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A NITRIC OXIDE DONOR REDUCES BRAIN INJURY AND ENHANCES RECOVERY OF CEREBRAL BLOOD FLOW AFTER HYPOXIA-ISCHEMIA IN THE NEWBORN RAT

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Abstract

Nitric oxide (NO) released in response to hypoxia-ischemia (HI) in the newborn brain may mediate both protective and pathologic responses. We sought to determine whether pharmacologic increase of NO using an NO donor would reduce neurologic injury resulting from HI in the postnatal day 7 rat. We measured NO levels and CBF in the presence of either a NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME) or an NO donor (Z)-1-[N-(2-aminoethyl)-N-(2-ammonio-ethyl)amino] diazen-1-ium-1,2-diolate (DETANONOate). Both inhibition of NOS and administration of an NO donor reduced neuropathologic injury after 7-day recovery. NO levels decreased in both ischemic and contralateral hemispheres during HI. This response was prevented by treatment with DETANONOate. Despite the decrease in NO, CBF increased during ischemia in the contralateral hemisphere but decreased when combined with brief hypoxia. Treatment with L-NAME abolished these increases, which were not altered by DETANONOate. Reduction of cellular metabolism by mild hypothermia also reduced both NO and CBF. Following prolonged HI, CBF remained decreased in the ischemic hemisphere up to 24-hour recovery. This decrease was prevented by treatment with DETANONOate. These data show that administration of an NO donor reduces neurologic injury following HI in the newborn rat. This mechanism of this protection, in part, is due to an increase in the rate of recovery of CBF compared to vehicle-treated animals. Augmentation of NO-dependent increases in CBF may serve to improve neurologic outcome after perinatal asphyxia.

Keywords

Hypoxia-ischemia; nitric oxide; cerebral blood flow; hypothermia; newborn

INTRODUCTION

Hypoxia-ischemia (HI) in the perinatal period may cause significant infant mortality and neurologic morbidity with limited therapeutic options available [8]. Studies using

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pharmacologic inhibition of nitric oxide synthase (NOS) isoforms or selective NOS gene knockout have suggested that excessive nitric oxide (NO) production by inducible (i)NOS and neuronal (n)NOS is neurotoxic, while endothelial (e)NOS activation serves to protect against neurologic injury by vasodilation and preservation of cerebral blood flow (CBF) [6].

The use of NOS inhibitors for neuroprotection in models of cerebral ischemic injury has yielded contradictory results [23]. Inhibition of eNOS has been shown to increase [10] or decrease [2] neurologic injury following cerebral ischemia. NO may exert beneficial and harmful effects in multiple diseases including cerebral ischemia [20].

NO is a pivotal regulator of CBF [12,16] and our previous studies have shown a reduction in NOS activity after neonatal HI [14]. The contribution of decrease in NO signaling to changes in CBF during HI and the effects of NO supplementation is not known [1,11,13]. Thus, in this study we sought to determine whether pharmacologic increase of NO using an NO donor would reduce neurologic injury resulting from HI.

The Institutional Animal Care and Use Committee of Children's Memorial Hospital, Chicago, approved all experimental procedures. Postnatal day (P)7 Wistar rats were used for the studies. All reagents were obtained from Sigma (Sigma, St. Louis, MO) unless otherwise stated.

Induction of HI injury in the P7 rat was performed using modifications of published methods [21]. To measure NO levels and cortical CBF in real time during HI, P7 rats were anesthetized under isoflurane (2.5% for induction, 1–1.5% for maintenance) in oxygen via a face-mask. The right common carotid was mobilized and a suture placed around the vessel without occluding flow. Core temperature was stabilized at $37.0 \pm 0.1^\circ\text{C}$ by surface heating and cooling. Temperature was monitored continuously using a rectal probe (IT-18, Physitemp, NJ). After a 5 min period of stabilization, carotid ligation was induced by tension on the suture and confirmed by a reduction in CBF signal. Following 5 min ischemia, hypoxia was induced by exposure to a mixture of 8% oxygen/balance nitrogen. Hypoxia was discontinued after 10 min and recovery performed in 100% oxygen for a further 10 min. All physiologic data (CBF, NO, temperature) was recorded continuously in digital format (Powerlab 4SP, AD Instruments, Colorado Springs, CO). For analysis of CBF response during long-term recovery, P7 rats underwent HI as previously described [21], including 70 min of hypoxia following unilateral carotid ligation.

Animals were treated with a NOS inhibitor or an NO donor as previously described [21]. The NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) or diluent (saline) was injected (30 mg/kg ip) 30 minutes prior to hypoxia. To increase exogenous NO, animals were administered (Z)-1-[N-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazen-1-ium-1,2-diolate (DETANONOate), at a dose of 0.4 mg/kg ip 24 hr and 1 hr prior to HI [21,25].

After 7-day recovery from injury, serial 10 μm coronal paraffin-embedded sections were prepared from the level of the genu of the corpus callosum to the posterior hippocampus. Alternate sections were stained with 0.2% cresyl violet acetate solution and assessed for the extent of infarct and neuropathologic injury. Scoring of pathologic injury was carried out using published criteria for such injury in the immature rodent [15].

Lipid peroxidation products were measured by immunofluorescent detection of 4-hydroxynonenal (HNE), a neurotoxic product of free radical-mediated lipid peroxidation [24]. After deparaffinization and antigen retrieval, non-adjacent sections (2 per animal; n = 4–5 animals per group) were incubated in anti-4-HNE primary antibody (1:50; Oxis International, USA) for 1hr at room temperature. HNE-immunoreactive cells were visualized using a goat anti-mouse secondary antibody (Molecular Probes, Inc.). A laser scanning confocal microscope (Carl Zeiss, LSM 510 Meta) was used to produce digital images of fluorescent

stained sections. Time lapsed images from the cortical region of each section were collected into a single file or t-series keeping the microscope setting same for all the slides, and fluorescence intensity quantified using LSM 510 software.

NO levels were measured using an NO electrode (tip diameter 700 μ m, AMINO-700, Innovative Instruments, Tampa, FL), single channel NO detector system (iNO-T, Innovative Systems). A craniotomy was performed and the probe inserted into the brain at a depth of 1.5 mm, 2 mm caudal and lateral to Bregma. Data was recorded in digital format simultaneously with laser-doppler flowmetry (LDF) and temperature.

Cortical cerebral blood flow was measured by LDF (v38 monitor with 1.5 mm MP3 probe, Moor Instruments, Wilmington DE) during HI [9,11]. To determine changes in cortical CBF during HI, experiments were performed with continuous LDF monitoring under isoflurane anesthesia [1,11]. The probe was positioned over the left (non-ischemic) or right (ischemic) hemisphere 3 mm posterior to Bregma and 3 mm lateral to the midline. Values from this study were expressed as percentage changes from pre-HI values. To determine the *long-term* changes in cortical CBF in response to HI, modifications of the 'scanning' laser doppler technique for measurement of CBF in the rodent were used [9]. Rats were placed in a stereotactic apparatus (Stoelting, Wood Dale, IL) under isoflurane anesthesia, the LDF probe placed 1 mm caudal and 1 mm lateral to Bregma on the skull surface and CBF recorded for 30 seconds. A total of three such measurements were obtained by moving the probe 2 mm a caudal direction. The probe was then moved 2 mm lateral and a further 3 measurements obtained beginning caudal to Bregma before repeating the procedure over the contralateral hemisphere. This procedure was performed at serial time points over a 7d recovery period after HI.

To distinguish between the effects of NO and cerebral metabolism on changes in CBF, P7 rats were subject to mild hypothermia during HI. After a 5 minute period of physiologic stabilization at 36.5°C, rectal temperature was reduced to 33.5 $\pm$ 0.1°C by surface cooling. Rats underwent HI with continuous LDF and NO monitoring as described above under normothermia. At the conclusion of HI, rats were re-warmed to 36.5°C over 5 minutes and monitored for a further 10 minutes at 36.5°C.

Data are presented as mean $\pm$ SEM except neurologic injury score, which is presented as median $\pm$ interquartile range (IQR). CBF and NO data are presented as the % of pre-HI values. For intra-HI studies of CBF and NO, the mean of the last 5 minute of recording under a given condition (I, HI, or recovery) were used for data analysis. One-way ANOVA with Dunnett's post test and repeated measures ANOVA were performed using GraphPad Prism (GraphPad Software, San Diego California USA). Statistical significance was assumed when $P < 0.05$.

Consistent with published data [22,23], inhibition of NOS during HI resulted in a significant reduction in neurologic injury after 7-day recovery. The neuropathologic injury score in cresyl-violet stained sections in the L-NAME treated group (n=12 per group) was significantly reduced (0 $\pm$ 1) compared to vehicle treatment (0 $\pm$ 3; L-NAME vs. VEH, $P < 0.001$). DETANONOate treatment also significantly reduced injury (0 $\pm$ 2; $P < 0.001$ vs. VEH) compared to vehicle-treated controls but was not as effective as L-NAME (DETA vs. L-NAME, $P < 0.001$).

To confirm that treatment with L-NAME or DETANONOate altered cortical NO levels, we measured NO prior to HI. The NO level (nmoles $\pm$ SEM) in control (vehicle-treated) rats was 66.1 $\pm$ 2.3 (n=53). Treatment with L-NAME resulted a significant reduction in NO (52.5 $\pm$ 4.5; n=17) compared to the control group ($P < 0.05$). Rats treated with DETA showed a significant increase in NO levels (80.7 $\pm$ 6.0; n=19) compared to the control group.

We measured changes in cortical NO levels (data expressed as mean change from baseline) during HI and recovery (Fig. 1A–C). In vehicle-treated animals (Fig. 1A) in both left (control) and right (ischemic) hemispheres, NO levels were significantly decreased compared to pre-ischemia baseline values during ischemia, HI, and recovery. The reduction in NO during ischemia was greater in the right than left hemisphere, but did not differ between hemispheres during HI or recovery. Treatment with the NOS inhibitor, L-NAME abolished this difference (Fig. 1B). In contrast, DETANONOate -treatment significantly protected against the reduction in NO levels in the right hemisphere during ischemia, HI and recovery (Fig. 1C). As for L-NAME, there were no inter-hemisphere differences in NO levels.

As an initial approach to understanding the conflicting effects of modulation of NO levels on neurologic injury, we measured changes in CBF during HI (Fig. 1D–F). In vehicle-treated animals (Fig. 1D), there were significant decreases in CBF compared to baseline values during ischemia, HI and recovery. CBF increased in the left (non-ischemic) hemisphere during ischemia and recovery, but was decreased during HI. During each phase, the CBF response in the left and right hemisphere was significantly different. In contrast, treatment with L-NAME (Fig. 1E), prevented the increase in CBF during ischemia and recovery, and abolished differences between the hemispheres. Treatment with DETANONOate (Fig. 1F) resulted in a response similar to that observed in the vehicle-treated group.

To determine whether changes in CBF during HI were linked to cellular metabolism [12,19] independent of NO, we performed HI under conditions of mild hypothermia (Fig. 2). Cooling resulted in a decrease in CBF (Fig. 2A). Systemic hypothermia prevented the increase in CBF seen in the left hemisphere during ischemia in normothermic animals. The decrease in CBF during ischemia was greater in the right than the left hemisphere. During resuscitation, CBF did not increase in the left hemisphere until normothermia was achieved. NO levels were decreased throughout the procedure under hypothermic conditions (Fig 2B) but increased in the left hemisphere after restoration of normothermia.

To determine whether pharmacologic modulation of NO levels affected CBF during recovery after HI, we next measured changes in CBF over a 7-day recovery period after HI in which hypoxia was administered for 70 minutes (Fig. 3). In vehicle-treated animals (Fig. 3A), there were significant differences between CBF in the left and right hemisphere at 2, 6, 12, and 24 hr after HI. CBF was significantly increased compared to pre-ischemia values in the left hemisphere at 2, 6 and 12 hr recovery after HI, but there was no difference between hemispheres at 7d recovery. Inhibition of NOS (Fig. 3B) prevented any increase in CBF in the left hemisphere until 12 hr after HI, which persisted until 24 hr recovery. Administration of the NO donor (Fig. 3C) prevented the decrease in CBF measured in the right hemisphere at 12- and 24-hr recovery in the vehicle-treated group.

Following HI, there was a significant increase in free radicals, measured by a change in lipid peroxidation after 7 day recovery (Fig. 4). This increase was prevented both by inhibition of NOS and administration of the NO donor.

We sought to address the divergent, beneficial and neurotoxic, effects of NO on neurologic injury following cerebral HI in the newborn. Consistent with previous studies in the newborn rodent administration of a NOS inhibitor during the insult reduced neurologic injury. Here, we show that administration of an NO donor reduces neurologic injury following HI as has previously been reported in adult animals models of cerebral ischemia [18,25].

The protective effect of NOS inhibition may be due to reduction in the NO-induced formation of free radicals [26] produced by neuronal or inducible NOS [17]. We also found that L-NAME treatment prevented the collateral (left hemisphere) increase in CBF seen in the vehicle-treated animals from 2 to 12 hours after HI while treatment with an NO donor led to an increase in the

rate of recovery of CBF in the ischemic brain. Our data suggests that the reduction in injury resulting from treatment with an NO donor is associated with a more rapid recovery in CBF in the ipsilateral hemisphere comparable to the mechanism in adult model of cerebral ischemia [18,25].

NO levels decreased during HI in contrast to other studies in which elevated levels of NO metabolites and NOS activity have been reported after HI [4]. This was a brief duration of HI in order to enable us to monitor CBF and NO during the insult. Second, we measured NO directly, not NOS activity, which may itself be decreased [14]. These studies are limited by the use of *ex vivo* measurements of the potential for NO production [3]. Studies using NO metabolites as an indicator of NO production have suggested an increase in NO following HI [7]. In addition, NOS activity may vary within the ischemic region, being increased in the core compared to the penumbra [3].

A number of mechanisms may contribute to the decrease in NO levels including a decrease in production of NO. Both increase [4], and decrease [14], in NOS activity after HI in the rat fetus and neonate have been reported. Alternatively, the reduction in NO may be due to increased NO metabolism associated with increases in free radical levels or the degree of reduction in CBF [5].

We used hypothermia to uncouple cellular metabolism and CBF [19]. The lack of precise correspondence between CBF and NO changes during hypothermia is consistent with the multifactorial regulation of CBF. This is further supported by our finding that CBF increased despite measured decreases in NO during normothermia.

The thresholds for induction of metabolic and histologic lesions following cerebral ischemia are fundamentally linked to CBF [12]. We found persistent changes in CBF in both the ischemic (decreased) and non-ischemic (increased) hemisphere which were present until 24hr recovery. This finding is in contrast to a previous study in which no differences were measured between ischemic and non-ischemic hemispheres up to 24 hr after HI [13]. The protective effect of L-NAME is associated with prevention of the prolonged collateral increase in CBF in the contralateral hemisphere up to 12 hours after HI. This finding suggests that a decrease in CBF following HI may be associated with a reduction in neurologic injury and is confirmed by our data showing a decrease in neuropathologic injury under the same conditions of L-NAME treatment. Conversely, DETANONOate produced a more rapid recovery of CBF in this experiment, which also resulted in improved neurologic outcome.

NO plays a complex role in free radical-mediated injury during cerebral reperfusion [23] and may contribute to this injury through the formation of peroxynitrite generated by reaction with superoxide. NO may also act as a free radical scavenger, depending on the time of administration during reperfusion [18]. The lack of increase in lipid peroxidation products in the NO donor-treated group suggests that pharmacologic increase in NO levels did not exacerbate the increase in free radical formation.

Several methodological issues must be considered in interpreting this data. The long-term changes in CBF cannot be directly correlated with the real-time monitoring data as they are obtained under different durations of hypoxia. While CBF was affected by treated with a NOS inhibitor, this is not specific to one NOS isoform [20]. Other studies have shown both an increase [10] and decrease [2] in neurologic injury associated with inhibition of eNOS. The relative lack of specificity for many of the NOS inhibitors for a specific NOS isoforms may account for these findings [23].

In conclusion, these data show that administration of an NO donor reduces neurologic injury following HI in the newborn rat. This mechanism of this protection, at least in part, is due to

an increase in the rate of recovery of CBF compared to vehicle-treated animals. Further study of the potential of NO augmentation as a therapy for HI is warranted.

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Figure 1.

(A–C) Cortical nitric oxide response and (D–F) cerebral blood flow (CBF) response to ischemia, hypoxia-ischemia (HI) and recovery in oxygen on postnatal day 7. Data (% change from baseline values $\pm$ SEM) is shown from left (contralateral; filled bars) and right (ischemic; no-fill) hemispheres. A. Vehicle controls ($n =$ at least 12 for each hemisphere); B. L-NAME treated ($n=8$ for each hemisphere) and; C. DETANONOate-treated (DETA, $n=8$ for each hemisphere). (D). Vehicle controls ($n =$ at least 12 for each hemisphere); E. L-NAME treated ($n = 8$ for each hemisphere) and; F. DETANONOate-treated (DETA, $n=8$ for each hemisphere). * $P < 0.01$ versus right (ischemic) hemisphere by ANOVA. # $P < 0.001$ versus Vehicle-treated by ANOVA.

Figure 2.

Effects of hypothermia on (A) cerebral blood flow and (B) NO levels during hypoxia-ischemia (HI) under hypothermia (rectal temperature 33.5°C) and normothermia (37.0°C). Data (% change from baseline values $\pm$ SEM) is shown from left (contralateral; filled bars) and right (ischemic; no-fill) hemispheres. * $P < 0.01$ or ** $P < 0.001$ versus right (ischemic) hemisphere by ANOVA ($n=10$).

Figure 3.

Cerebral blood flow (CBF) response to hypoxia-ischemia measured over 7 day recovery. CBF in absolute Laser Doppler Units (LDF) was quantified as described in Methods using multiple measures from the left (non-ischemic) and right (ischemic) hemisphere. (A) Vehicle treated; (B) L-NAME and (C) DETANONOate-treated. For all groups, $n =$ at least 8. $*P < 0.05$ compared to contralateral hemisphere by ANOVA. $\#P < 0.05$ compared to pre-ischemia values by repeated measures ANOVA.

Figure 4.

Lipid peroxidation products measured by immunofluorescence after 7 day recovery from HI or sham procedure. (A) Representative confocal microscopic images showing localization of HNE in the right cortex (top) and in the left cortex (bottom) of Vehicle, DETANONOate, L-NAME and SHAM animals after 7 day recovery. Images were taken as time-series at 400x magnification. (B) Quantification of image intensity shows increased fluorescence in the right cortex of vehicle-treated animals, but not L-NAME, DETANONOate-treated or sham controls. $^{\#}P < 0.05$ vs left hemisphere (n = 4–5 animals per group).