Intermittent hypoxia conditioning prevents behavioral deficit and brain oxidative stress in ethanol-withdrawn rats

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Interruption hypoxia (IH) has been found to protect brain from ischemic injury. We investigated whether IH mitigates brain oxidative stress and behavioral deficits in rats subjected to ethanol intoxication and abrupt ethanol withdrawal (EW). The effects of IH on overt EW behavioral signs, superoxide generation, protein oxidation, and mitochondrial permeability transition pore (PTP) opening were examined. Male rats consumed dextrin or 6.5% (wt/vol) ethanol for 35 days. During the last 20 days, rats were treated with repetitive (5–8 per day) 1-h cycles of hypoxia (9.5–10% inspired O2) separated by 4-min normoxia exposures. Cerebellum, cortex, and hippocampus were biopsied on day 35 of the diet or at 24 h of EW. Superoxide and protein carbonyl contents in tissue homogenates and absorbance decline at 540 nm in mitochondrial suspensions served as indicators of oxidative stress, protein oxidation, and PTP opening, respectively. Although IH altered neither ethanol consumption nor blood ethanol concentration, it sharply lowered the severity of EW signs including tremor, tail rigidity, and startle response. Compared with dextrin and ethanol per se, in the three brain regions, EW increased superoxide and protein carbonyl contents and accelerated PTP opening in a manner ameliorated by IH. Administration of antioxidant N-acetylcysteine throughout the IH program abrogated the reductions in EW signs and superoxide content, implicating IH-induced ROS as mediators of the salutary adaptations. We conclude that IH conditioning during ethanol consumption attenuates oxidative damage to the brain and mitigates behavioral abnormalities during subsequent EW. IH-induced ROS may evoke this powerful protection.

hypoxia, the reduction of O2 supply below physiological levels, is generally considered harmful, especially to tissues with high O2 requirements (13). Hypoxia occurs naturally at high altitude, during strenuous exercise, and with various respiratory diseases. The fact that tissues survive such hypoxic stresses suggests that adaptive responses are mobilized to compensate for limitations in O2 supply. Indeed, recent research demonstrates that cyclic exposure to moderate hypoxia with intervening periods of normoxia bolsters cellular resistance to ischemic stress in several organs (6, 7, 9, 25, 26, 31, 48), including brain (3, 33, 40). These studies suggest that intermittent hypoxia (IH) may stimulate endogenous defense mechanisms, affording significant cytoprotection.

Brain mitochondria appear to be the principal targets of oxidative stress engendered by ethanol intoxication and withdrawal (20). The inner mitochondrial membrane’s highly selective permeability to electrolytes, nucleotides, and metabolic substrates is essential for mitochondrial ATP production. The opening of nonselective mitochondrial permeability transition pores (PTP) in the inner membrane disrupts ATP production by collapsing the electrochemical gradient for H+ and the driving force for oxidative phosphorylation. During ethanol withdrawal (EW), excessive glutamate-induced neuronal excitation increases intracellular concentrations of Ca2+ and reactive oxygen species (ROS), factors that provoke PTP opening (5, 11, 14, 15, 35, 43, 48). These pores permit nonspecific passage of solutes and water, leading to mitochondrial swelling and possible rupture and to decreased efficiency of mitochondrial respiration (15, 48). Zhu et al. (49) reported that IH protected against Ca2+-induced PTP opening in rat ventricular myocardium.

Meerson et al. (27, 28) reported that exposure of rats to high-altitude-induced IH attenuated various comorbidities associated with EW, such as pain, liver damage, and cardiac arrhythmias. We previously reported that EW caused oxidative stress in brain areas vulnerable to ethanol, such as cerebellum and cortex (19, 34). Accordingly, the current study was conducted to determine whether IH can prevent brain oxidative stress and behavioral impairment during EW. First, we tested whether IH indeed protects against overt behavioral signs of EW and whether such effects of IH are due to mechanisms activated during the antecedent alcohol consumption regimen. At the cellular level, we tested whether IH protects against EW’s pro-oxidant effects and mitochondrial PTP opening. The cerebellum, cortex, and hippocampus were specifically examined because of the known susceptibility of these brain regions to ethanol and EW insult and to oxidative stress (12, 19, 38).

METHODS

Chemicals. Analytic grade reagents were purchased from Sigma Aldrich (St. Louis, MO) or Calbiochem (San Diego, CA). Diet ingredients were obtained from Research Organics (Cleveland, OH) or MP Biomedicals (Irvine, CA).

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA), 3.5 mo old at the beginning of the study, were housed individually at controlled temperature (22–25°C) and humidity (55%) with ad libitum access to water. A 12:12-h light-dark cycle was maintained with lights on between 7:00 AM and 7:00 PM. Each rat’s weight was recorded daily until death. All animal experimentation was conducted in accordance with the Guide to the Care and Use of Laboratory Animals. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Animals [DHHS Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205] and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Chronic ethanol administration. Experimental procedures were conducted according to the protocol depicted in Fig. 1. Rats were divided into six groups (5–7 rats/group) according to diet (ethanol vs. control dextrin), treatment (IH vs. non-IH sham), and time of death (ethanol exposure vs. EW). The groups were dextrin sham, dextrin/IH, ethanol sham, ethanol/IH, EW sham, and EW/IH. Ethanol and EW rats were administered a liquid diet containing 6.5% (wt/vol) ethanol (8). Control animals were fed a liquid diet with dextrin isocalorically substituted for ethanol. Each liter of these diets contains 42 g of pulverized casein, 0.6 g of L-methionine, 2.1 g of vitamin mixture, 7.3 g of mineral mixture, 25 g of sucrose, 3 g of xanthum gum, 0.4 g of choline bitartrate, 1 g of cellulose, 10.5 g of corn oil, and either ethanol or dextrin in aqueous suspension. One hundred milliliters of diet were placed in each cage daily for 5 wk. These diets were of choline bitartrate, 1 go fCelufil cellulose, 10.5 g of corn oil, and either ethanol or dextrin in aqueous suspension. One hundred milliliters of diet were placed in each cage daily for 5 wk. These diets were abruptly terminated after 35 days and replaced with conventional Chow diet to initiate withdrawal.

Evaluation of EW signs. Behavioral deficit was assessed 24 h after withdrawing ethanol or dextrin control diet by scoring seven criteria as previously described (19): 1) vocalization, urination, and defecation on handling (score: 0–3); 2) tail rigidity when the tail was drawn between the rater’s fingers (0–3); 3) frequency and severity of tremor during handling (0–3); 4) startle (swinging, jumping, or freezing) observed for 15 s after auditory stimulus (0–3); 5) convulsion during and after handling (0 or 1); 6) spontaneous seizure (0 or 2); and 7) death (0 or 10). The sum of the seven scores equaled each animal’s total score. Scoring was done by an evaluator blinded to each rat’s diet and IH vs. sham program. All tests were conducted in a designated room in the vivarium to limit auditory stimulation. Rats were killed immediately after the sign test (19), and tissues were harvested for assessment of oxidative stress and mitochondrial resilience.

IH conditioning. Rats were hypoxia- or sham-conditioned in custom-made 267-liter acrylic chambers. These chambers are large enough to accommodate multiple rat cages and to allow the atmosphere within the chamber to be adjusted quickly and precisely using compressed gas. The IH program (Table 1; Ref. 50) was administered each morning during the last 20 days of the 35-day ethanol diet but not during EW. The program consisted of brief (5–10 min) hypoxic exposures (5–8 per day) with intervening 4-min periods of reoxygenation. Percent O2 in the chamber was monitored with a precision O2 sensor (Alpha Omega Instruments model 2000). Compressed N2 was introduced into the chamber to lower percent O2 to the prescribed value (Table 1) within 90 s. Abrupt reoxygenation was achieved by opening the top and ends of the chamber. Non-IH groups underwent sham conditioning protocols in which compressed air instead of N2 was introduced to maintain the fractional inspired O2 (FiO2) at 21%. The rats exhibited no discomfort or distress during the hypoxia or sham sessions. To examine the role of oxyradicals in IH-induced adaptations, we administered an additional four ethanol-consuming rats the antioxidant N-acetylcysteine (100 mg/kg ip) 2 h before each IH session throughout the 20-day program.

Blood ethanol concentrations. Blood ethanol concentrations were measured in additional (n = 3) dextrin, ethanol, and ethanol/IH rats. After completion of the day 10 IH conditioning session, i.e., 3 h after placement of fresh diet bottles, the rats were secured in a Plexiglas restraint device and a syringe fitted with a 25-gauge needle was inserted at a 45° angle toward the vein. Blood (200 μl) was withdrawn and immediately mixed with 90 μl of ice-cold 0.55 M HClO4. Samples were centrifuged at 1,500 g for 10 min to sediment protein precipitate. Supernatants were adjusted to pH 5 with 200 μl of a solution containing 0.6 M KOH and 50 mM acetic acid and then centrifuged to sediment KClO4 precipitate. Ethanol in the supernatant was measured by colorimetric assay (42) in which NAD+ reduction to NADH is coupled to ethanol oxidation by alcohol dehydrogenase (extinction coefficient ε = 6.2 mM−1 cm−1) in a Beckman DU 640 spectrophotometer.

Assessment of oxidative markers. Superoxide (O2−) content in brain homogenates was measured as the superoxide dismutase-inhibitable reduction of acetylated ferricytochrome c (1). Ferricytochrome c reduction was monitored at 550 nm in a Beckman DU 640 spectrophotometer after addition of 7.5 mM succinate in the absence and presence of 100 U/ml superoxide dismutase. Ferricytochrome c concentration was obtained by applying an extinction coefficient of 27.7 mM−1 cm−1 to the absorbance values (1).

Protein carbonyls were measured (23) using 2, 4-dinitrophenylhydrazine (DNPH), which combines with aldehyde or ketone moieties in proteins to form DNP-protein adducts. The rats were anesthetized with xylazine (20 mg/kg ip) and ketamine (100 mg/kg ip) and decapitated. Cerebellum, cerebral cortex, and hippocampus were homogenized in 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Calbiochem, San Diego, CA). To 1 ml of homogenate, we added 0.2 ml of 10 mM DNPH in 2 N HCl and 0.2 ml of 2 N HCl was added to another 1 ml of homogenate to provide a blank. Mixtures were incubated for 60 min at room temperature. The protein was precipitated with an equal volume of 20% trichloroacetic acid and was washed three times with ethanol-ethyl acetate (1:1 vol/vol). The final precipitate was dissolved in 2 ml of 6 M guanidine hydrochloride (pH 2.3), and insoluble debris was removed by centrifugation. Ab-

Table 1. IH conditioning program

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Intermittent hypoxia (IH) conditioning was administered during the last 20 days of the 35-day ethanol diet and then discontinued during ethanol withdrawal. Non-IH rats were sham conditioned by introduction of compressed air (21% O2) instead of N2 into the chamber.
sorbance of DNP-protein adducts was measured at 360 nm (ε = 22 mM⁻¹·cm⁻¹; Ref. 23) in a Beckman DU 640 spectrophotometer. Carboxyl contents were expressed as nanomoles of carbonyl per milligram of protein.

**Assessment of mitochondrial membrane swelling.** Mitochondria were isolated from brain homogenates by differential centrifugation (41). Tissue biopsies were dissected, rinsed, and homogenized in ice-cold isolation buffer (320 mM sucrose, 1 mM K₂EDTA, 10 mM Tris·HCl). Homogenates were centrifuged at 1,330 g for 5 min at 4°C. The pellet was resuspended in 0.5 volume of isolation buffer and recentrifuged. The two supernatants were combined and centrifuged at 21,200 g for 5 min. The pellet was resuspended in 12% Percoll solution and centrifuged at 6,900 g for 10 min, yielding a soft pellet that was washed once with mitochondrial isolation buffer and centrifuged again at 6,900 g for 10 min. The resulting mitochondrial pellet was used for the assay of mitochondrial swelling and rupture as a marker of PTP opening (48).

Mitochondrial membrane swelling was assessed by suspending mitochondria in medium containing phosphate, which induces swelling and rupture more rapidly in vulnerable mitochondrial membranes than healthy membranes (29). Mitochondria (0.25 mg protein) were suspended in medium containing 250 mM sucrose, 10 mM Tris-MOPS, 0.05 mM EGTA, 5 mM pyruvate, 5 mM malate, and 1 mM phosphate (pH 7.4), and absorbance by this suspension was measured at 540 nm in a Beckman DU 640 spectrophotometer. Intact mitochondria scatter light at 540-nm wavelength; mitochondrial swelling and rupture due to prolonged or excessive mitochondrial PTP opening reduces mitochondrial light scattering and, thus, absorbance (48). The time required for absorbance to fall to the midpoint between initial and final values was determined to assess how readily PTP open in different treatment groups.

**Statistical analysis.** Measurements were done in duplicate or triplicate, and mean values were computed for each rat. Values are means ± SE. Single-factor analyses of variance (ANOVA) were performed to identify group differences among EW sign scores, blood ethanol concentrations, or ethanol consumption. Two-factor ANOVA performed to identify group differences among EW sign scores, blood means, and mean values were computed for each rat. Values are different treatment groups.

**Fig. 3.** Behavioral signs of ethanol withdrawal. These data and those depicted in Figs. 4–7 are from rats (5–7 rats/group) that received dextrin or 6.5% (wt/vol) ethanol diet for 35 days, with sham (dextrin and EW groups) or IH treatment (EW/IH group) for the last 20 days of the diet. The ethanol diet was then abruptly terminated to produce EW, and the IH program was also stopped. Rats were tested for behavioral signs at 24 h of EW. Antecedent IH treatment sharply attenuated the severity of the EW signs. *P < 0.001 vs. dextrin. †P < 0.01 vs. EW.

**RESULTS**

At the end of the diet regimen, there were no significant differences in body weights between the dextrin (320 ± 9 g), ethanol (311 ± 11 g), and ethanol/IH (321 ± 3 g) groups. The rats that received IH during ethanol diet appeared to be more active and cleaner than the non-IH ethanol rats. No abnormal behaviors or signs were observed during the IH sessions.

**Ethanol consumption and blood ethanol concentrations.** Figure 2 presents the daily ethanol intake in non-IH (ethanol group) or IH-treated rats (ethanol/IH group). The ethanol and ethanol/IH rats drank 57 ± 1.2 and 58 ± 1.3 ml of liquid ethanol diet (6.5% wt/vol) per day, respectively. When normalized to body weights, the daily ethanol intakes averaged 12.0 ± 0.2 g/kg in ethanol rats and 12.6 ± 0.3 g/kg in ethanol/IH rats. There were no significant differences between the two groups, indicating that IH did not alter ethanol consumption.

**Blood ethanol concentration was measured at day 10, i.e., the midpoint of the IH or sham program, to test whether IH**
alters circulating ethanol concentrations. As expected, blood ethanol was undetectable in the dextrin group. Ethanol concentrations were similar in ethanol-consuming rats completing sham (1.00 ± 0.06 mg/ml) and IH (0.96 ± 0.04 mg/ml) protocols, indicating that IH did not influence ethanol exposure.

Behavioral signs of EW. Figure 3 presents the severity of overt behavioral signs of EW, measured 24 h after withdrawal of ethanol or dextrin diets. ANOVA detected statistically significant differences in EW sign scores among dextrin rats, EW rats with IH, and EW rats without IH treatment \([F(2, 14) = 37, P < 0.001]\). A post hoc Tukey comparison revealed that, as expected, the EW group had higher sign scores (7.2 ± 0.8) than the dextrin group (1.0 ± 0.3, \(P < 0.001\)). Sign scores in the EW/IH group were sharply lower (2.0 ± 0.3) than those in the EW group (\(P < 0.01\)) and did not significantly differ from those in the dextrin group, demonstrating that IH alleviated EW-induced behavioral signs.

O\(_2^•\) generation during EW. The finding that IH markedly attenuated EW behavioral signs raised the possibility that IH might have dampened oxidative stress imposed by EW. Superoxide contents were measured to index ROS formation in brain. ANOVA revealed significant treatment effects on O\(_2^•\) contents in cerebellum \([F(5, 23) = 277, P < 0.001]\), hippocampus \([F(5, 23) = 384, P < 0.001]\), and cortex \([F(5, 23) = 451, P < 0.001]\). Ethanol exposure sharply increased O\(_2^•\) contents in all three regions compared with dextrin controls (Fig. 4; \(P < 0.001\)). In non-IH rats, O\(_2^•\) increased further during EW in all three regions (\(P < 0.05\)). During ethanol exposure, IH only reduced O\(_2^•\) accumulation in cortex. During EW, IH treatment attenuated O\(_2^•\) in all three brain regions (\(P < 0.001\)).

Protein carbonyl content. The effects of ethanol exposure, EW, and IH on protein oxidation were assessed from protein carbonyl contents in brain homogenates. In general, carbonyl contents (Fig. 5) paralleled O\(_2^•\) contents (see Fig. 4). ANOVA revealed significant treatment effects on carbonyl contents in cerebellum \([F(5, 25) = 74, P < 0.001]\), cortex \([F(5, 25) = 76, P < 0.001]\), and hippocampus \([F(5, 25) = 71, P < 0.001]\). The ethanol diet sharply increased carbonyl contents in all three brain areas (\(P < 0.01\)). During EW in the non-IH rats, carbonyl content increased even further (\(P < 0.01\) vs. ethanol exposure), indicating that EW inflicted appreciable oxidative stress on brain proteins. IH protection against carbonyl production was observed during EW (\(P < 0.01\)) but not during ethanol exposure, indicating more effective IH protection against the protein oxidation inflicted by EW than that caused by ethanol per se.

Mitochondrial membrane swelling. The effects of ethanol exposure, EW, and IH on mitochondrial membrane swelling, an indicator of PTP opening, were assessed by measuring light scattering by mitochondrial membranes. In general, light scattering declined most rapidly in mitochondria from brains of non-IH EW rats (Fig. 6A). Two-factor ANOVA revealed significant treatment-dependent \([F(5, 10) = 179, P < 0.001]\) and brain region-dependent \([F(2, 10) = 7.8, P = 0.001]\) differences in time to 50% absorbance change. In addition, there was a significant interaction between treatment and brain region \([F(10, 75) = 12, P = 0.001]\), suggesting that the different brain regions are not equally vulnerable to PTP opening under specific treatment conditions. Indeed, a post hoc Tukey test revealed that cerebellar mitochondria were more vulnerable to...
swelling and rupture during EW than were cortical and hippocampal mitochondria. In cortical mitochondria, rates of swelling and rupture during ethanol exposure and EW did not differ significantly. Hippocampal mitochondria were the least affected by ethanol exposure or EW (Fig. 6B). IH produced significant protection only in cerebellar mitochondria during EW (P = 0.01); however, there was a tendency to protection in the cortical and hippocampal regions. Collectively, these results indicate that EW increases vulnerability of brain mitochondrial membranes and that IH minimizes this effect of EW.

**Bivariate correlations.** Bivariate Pearson analyses were performed to test correlations between EW-induced behavioral signs and oxidative injury (Fig. 7). Positive correlations (P < 0.001) were detected among EW sign scores, O$_2^•$ contents, and carbonyl contents, all of which were negatively correlated (P < 0.001) with mitochondrial membrane resilience (time to 50% absorbance decrease). The correlations between EW signs and oxidative markers were less robust than those between oxidative markers per se. Indeed, EW sign scores did not significantly correlate with O$_2^•$ contents in cortex or hippocampus. These analyses indicate that O$_2^•$ contents, carbonyl contents, and mitochondrial PTP collectively determine EW damage and/or IH neuroprotection.

**IH induction of moderate, brief O$_2^•$ formation.** IH may protect brain from EW by inducing moderate, brief bursts of oxyradical formation that evoke beneficial adaptations. To test this possibility, we measured cerebellar O$_2^•$ formation 4 min or 24 h after the final cycle of hypoxia or sham conditioning in rats consuming dextrin diet. IH did indeed increase cerebellar O$_2^•$ content at 4 min of reoxygenation, but O$_2^•$ content returned to baseline by 24 h (Fig. 8). Thus IH evoked moderate but transient oxyradical formation in cerebellum.

**Antioxidant blockade of IH-induced neuroprotection.** To investigate further the salutary role of IH-induced oxyradicals, we injected the broad-spectrum sulfhydril antioxidant N-acetylcysteine (NAC) intraperitoneally 2 h before each IH conditioning session throughout the 20-day program. EW sign scores and cerebellar O$_2^•$ content were measured at 24 h of EW. NAC abrogated IH protection against EW signs (Fig. 9A). Moreover, the antioxidant treatment paradoxically increased cerebellar O$_2^•$ content at 24 h of EW, 1 day after the final NAC injection and IH session (Fig. 9B). Thus antioxidant treatment during IH blunted development of protection against EW-induced brain oxidative stress, implicating IH-induced ROS as pivotal elements of the antioxidative mechanism.

**DISCUSSION**

This study examined for the first time whether normobaric IH could evoke protection against behavioral deficit, cellular oxidative stress, and mitochondrial membrane fragility in rat brain during EW. The main findings are that daily administration of a program of brief, intermittent bouts of hypoxia and reoxygenation to rats consuming ethanol 1) prevents overt behavioral signs during subsequent EW, 2) decreases brain contents of the oxidative stress markers O$_2^•$ and protein carbonyls, and 3) preserves the resilience of mitochondrial membranes in the face of EW. Daily antioxidant (NAC) administration throughout the IH program abrogated IH protection against EW behavioral signs and oxidative stress. Collectively, these results demonstrate that the IH program afforded significant protection against EW stress. These findings extend to the brain the studies of Meerson et al. (27, 28), who reported that adaptation to hypobaric IH attenuated hepatic lipid peroxidation and cardiac dysfunction in rats during EW.

Because the IH treatment prevented the overt behavioral signs of EW, we tested whether such protection was due to reduced ethanol consumption and/or blood ethanol concentration. If IH decreased ethanol intake or accelerated clearance of blood ethanol, IH conditioned rats would likely be less addicted to ethanol and, thus, suffer less EW stress. However, neither intake nor blood concentration of ethanol significantly...
differed between IH and non-IH rats. These results argue against the possibility that the reduction in EW signs was due to altered ethanol intake or clearance in IH-treated rats. These results seem to contradict the findings of Meerson et al. (27), where hypobaric hypoxia exposures of several hours per day decreased ethanol consumption. Conceivably, IH cycles longer than the 5–10 min of this study, IH programs longer than 20 days, or different modes of IH administration such as hypobaric vs. normobaric may account for the different outcomes.

Our previous findings that EW induces oxidative stress in the rat brain (18, 34) prompted us to test whether IH protects against EW-induced free radicals and protein oxidation. In the current study, EW consistently produced higher O$_2^•^−$ and protein carbonyl contents than ethanol exposure per se, and IH treatment dampened these markers of oxidative stress. Rats experiencing EW suffer acute distress due to an abrupt transition from ethanol-induced suppression of glutamate-mediated excitation to withdrawal-induced neuronal hyperexcitability (10, 16). Also, oxidative stress is more intense during EW than during antecedent ethanol exposure (18, 34, 47). The pro-oxidant nature of ethanol intoxication-withdrawal also has been demonstrated in a clinical study where cerebrospinal fluid of withdrawn alcoholics contained higher concentrations of O$_2^•^−$ and excitatory amino acids than control subjects (46).

How IH protects against EW remains uncertain. We (24, 37) recently found that the protection against cardiac ischemia-reperfusion injury in dogs conditioned by the current IH regimen was abrogated by antioxidant NAC treatment during the IH program, suggesting that ROS play a pivotal role in producing the protection. Excess ROS cause neurological damage (46), but moderate levels of ROS such as those produced during IH may augment cellular antioxidant mechanisms. In this study, IH transiently increased cerebellar O$_2^•^−$ content. Moreover, administration of NAC to scavenge ROS during the IH sessions abrogated protection against neurological impairment and O$_2^•^−$ accumulation during subsequent EW. At physiological concentrations, ROS function as signaling molecules that activate transcription and may eventually increase synthesis of cytoprotective proteins (26). Indeed, Fiskum et al. (11) reported that an increase in basal ROS production evokes expression of antioxidant enzymes, bolstering resistance to oxidative stress in brain.

The possibility that IH protection is expressed at the mitochondrial level is crucial, because oxidative phosphorylation requires O$_2$, and thus, alteration in O$_2$ concentration during IH directly impacts mitochondrial ATP production. Furthermore, we have demonstrated that EW inactivates the key respiratory complex cytochrome c oxidase (17) and accelerates swelling and rupture of brain mitochondria from ovariecctomized female rats experiencing EW (20). Moreover, the identical IH regimen attenuated EW signs and oxidative stress in female rats (18). The current study demonstrated that EW increased mitochondrial membrane fragility in male rats, too, and extended this finding to IH protection against mitochondrial fragility during EW. Consistent with these results, a moderate IH regimen suppressed mitochondrial permeability transition in ischemic rat myocardium (49). In contrast, reintroduction of O$_2$ following anoxia (i.e., extreme hypoxia) elicited a burst of excess O$_2^•^−$ formation that provoked mitochondrial permeability transition (30). IH may directly or indirectly regulate intracellular Ca$^{2+}$, which in excess opens PTPs (14, 43, 48, 49). Alternatively, IH may increase synthesis of cytoprotective proteins, such as heat shock proteins, that are produced under stressful conditions (4, 21).

Finally, bivariate correlation analyses revealed robust correlations between EW sign scores and amounts of oxidative markers. This observation is consistent with a previous report of a strong association between ROS and EW-induced seizure activity in rats (47). Nevertheless, the magnitude of IH protection against EW signs is much greater than its protection against oxidative stress or mitochondrial fragility. EW signs are a global manifestation of hyperexcitability mediated by multiple factors, although the contribution of an individual factor to EW signs or IH protection may be modest. Indeed, Pearson correlation coefficients between EW signs and oxidative markers were less in value than those between oxidative markers per se. Moreover, our data suggest that the degree of association between the oxidative markers in response to EW and IH varies among different brain regions. In this study, cerebellar mitochondria were more vulnerable to EW than hippocampal or cerebrocortical mitochondria. Cerebellum normally contains large amounts of parvalbumin, a Ca$^{2+}$ binding protein that is depleted during EW (34). Because Ca$^{2+}$ is directly associated with mitochondrial permeability transition (14, 35, 43), the depletion of Ca$^{2+}$ binding proteins by EW might have permitted excess cytosolic Ca$^{2+}$, resulting in Ca$^{2+}$ entry into cerebellar mitochondria.

The duration of the IH regimen and the length and intensity of the hypoxia bouts are important determinants of whether IH is protective or harmful. Detrimental hypoxia programs, such as those that model sleep apnea or brain ischemia, typically utilize multiple, very brief cycles (e.g., 30–60 s) of severe hypoxia (FiO$_2$ 2–5%) (13, 36, 39, 49) or sustained hypoxia (44, 45). On the other hand, more moderate hypoxia is reported to evoke adaptations that protect the brain from subsequent ischemic insults (2, 22, 40). Thus, depending on the regimen, hypoxia can either harm or protect the brain. Protection is reliably achieved when hypoxia is moderately severe, divided by intermittent reoxygenation into multiple, brief (several minutes) bouts and of a cumulative duration much shorter than that of normoxia, e.g., 1 h/day (24, 26). The current IH regimen included all of these essential features. Behavioral and antioxidative protection during EW by this IH regimen may provide new insights into endogenous defense mechanisms against EW.

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GRANTS

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