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# Corticosteroid responses following hypoxic preconditioning provide neuroprotection against subsequent hypoxic-ischemic brain injury in the newborn rats

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ABSTRACT

Limited research has evaluated the corticosteroids (CS) response in hypoxic preconditioning (PC) induced neuroprotection against subsequent hypoxic-ischemic (HI) brain injury in newborns. To measure, CS response to hypoxic PC, at postnatal day 6 (P6), rat pups were randomly divided into sham, NoPC (exposure to 21% $O_2$) and PC (exposure to 8% $O_2$ for 3 h) groups. In a separate experiment, at P6, rat pups were randomly divided into three groups (sham, NoPC + HI, PC + HI). Rat pups in NoPC + HI and PC + HI groups, respectively had normoxic or hypoxic exposure for 3 h at P6 and then had the right carotid artery permanently ligated followed by 140 min of hypoxia at P7 (HI). Plasma CS levels were measured at 0.5, 1, 3, 6 and 12 h after hypoxic PC and hypoxic PC followed by HI. To investigate whether CS response to hypoxic PC provides neuroprotection against HI, at P6, rat pups were randomly divided into five groups. Fifteen minutes prior to PC or normoxic exposure, rat pups in DMSO + PC + HI and DMSO + NoPC + HI groups received DMSO while in RU486 + PC + HI and RU486 + NoPC + HI groups received RU486 (glucocorticoid receptor blocker, 60 mg/kg) s.c., respectively. Afterwards, rat pups were exposed to normoxia (DMSO + NoPC + HI, RU486 + NoPC + HI) or hypoxia (DMSO + PC + HI, RU486 + PC + HI) for 3 h and then HI 24 h later (P7). Rat pups at the corresponding age without any exposure to PC or HI or RU486/DMSO were used as sham. We found that hypoxic PC caused CS surge as well as augmented CS surge and preserved the glucocorticoid feedback regulation after HI. Hypoxic PC reduced HI induced early and delayed brain damage. RU486 partially but significantly inhibited hypoxic PC induced neuroprotection.



## 1. Introduction

Ongoing brain injury following hypoxia-ischemia (HI) provides a "therapeutic window" for intervening in the pathogenesis of HI brain injury in the developing brain. Promising new neuroprotective strategies, including moderate hypothermia designed to limit the extent of brain injury, have renewed interest in further research for innovative therapeutic approaches to improve outcome. The neonatal rat model of HI has been well characterized (Rice et al., 1981) and used extensively to assess the efficacy of neuroprotective strategies (Ashwal and Pearce, 2001). This well-established model of combining unilateral carotid artery occlusion and systemic hypoxia with 8% oxygen produces brain damage in the form of selective neuronal necrosis or infarction predominantly of the cerebral hemisphere ipsilateral to the arterial ligation (right hemisphere–RH). The opposite hemisphere (left hemisphere–LH) endures hypoxia but escapes any significant injury (Vannucci et al., 1998).

Brain preconditioning is a phenomenon in which the brain protects itself against lethal injury by adapting to sub-lethal insults. A hypoxic PC model has been developed for neonatal rat brain (Gidday et al., 1994). Stress induced by hypoxic PC results in an adaptive response which involves expression of multiple genes which ultimately counteract the pathways that cause HI induced neuronal death (Gidday et al., 1994). Extensive research has been done in the field of PC because the cellular mechanisms of neuroprotection induced by PC offer attractive targets for the development of therapeutic approaches. PC can be induced by a variety of stimuli. PC occurs even after stimuli to distant organs (remote PC) and can be cross-tolerant, protection to one type of

*Abbreviations:* Akt, protein kinase B; CS, corticosteroids or corticosterone; GR, glucocorticoid receptor; HI, hypoxia-ischemia or hypoxic-ischemic; PC, hypoxic preconditioning.

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injury (HI) by another type of stimulus (endotoxin). Thus, possibility of an overlapping mechanism of PC by different stimuli is likely. The pituitary–adrenal axis (PAA) plays a vital role in adaptive response to stress, but minimal research has examined its role in PC induced neuroprotection (Rybnikova et al., 2008). A better understanding of the role of PAA in the neuroprotection afforded by hypoxic PC may provide a foundation for the development of newer therapeutic strategies for the prevention and treatment of HI brain injury in newborns. Our first objective was to investigate the hormonal response involving corticosteroid (CS) to hypoxic PC and hypoxic PC followed by HI. Our second objective was to investigate whether CS response to hypoxic PC provide neuroprotection against subsequent HI brain injury in neonatal rats.

To investigate the first objective we measured plasma CS levels at different time points following hypoxic PC and hypoxic PC followed by HI in newborn rats. To investigate the second objective we blocked the glucocorticoid receptors (GR) by RU486 and then measured hypoxic PC induced neuroprotection against subsequent HI in newborn rats.

## 2. Methods

### 2.1. Animal

Timed pregnant Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) and pups were cared for in accordance with the National Institutes of Health guidelines and protocol approved by the University of Mississippi institutional committee on animal use. We used 6-day-old rat pups of either sex, weighing between 10–14 g from a total of 28 litters for our experiments. We divided male and female pups equally in each group.

### 2.2. Hypoxic preconditioning

PC was performed as described by Gidday et al. (1994). On postnatal day (P) 6, rat pups from each litter were culled to 12 newborns and randomly divided into different groups, placed in 1.5 L sealed jars immersed 5.5 cm deep in a 37 °C water bath and subjected to a warmed, humidified mixture of either 21% $O_2$ (normoxic exposure) or 8% $O_2$ (hypoxic exposure) in nitrogen bubbled through 37 °C water and delivered at 4 L/min for 3 h.

### 2.3. HI Exposure

The neonatal rat HI procedure was performed as described by Vannucci et al. (1998) with minor modifications. At P7, the rat pups were anesthetized with isoflurane (3% for induction and 1.5% for maintenance). The right common carotid artery was exposed, isolated and permanently doubly ligated. After surgery, the rat pups were returned to their dams for 2–3 h recovery. Hypoxic exposure was achieved by placing the rat pups in 1.5 L sealed jars immersed 5.5 cm deep in a 37 °C water bath and subjected to a warmed, humidified mixture of 8% $O_2$/92% nitrogen bubbled through 37 °C water and delivered at 4 L/min for 140 min. After this hypoxic exposure, the pups were returned to their dams and allowed to recover and grow.

### 2.4. CS response following hypoxic PC and hypoxic PC followed by HI

To measure, adrenal hormonal response to hypoxic PC, at P6 rat pups from each litter were culled to 12 newborns and randomly divided into sham, NoPC (exposure to 21% $O_2$) and PC (exposure to 8% $O_2$ for 3 h) groups. Plasma CS levels were measured at 0.5, 1, 3, 6 and 12 h after reoxygenation.

In a separate experiment, at P6, rat pups from each litter were culled to 12 newborn pups and randomly divided into three groups (sham, NoPC + HI, PC + HI). Rat pups in NoPC + HI and PC + HI groups, respectively had normoxic or hypoxic exposure for 3 h and then HI 24 h later at P7 as described above. Plasma CS levels were measured at 0.5, 1, 3, 6 and 12 h after HI.

Rat pups were decapitated with sharp scissor and blood was taken from both the artery and vein in the trunk in tubes coated with heparin. The blood was centrifuged at 2500 × *g* for 10 min at 4 °C and plasma CS was measured with a commercially available an enzyme immune assay kit (Assay Design, Ann Arbor, MI) following the manufacturer's instructions.

### 2.5. Effect of a RU 486, GR blocker on hypoxic PC induced neuroprotection against HI in newborn rats

On P6, rat pups from each litter were culled and randomly divided into five groups (Sham, DMSO + NoPC + HI, DMSO + PC + HI, RU486 + NoPC + HI and RU486 + PC + HI). Fifteen minutes prior to PC or normoxic exposure, rat pups in DMSO + PC + HI and DMSO + NoPC + HI groups received DMSO while in RU486 + PC + HI and RU486 + NoPC + HI groups received RU486 (GR blocker, 60 mg/kg) s.c., respectively. Afterwards, rat pups were exposed to normoxia (DMSO + NoPC + HI, RU486 + NoPC + HI) or hypoxia (DMSO + PC + HI, RU486 + PC + HI) for 3 h and then HI 24 h later at P7 as described above. After HI exposure, the pups were returned to their dams and allowed to recover and grow. Brains from rat pups at the corresponding age without any exposure to PC or HI or RU486/DMSO were examined for comparison (Sham). The extent of brain injury in the ipsilateral hemisphere was evaluated by changes in right hemisphere water content, caspase-3 activity, cleved-Caspase-3 staining at 24 h, microscopic brain damage score at 72 h, and delayed gross brain damage at 22 days following HI.

#### 2.5.1. Measurement of brain water content.

The rat pups were decapitated by cervical dislocation at 24 h after HI exposure ($n$ = 9–13/group). After removing cerebellum, medulla oblongata and pons, the brains were dissected, cerebral hemispheres were separated from each other and water content was determined using the previously described method (Feng et al., 2008). The brain samples were weighed and then dried at 95 °C for 48 h to obtain the dry weight. The brain water content was calculated as [(wet weight–dry weight)/wet weight × 100].

#### 2.5.2. Measurement of caspase-3 activity

At 24 h after HI, caspase-3 activity in cerebral hemispheres was measured as previously described ($n$ = 9–13 in each group) (Feng et al., 2009). Each cerebral hemisphere was homogenized and caspase-3 activity was measured using commercially available assay kits (Calbiochem, Inc., San Diego, CA, USA), following the manufacturer's instructions. Caspase-3 activity was determined colorimetrically with a microplate reader at 405 nm. A p-nitroanilide (pNA) calibration curve was established to quantify units of caspase-3 activity. The protein concentration was determined by the method of Bradford (Bradford, 1976).

#### 2.5.3. Immunofluorescence analysis.

Brain specimens were prepared as described previously (Feng et al., 2009; LeBlanc et al., 2000). At 24 h after HI, the pups were anesthetized with pentobarbital and given transcardiac perfusion with ice-cold saline and then with 4% paraformaldehyde in PBS. Brains were removed and immersed in 10% formalin and then cryoprotected and rapidly frozen by 2-methylbutane chilled in liquid nitrogen. Serial coronal brain sections of 10 μm thickness were cut and stored at −80 °C for future analysis. Immunofluorescence analysis for cleaved caspase-3 was performed under standard protocols