

Acute Hypoxia-Ischemia Results in Hydrogen Peroxide Accumulation in Neonatal But Not Adult Mouse Brain

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ABSTRACT: The neonatal brain responds differently to hypoxic-ischemic injury and may be more vulnerable than the mature brain due to a greater susceptibility to oxidative stress. As a measure of oxidative stress, the immature brain should accumulate more hydrogen peroxide (H_2O_2) than the mature brain after a similar hypoxic-ischemic insult. To test this hypothesis, H_2O_2 accumulation was measured in postnatal day 7 (P7, neonatal) and P42 (adult) CD1 mouse brain regionally after inducing HI by carotid ligation followed by systemic hypoxia. H_2O_2 accumulation was quantified at 2, 12, 24, and 120 h after HI using the aminotriazole (AT)-mediated inhibition of catalase spectrophotometric method. Histologic injury was determined by an established scoring system, and infarction volume was determined. P7 and P42 animals were subjected to different durations of hypoxia to create a similar degree of brain injury. Despite similar injury, significantly less H_2O_2 accumulated in P42 mouse cortex compared with P7 at 2, 12, and 24 h after HI. In addition, less H_2O_2 accumulated in P42 mouse hippocampus compared with P7 hippocampus at 2 h. Since immature neurons are more vulnerable to the toxic effects of H_2O_2 than mature neurons, this increased accumulation in the immature brain may explain why the neonatal brain may be more devastated, even after a milder degree of acute hypoxic-ischemic injury. (*Pediatr Res* 59: 680–683, 2006)

Hypoxic-ischemic injury to the prenatal and perinatal brain causes devastating brain damage associated with significant morbidity and mortality (1). Neonatal HI often leads to neurologic dysfunction, cerebral palsy, and epilepsy later in life (2).

Previous studies have determined that the response of the immature central nervous system (CNS) to hypoxia-ischemia (HI) differs from that of the mature CNS. In both age groups, the pathogenesis of injury is complex and involves energy depletion, release of excitatory amino acids such as glutamate, activation of *N*-methyl-D-aspartate (NMDA) receptors, accumulation of reactive oxygen species, and initiation of apoptosis (3). However, the extent to which these events promote injury is greatly influenced by age. Primarily, it appears that the neonatal brain may be more vulnerable to HI than the mature brain because of a greater susceptibility to oxidative

stress (4). Several cellular and molecular mechanisms may be responsible for this increased susceptibility, including the enzymatic activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. After a hypoxic-ischemic insult, these defense mechanisms can become overwhelmed, resulting in accumulation of oxygen free radicals and neuronal death through reactions involving lipid peroxidation, protein oxidation, and DNA damage (5). In addition, the neonatal brain is particularly susceptible to oxidative damage because of its high concentration of unsaturated fatty acids, high rate of oxygen consumption, and availability of redox-active iron (2). Several lines of evidence suggest that the activities and the responses of these enzymes to oxidative stress are age dependent. For instance, GPx levels are low embryonically and neonatally and then gradually increase to reach their maximum levels during adulthood (6). In addition, GPx activity falls dramatically at 2 and 24 h after HI in neonatal mice (7). Furthermore, SOD1 overexpression results in marked neuroprotection in adult rats after ischemia-reperfusion (8), whereas SOD1 overexpression in the neonatal animal exacerbates hypoxic-ischemic brain injury (5). A possible explanation for the variable effect of SOD in the brain during different stages of development is that SOD1 transgenic adult mice show an adaptive rise in catalase activity (9), whereas neonatal SOD1 transgenic brains do not show an adaptive increase in either GPx or catalase (7). The consequent imbalance in antioxidant enzyme activities would allow for greater H_2O_2 production after HI and thus greater injury. In fact, the brains of P7 mice that overexpress SOD1 have a significantly higher H_2O_2 concentration at 24 h after HI compared with wild-type P7 mice (7). H_2O_2 has been shown to cause apoptosis in cultured oligodendroglia (10) and neurons (11) and is more toxic to immature neurons *in vitro* than to mature cells (12).

Therefore, the hypothesis is that given a similar acute ischemic injury, immature brain will accumulate more H_2O_2 than the mature brain in regions of damage. To address this hypothesis, conditions were determined that would result in a

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Abbreviations: AT, aminotriazole; GPx, glutathione peroxidase; H_2O_2 , hydrogen peroxide; HI, hypoxia-ischemia; OL, oligodendrocyte; P, postnatal day; SOD, superoxide dismutase

similar degree of acute injury at P7 and P42 in the mouse brain and relative H₂O₂ accumulation during the evolution of this hypoxic-ischemic injury in the cortex and hippocampus was ascertained.

METHODS

Animals. All experiments were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco and carried out with the highest standards of care and housing, according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. A total of 195 CD1 male mice were used for these experiments. Only male mice were used for these experiments as previous data illustrate that female adult mice are more resistant to damage (13). The CD1 strain was chosen because previous research suggests these mice are particularly susceptible to brain damage in the HI model (14).

Hypoxic-ischemic injury. HI was induced with an adaptation of the Rice-Vannucci procedure (5,15). On P7 or P42, mice were anesthetized with 2.5% halothane, 30% nitrous oxide, balance oxygen. The right common carotid artery was exposed and permanently ligated by electrical coagulation. After a 2-h recovery period, P7 and P42 mice were placed in a hypoxic chamber (8% oxygen/balance nitrogen) in a 37°C water bath for 30 or 40 min, respectively. It has been shown that mortality is high in adult mice after HI (13), which we found to be true as well, so we performed preliminary experiments that demonstrated that durations of 30 min of hypoxia in P7 mice and 40 min of hypoxia in P42 mice would produce a similar degree of injury between the two age groups, while minimizing mortality.

Histology and volume of infarction. For examination of the degree of injury after HI, animals were anesthetized with a lethal dose of pentobarbital at either 24 h or 5 d after HI. Brains were removed after perfusion fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Fifty-micrometer sections were cut on a vibrating microtome, and sections were stained with cresyl violet or iron stain for injury scoring. Analysis was performed in a blinded fashion on P7 ($n = 6$) and P42 ($n = 6$) brain sections at 5 d post-HI. Scoring was based on a previously described method in which brains are scored from 0 to 24, with 0 = no histologic injury and 24 = large cystic infarction (14).

The percentage of volume of brain infarction was determined for P7 ($n = 6$) and P42 ($n = 6$) mouse brains at 5 d after HI by photographing and measuring the area of surviving hemisphere in consecutive sections with a video image analysis system. Using NeuroLucida and Neuroexplorer software (MicroBrightfield, Williston, VT), the contralateral and surviving ipsilateral hemispheres of eight coronal sections from each brain were measured at the level of the anterior hippocampus by tracing the image. The hemispheric infarct volume in each brain was calculated as previously described (16).

P7 ($n = 5$) and P42 ($n = 5$) mouse brains were also prepared histologically at 24 h after HI to show the extent of injury at this early stage in the progression of injury.

Catalase assay and AT treatment. H₂O₂ can be converted to H₂O and O₂ by catalase or GPx. Catalase binds to H₂O₂ in the process of decomposing it. AT selectively and irreversibly inhibits catalase that is bound to H₂O₂ (17,18). Thus, inhibition of catalase activity by AT is directly proportional to the H₂O₂ concentration at the time of AT exposure (19). This is an indirect measurement, and we therefore refer to results as H₂O₂ accumulation rather than concentration. To assess the accumulation of H₂O₂ after HI, P7 ($n = 64$) and P42 ($n = 60$) mice were killed at 2, 12, and 24 h and 5 d after HI and the cortices and hippocampi were dissected free on ice. Brains were flash frozen and stored at -80°C. Two hours before sacrifice, mice were injected intraperitoneally with AT (200 mg/kg in normal saline; $n = 60$) or an equivalent volume of vehicle ($n = 64$). This time point was chosen for P42 mice based on a time curve of inhibition of catalase after injection of AT demonstrating 50% inhibition at 2 h (Fig. 1). This value agrees with previously published results for P7 CD1 mice that also showed 50% inhibition of catalase 2 h after injection of AT (7), although absolute values differed.

Catalase activity was then measured in the brains of these mice as described previously with slight modification (7,9,19). Activity was determined in duplicate samples by kinetic colorimetric assay following the decrease in absorbance of a known concentration of H₂O₂ at 240 nm over 1 min and expressed as units per milligram of protein, with 1 unit defined as 1 μ mol of H₂O₂ reduced per minute. All values were normalized to an internal control that consisted of several homogenized cortices and hippocampi. Data are expressed as the percentage of inhibition of catalase activity by AT.

Tissue levels of AT in P7 ($n = 12$) and P42 ($n = 12$) were assayed by the colorimetric method of Green and Feinstein (20) using a protocol previously described in detail (19). In brief, AT is first diazotized by sodium nitrate and

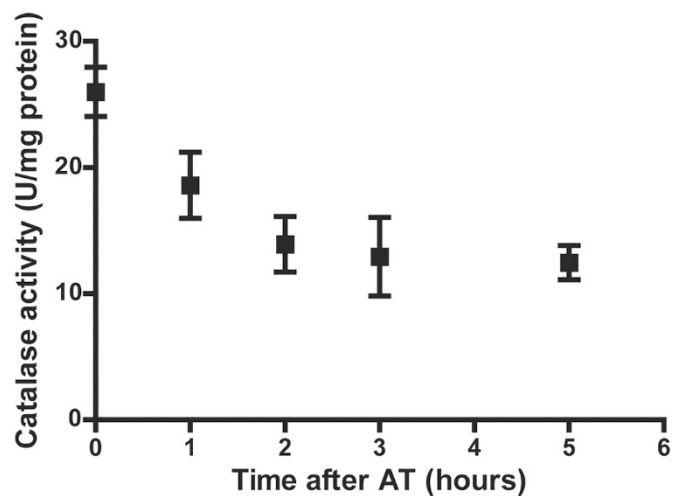


Figure 1. Time curve for inhibition of catalase by AT. P42 CD1 mice were injected intraperitoneally with AT (200 mg/kg of body weight) and killed at 1, 2, 3, and 5 h after injection ($n = 10, 8, 14,$ and $14,$ respectively). Control mice were injected with an equivalent volume of normal saline ($n = 6$). Catalase activity in the cortex and hippocampus is expressed in units per milligram of protein, where 1 unit equals 1 μ mol of H₂O₂ reduced per minute. Results are expressed as mean \pm SD, $p < 0.0001$ at all time points, compared with saline. Data from the cortex and hippocampus are combined because they were not significantly different.

then coupled to chromotropic acid to form a colored derivative, the absorbance of which is read at 525 nm. All samples were run as a single assay. Results were expressed as micrograms of AT per milligram of protein.

Statistical analysis. The volume of infarction and histologic damage score were analyzed by the Mann-Whitney test. The catalase activity time curve was analyzed with a one-way analysis of variance (ANOVA) comparing each time point to uninhibited catalase activity in naïve (no HI) brain followed by *post hoc* Dunnett's test. AT concentration and percentage inhibition of catalase after HI in hippocampus and cortex were analyzed with two-way ANOVA followed by Bonferroni *post hoc* test. Significance was established at $p < 0.05$. All statistical analyses were performed with Prism Version 4 software (Graphpad Software, San Diego, CA).

RESULTS

Brain injury, histologic damage, and volume of infarction.

Exposing P7 mice to 30 min of hypoxia and P42 mice to 40 min of hypoxia resulted in similar injury at 5 d post-HI as measured by histologic damage score and volume of infarction. By 24 h after HI, severe unilateral injury was obvious in both P7 and P42 mouse brains. Neuronal loss and disorganization of the infarcted region were evident to a similar extent. At 5 d after HI, P7 damage had progressed to cystic infarction, with considerable tissue loss. At 5 d after HI, P42 mice had a similar degree of injury. However, the neonatal brain had cavitated, whereas the adult brain had scarred with no remaining neurons in the core and penumbral regions.

Overall, P7 and P42 mice had similar injuries as measured by histologic scoring of damage [Fig. 2A: P7 median injury score = 15.5 (range, 5–24) versus P42 median injury score = 19 (range, 1–24), $p = 0.9372$] and percentage of volume of infarction (Fig. 2B: P7 mean \pm SD = 42.0 \pm 17.4 versus P42 mean \pm SD = 48.7 \pm 12.2, $p = 0.9372$).

H₂O₂ production. The AT method to identify H₂O₂ accumulation *in vivo* was used (7). It was first demonstrated that there was no significant difference in brain tissue levels of AT measured at 2, 12, and 24 h and 5 d after HI. Average

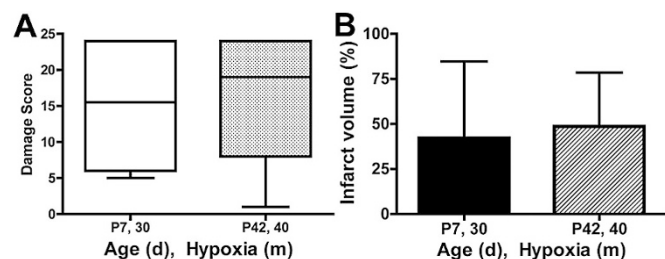


Figure 2. Brain injury after HI in the neonatal and adult mouse brain. (A) Histologic injury score 5 d after HI. The horizontal line represents the median score. P7 median injury score = 15.5 (range, 5–24; $n = 6$) vs P42 median injury score = 19 (range, 1–24; $n = 6$, $p = 0.9372$). (B) The percentage of volume of infarction 5 d after HI (P7 mean \pm SD = 42.0 \pm 17.4 vs P42 mean \pm SD = 48.7 \pm 12.2, $p = 0.9372$).

concentrations were 11.5 \pm 1.0 $\mu\text{g}/\text{mg}$ protein (P7) versus 13 \pm 4 $\mu\text{g}/\text{mg}$ protein (P42). At all other time points, the concentrations were 15.0 \pm 2.0 in both groups. Therefore, the time points chosen for measurement of H_2O_2 , by using the AT inhibition of catalase assay, were justified based on the appearance and the half-life of the drug in the brain (Fig. 1).

Less H_2O_2 accumulated in P42 mouse cortex at 2, 12, and 24 h after HI compared with P7 mouse cortex (Fig. 3A, $p < 0.001$ at all time points). In addition, less H_2O_2 accumulated in the P42 mouse hippocampus at 2 h ($p < 0.001$), 12 h ($p < 0.01$), and 24 h ($p < 0.001$) after HI compared with P7 hippocampus (Fig. 3B). H_2O_2 accumulation in contralateral to ligation, nonischemic hemispheres in both P7 and P42 mice was not significantly different from the accumulation found in uninjured brains (data not shown); in addition, H_2O_2 accumulation was similar between P7 and P42 mouse in naïve brain in both the cortex and hippocampus.

While there was a trend toward greater mortality in the adult mice after HI compared with the neonatal mice, it was not significant (P7 mortality = 10% versus P42 mortality = 33%; $p = 0.06$).

DISCUSSION

The major finding in this study is that, given a similar ischemic injury, the adult mouse brain accumulates less H_2O_2

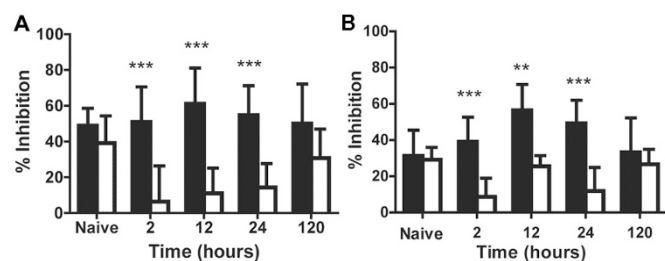


Figure 3. H_2O_2 accumulation, as measured indirectly by the AT inhibition of catalase activity, in P7 (■; $n = 64$) and P42 (□; $n = 60$) mouse brain. (A) Less H_2O_2 accumulated in ipsilateral to ligation P42 cortex at 2, 12, and 24 h ($p < 0.001$ at all time points) after HI compared with P7 cortex. There is no difference between the naïve and postischemic states at P7. P42, however, has less H_2O_2 accumulation at 2h after HI than naïve. (B) Less H_2O_2 accumulated in ipsilateral to ligation P42 hippocampus at 2 h ($p < 0.001$), 12 h ($p < 0.01$), and 24 h ($p < 0.001$) after HI compared with P7 hippocampus. Data are expressed as the percentage of inhibition of catalase activity by AT. Error bars are \pm SD.

than the neonatal brain in damaged regions. The importance of this finding is that H_2O_2 may be the critical mediator in determining whether downstream signaling will favor cell death or repair. While it is well-known that H_2O_2 is toxic to developing neurons and oligodendrocytes (OLs) (10–12), recent studies suggest that H_2O_2 induces preconditioning neuronal protection *in vitro* and *in vivo* (21,22). Therefore, critical control of these levels will determine the ultimate evolution of brain damage. The accumulation of H_2O_2 in the first 24 h after HI in the neonatal mouse brain versus similar brain regions in the adult can be explained by an imbalance between H_2O_2 production and consumption and better consumption of H_2O_2 after injury in the adult mouse. A tenuous balance exists between the H_2O_2 -producing and -consuming enzymes in the uninjured state, and the balance is disturbed when an injury occurs. For example, an increase in GPx activity might be expected after an ischemic insult as a compensatory mechanism in response to accumulation of H_2O_2 , and this has been shown to be the case in adult models of stroke (23). However, we did not find an increase in GPx activity in P7 mice after hypoxic-ischemic injury in a recent study (24). It is possible that the decrease in inhibition in the mature mice does not reflect a decrease in the rate of H_2O_2 production, but rather is due to diversion of H_2O_2 away from catalase to GPx or other scavenging enzymes. This is supported further by the fact that catalase activity does not change after ischemia in the neonatal brain (7).

Support of our hypothesis that the balance of scavenging enzymes is critical in an age-dependent manner is documented in a study of immature rat OLs found to be more sensitive to H_2O_2 than mature rat OLs. The mature OLs were able to degrade H_2O_2 faster than developing OLs, and this increased degradation was likely secondary to the 2- to 3-fold increase in GPx expression and activity observed in these cells. Inhibition of GPx by mercaptosuccinate made the mature OLs vulnerable to H_2O_2 (25). Additional support is provided by the finding that immature transgenic mice that overexpress GPx have less injury after HI than wild-type littermates (24). Also, neurons cultured from mice that overexpress GPx, when exposed to H_2O_2 , are resistant to injury (26).

In conclusion, this study shows that given a similar degree of initial injury, the neonatal mouse brain accumulates more H_2O_2 than the adult mouse brain in regions of damage. It is currently unclear whether this increased accumulation of H_2O_2 is responsible for injury propagation or whether H_2O_2 is the critical mediator of downstream signaling determining whether the brain is capable of repair or is destined for further cell death. Further studies on the neuromodulatory role of H_2O_2 are needed to fully understand its role in hypoxic-ischemic brain injury.

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