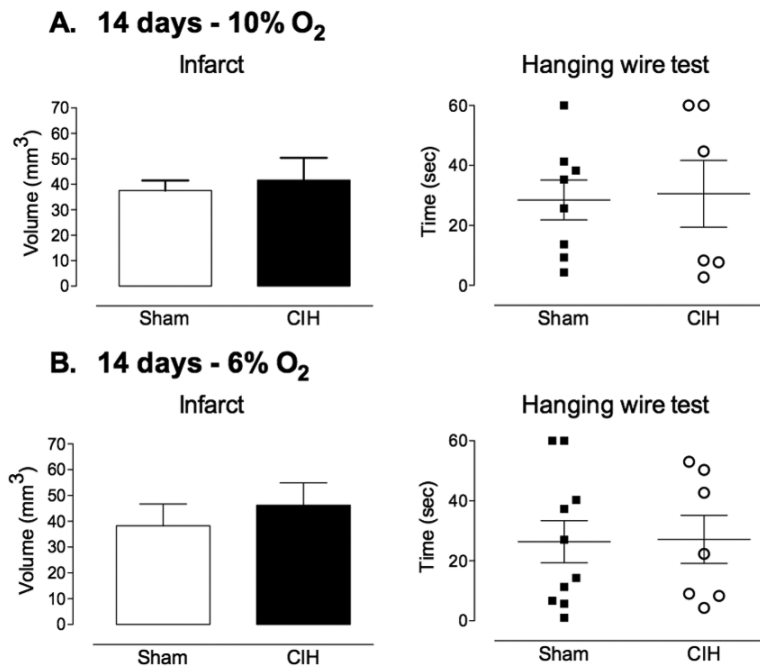


Figure 1. Influence of 14 days CIH on focal cerebral ischemic injury

A. Exposure to 10% CIH for 14 days prior to induction of ischemia had no effect on infarct volume or functional impairment (hanging-wire test) evaluated at 72 hrs (n=6-8/group). **B.** Similarly, exposure to 6% CIH for 14 days did not alter infarct volume or functional impairment evaluated at 72 hrs (n=7-10/group).

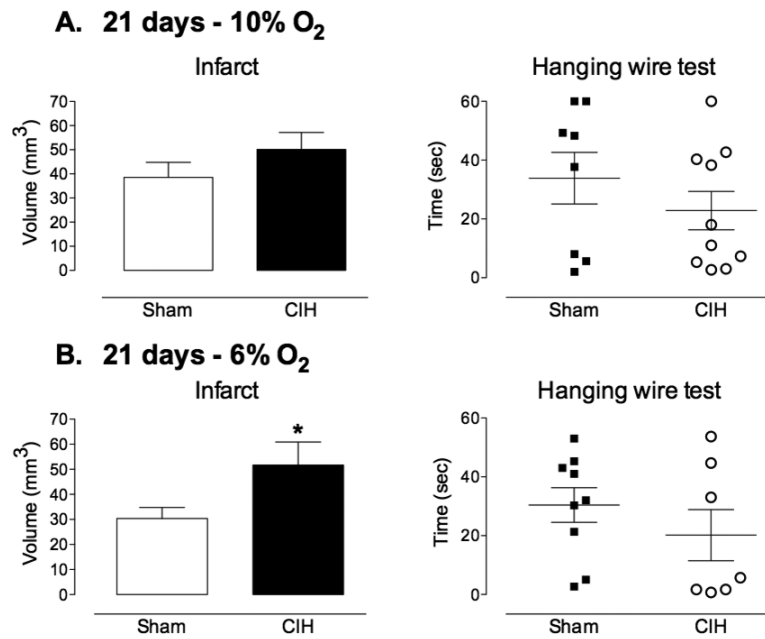


Figure 2. Influence of 21 days CIH on focal cerebral ischemic injury

A. Exposure to 10% CIH for 21 days prior to induction of ischemia had no effect on infarct volume or functional impairment (hanging-wire test) evaluated at 72 hrs (n=8-10/group). **B.** Exposure to 6% CIH significantly increased infarct volume compared to sham treated mice, while functional impairment was not affected (n=7-9/group; *p<0.05, t-test).

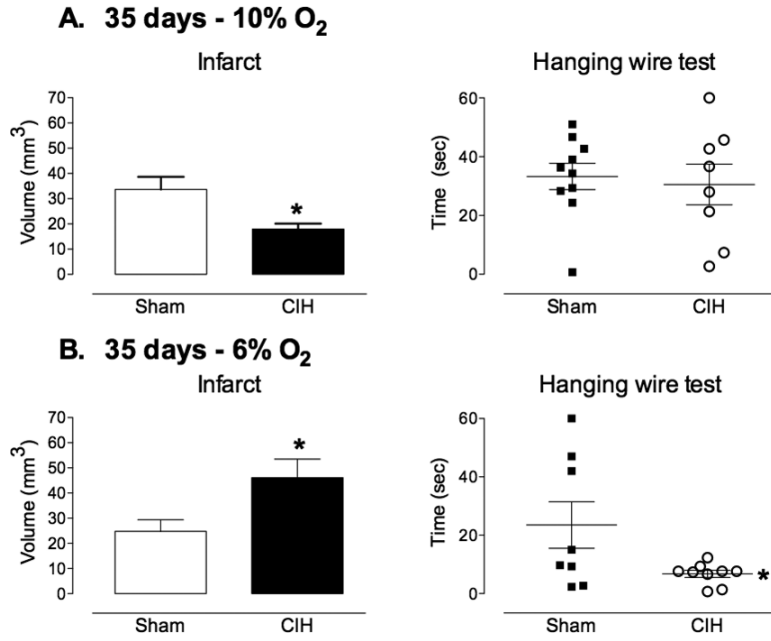


Figure 3. Influence of 35 days CIH on focal cerebral ischemic injury

A. Exposure to 10% CIH for 35 days prior to induction of ischemia resulted in a significant reduction in infarct volume (n=8-10/group; *p<0.05, t-test). Functional impairment (hanging-wire test), was not affected (n=8-10/group). **B.** Exposure to 6% CIH increased both infarct volume and functional impairment 72 hrs after induction of cerebral ischemia (n=8-10/group; *p<0.05, t-test).

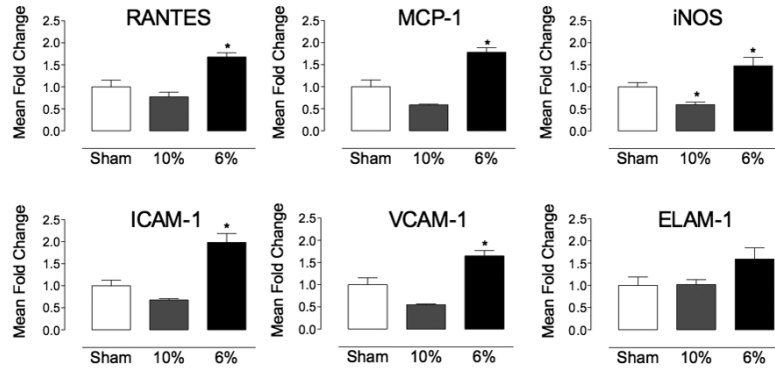


Figure 4. CIH induced changes in pro-inflammatory gene expression in non-ischemic mice

mRNA expression of pro-inflammatory genes RANTES, MCP-1, iNOS, ICAM-1, VCAM-1 and ELAM-1 tended to be decreased in non-ischemic mice exposed to 10% CIH (35 days; n=5-15/group; *p<0.05 from sham, ANOVA), while expression was increased following exposure to 6% CIH (35 days; n=5-15 /group; *p<0.05 from sham, ANOVA).

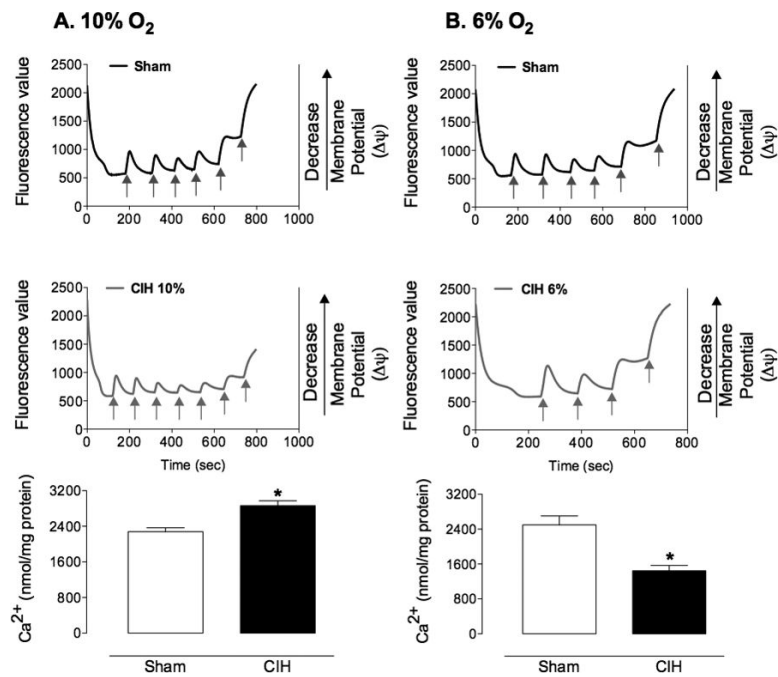


Figure 5. Effect of CIH on mitochondrial Ca²⁺-induced membrane depolarization in non-ischemic mice

A. Representative trace and group data illustrating exposure to 10% CIH decreased susceptibility of mitochondria to Ca²⁺ induced loss of membrane potential (n=9/group; *p<0.05, t-test). **B.** In contrast, exposure to 6% CIH increased susceptibility to Ca²⁺ induced membrane depolarization (n=9/group; *p<0.05, t-test). Additions of Ca²⁺ are indicated by arrows.

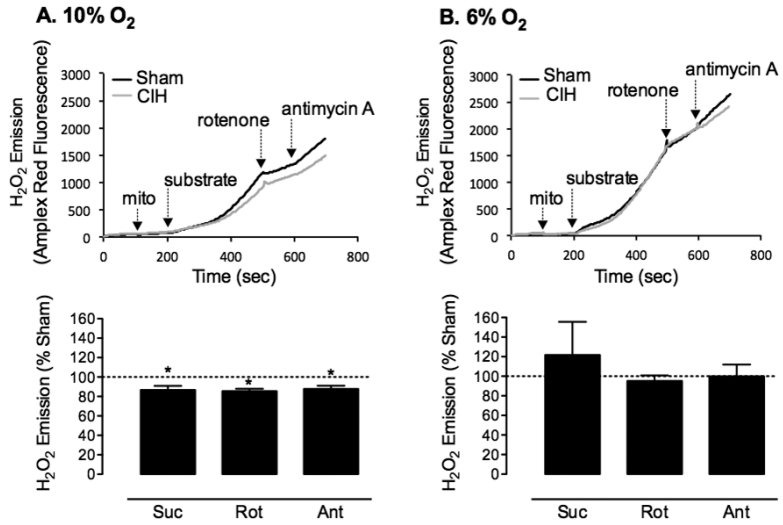


Figure 6. Effect of CIH on mitochondrial ROS production in non-ischemic mice

A. Representative Amplex Red fluorescence traces (top panel) and group data (bottom panel) showing that exposure to 10% CIH decreased the rate of H₂O₂ emission from isolated mitochondria, following sequential additions of substrate (succinate), rotenone, and antimycin A, relative to sham treated controls (n=5-6/group; *p<0.05, t-test). **B.** Representative trace (top panel) and group data (bottom panel) showing that exposure to 6% CIH did not attenuate mitochondrial H₂O₂ production relative to sham treated controls (n=5/group,). The additions of mitochondria (mito), substrate (succinate), rotenone and antimycin A are indicated by arrows.