Oxidative Stress during Post-Hypoxic-Ischemic Reperfusion in the Newborn Lamb: The Effect of Nitric Oxide Synthesis Inhibition

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ABSTRACT

Post-hypoxic-ischemic (HI) reperfusion induces endothelium and neurons to produce excessive amounts of nitric oxide and superoxide, leading to peroxynitrite formation, release of protein-bound metal ions (i.e., iron), and cytotoxic oxidants. We produced severe HI in 18 newborn lambs and serially determined plasma prooxidants (non-protein-bound iron), lipid peroxidation (malondialdehyde), and antioxidative capacity (ratio of ascorbic acid/dehydroascorbic acid (AA/DHA), α-tocopherol, sulfhydryl groups, allantoin/uric acid ratio, and vitamin A) in blood effluent from the brain before and at 15, 60, 120, and 180 min after HI. The lambs were divided in three groups: six received a placebo (CONT), six received low dose (10 mg/kg/i.v.) N\textsuperscript{ω}-nitro-L-arginine (NLA-10) to block nitric oxide production, and six received high dose NLA (40 mg/kg/i.v.; NLA-40), immediately after completion of HI. Non-protein-bound iron increased in all groups after HI but was significantly lower in both NLA groups at 180 min post-HI (p < 0.05), the AA/DHA ratio showed a consistent decrease in CONT (at 60 min post-HI, p < 0.05), but remained stable in NLA lambs. α-Tocopherol decreased steadily in the CONT, but not in the NLA lambs [180 post-H: 1.9 ± 0.9 versus 4.2 ± 0.7 μM (NLA-40), p < 0.05]. Malondialdehyde was significantly higher in CONT lambs 120 min post-HI compared with NLA groups [0.61 ± 0.17 versus 0.44 ± 0.05 μM (NLA-40), p < 0.05]. Vitamin A and sulfhydryl groups did not differ among groups. We conclude that post-H inhibition of nitric oxide synthesis diminishes non-protein-bound iron increment and preserves antioxidative capacity. (Pediatr Res 41: 321–326, 1997)

Abbreviations

AA/DHA ratio, ascorbic acid/dehydroascorbic acid ratio
CONT, control
HI, hypoxic-ischemic
MABP, mean aortic blood pressure
NLA, N\textsuperscript{ω}-nitro-L-arginine
ANOVA, analysis of variance

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would diminish production of prooxidants and consumption of antioxidants, and thus limit lipid peroxidation.

**METHODS**

**Animal preparation.** Surgical and experimental procedures used were reviewed and approved by the Committee on Animal Experiments at the University of Leiden and the Scientific Board of the Department of Pediatrics.

Eighteen newborn lambs with weights ranging from 3.5 to 5.3 kg (median 4.7 kg) and ages ranging from 2 to 11 d (median 7 d) were studied. General anesthesia was induced with ketamine hydrochloride (3 mg/kg i.v.) and supplemented by xylazine (1 mg/kg i.m.). In addition, local anesthesia was accomplished with 1% lidocaine hydrochloride before each skin incision. During the actual study, the wounds were sprayed with 1% lidocaine at regular intervals. After intubation, the lambs were ventilated with oxygen and air using a pressure-regulated ventilator, which was adjusted to maintain arterial Po$_2$ and Pco$_2$ in the normal range. Pancuronium (0.2 mg/kg i.v.) was administered for muscle relaxation. The animals were nursed on a heating pad to maintain normal body temperature. An i.v. infusion of 5% glucose in 0.9% NaCl was maintained throughout the study at about 15 mL/kg/h. NaHCO$_3$ was administered if the arterial pH was lower than 7.30 with a base excess of >5 mmol/L.

Into the right and left femoral arteries and veins 5 or 6 Fr self-sealing sheaths were placed using a percutaneous technique. Into the left femoral artery a 5 Fr micromanometer catheter (Millar Instruments, Houston, TX) was advanced into the descending thoracic aorta for continuous measurement of instantaneous mean aortic pressure. The right femoral artery was used for sampling of arterial gases and pH. Both femoral venous catheters were used for blood withdrawal and reperfusion, and for infusion of drugs. Via the right jugular vein a 4 Fr catheter was advanced retrogradely toward the head and positioned in the internal maxillary vein, into which the cerebral sinuses drain. Blood from this vessel was used to sample venous blood effluent from the brain to determine the redox status. After the carotid arteries in the neck were exposed, appropriately sized transonic flow transducers (Transonic Systems Inc., Ithaca, NY) were applied to fit around the vessels to measure carotid artery blood flow (mL/min) for assessment of changes in actual brain blood flow. This was done to be sure that there was indeed cerebral ischemia during the HI insult (see also “Experimental Protocol” below).

**Physiologic measurements.** Arterial blood gases and pH were measured using a Corning 178 pH/blood gas analyzer (Corning, Halstead, UK). Blood gases, pH, and Hb were determined at regular intervals and adjusted if necessary. Instantaneous aortic pressure and ECG were continuously displayed on a memory oscilloscope (Gould OS 4100, Hainault, UK), digitized with a sample frequency of 200 Hz, and stored on hard disk using a personal computer.

**Measurement of prooxidant activity, antioxidant capacity and lipid peroxidation.** Blood was collected into heparinized glass tubes and immediately centrifuged (750 g, 10 min); the plasma was stored under argon at −70°C until analysis. Plasma samples that showed pink discoloration (hemolysis) were excluded from the study. Non-protein-bound iron, a prooxidant, was measured using the bleomycin assay (21). Using this assay, the absence of non-protein-bound iron, i.e. the presence of iron binding capacity, can be measured as well as the presence of non-protein-bound iron, i.e. the lack of iron binding capacity due to saturation or dysfunction of transferrin (22). If non-protein-bound iron is present, the lower detection limit is 0.6 μM. The glass tubes used to collect the blood did not contain detectable amounts of iron. The intra- and interassay coefficients of variation of the bleomycin assay were 6.6 and 7.4%, respectively.

HPLC techniques were used to determine the following antioxidants: reduced and oxidized ascorbic acid (ascorbic acid and dehydroascorbic acid, respectively) (23), uric acid and its oxidation product allantoin (24), and α-tocopherol and retinol (25). Plasma sulfhydryl content was determined spectrophotometrically (25). The lipid peroxidation product malondialdehyde was measured using high performance liquid chromatography (26).

**Experimental protocol.** After completion of the surgical preparation, the lambs were allowed to achieve hemodynamic stability and wash out their ketamine, to exclude an effect of ketamine on the brain (27). The period between ketamine medication and the start of the experiment was always at least 3 h. The skin incisions were sprayed with 1% lidocaine at regular intervals. After having reached steady state (MABP, heart rate), blood samples were taken from the jugular vein catheter to determine the various indicators of the redox status, from the aorta to determine the arterial blood gases and pH, and from the femoral vein to determine the Hb, before and after 15, 60, 120, and 180 min after the HI insult. An additional arterial blood sample was taken at 2 min after completion of the HI insult to determine pH and blood gases to assess the magnitude of HI-induced metabolic acidosis. The HI insult was established by ventilating the lamb with 6–8% oxygen and 10% carbon dioxide (supplemented with nitrogen) for 30 min, followed by 5 min of hypotension (MABP < 35 mm Hg) achieved by careful withdrawal of blood. Upon completion of the HI insult, six lambs received an i.v. infusion with a placebo (0.1 N HCl supplemented with 30 mL of 0.9% NaCl; CONT group), six lambs received low dose NLA (10 mg/kg of NLA with 0.1 N HCl dissolved in 30 mL of 0.9% NaCl; NLA-10 group), and six lambs received high dose NLA (40 mg/kg of NLA with 0.1 N HCl dissolved in 30 mL of 0.9% NaCl; NLA-40 group). Resuscitation was performed in principle in a way similar to that used routinely in our neonatal intensive unit. Extraambient oxygen was progressively decreased, depending on the color of the tongue and the arterial blood, and blood gases were determined 2 min after completion of the HI insult; cardiac arrest and hypotension were treated with adrenalin (1:10 000) and/or dopamine when appropriate. The blood withdrawn to achieve hypotension was reinfused immediately after the completion of the HI period. Metabolic acidosis was corrected with NaHCO$_3$ (see above).

**Statistical analysis.** Data are summarized as means ± 1 SD or ± SEM. Differences between pre-HI values and values of MABP and blood gases immediately after completion of the HI...
RESULTS

Physiologic measurements. There were no differences between the three study groups with respect to animal weight or postnatal age. MABP and carotid artery blood flow decreased to extremely low values at the end of the 5-min hypotensive period. Lowest MABP and carotid artery blood flow values (means ± SEM) during this 5-min period did not differ between groups [CONT group: 28 ± 2 mmHg/12 ± 3 mL.min⁻¹ (pre-HI: 70 ± 8 mL.min⁻¹); low dose NLA group: 27 ± 1 mmHg/13 ± 3 mL.min⁻¹ (pre-HI: 66 ± 4 mL.min⁻¹); and high dose NLA group: 24 ± 2 mmHg/11 ± 2 mL.min⁻¹ (pre-HI: 62 ± 7 mL.min⁻¹)]. Table 1 summarizes the mean values (±1 SD) of MABP, arterial pH, Pco₂, Po₂, and base excess during the respective time points, including 2 min post-H. Although MABP and Po₂ did not differ between groups during the study, MABP in the NLA-40 lambs increased, as expected, to 89 ± 15 mm Hg 60 min post-H, compared with pre-HI values.

There were no relevant differences among groups for hemodynamic parameters or pH and blood gas values, except the rather low Pco₂-values in the NLA-10 group at 180 min post-H. There was no difference among the groups with respect to the amount of bicarbonate infused to treat the severe metabolic acidosis in the immediate post-H period. Hemoglobin was not different within or among groups during the various time points.

Prooxidative activity. The patterns of non-protein-bound iron concentrations of the three groups (mean values ± SEM) are shown in Figure 1. Although extremely low, non-protein-bound iron was detectable during the pre-HI condition in five, four, and five animals of the CONT, NLA-10, and NLA-40 groups, respectively. Non-protein-bound iron increased significantly in all three groups post-HI with highest values at 60 and 120 min post-H, but remained significantly higher in the CONT group compared with pre-HI values and the NLA-10 and NLA-40 groups at 180 min post-H. The two NLA groups showed no significant difference at 180 min post-HI compared with the pre-HI values.

Antioxidative capacity. Figure 2 summarizes the patterns of the mean values (± SEM) of the Ascorbic/dehydroascorbic acid-ratio (AA/DHA-ratio), sulfhydryl groups and α-tocopherol during the study periods. Contrary to the pattern of both NLA groups, the patterns of AA/DHA ratio and α-tocopherol in the post-HI period of the CONT group indicate consumption of these antioxidants: Ascorbic acid decreased and dehydroascorbic acid increased significantly in the CONT group post-HI compared with the pre-HI values, indicated by a consistent decrease of the AA/DHA-ratio during the post-HI period [pre-HI: 5.1 ± 0.9; 60 min post-HI: 2.2 ± 0.9 (p < 0.05 versus pre-HI), and 2.9 ± 0.7 180 min post-HI]. This was not the case for the NLA groups, in which the ascorbic acid, dehydroascorbic acid, and AA/DHA ratio remained stable during the post-HI period, and were not significantly different.

Table 1. Mean values ± 1 SD of MABP, pH, and blood gases, and base excess (BE) of the CONT, NLA-10, and NLA-40 groups during the respective conditions, including 2 min after HI insult.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-HI</th>
<th>2 min</th>
<th>15 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
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<tr>
<td>MABP (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CONT</td>
<td>81 ± 13</td>
<td>70 ± 34</td>
<td>81 ± 16</td>
<td>79 ± 28</td>
<td>78 ± 20</td>
<td>76 ± 17</td>
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<tr>
<td>NLA-10</td>
<td>79 ± 20</td>
<td>69 ± 25</td>
<td>84 ± 16</td>
<td>76 ± 17</td>
<td>85 ± 14</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>NLA-40</td>
<td>71 ± 11</td>
<td>67 ± 20</td>
<td>80 ± 16</td>
<td>89 ± 15*</td>
<td>76 ± 14</td>
<td>83 ± 11</td>
</tr>
<tr>
<td>pH</td>
<td>7.38 ± 0.07</td>
<td>6.99 ± 0.09*</td>
<td>7.02 ± 0.19*</td>
<td>7.19 ± 0.20</td>
<td>7.23 ± 0.16</td>
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<td>NLA-10</td>
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<td>6.90 ± 0.11*</td>
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<td>7.23 ± 0.07</td>
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<td>7.05 ± 0.09*</td>
<td>7.10 ± 0.13*</td>
<td>7.16 ± 0.14</td>
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<td>Pco₂ (kPa)</td>
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<td>7.8 ± 7.3</td>
<td>5.3 ± 2.1</td>
<td>4.3 ± 1.4</td>
<td>4.4 ± 1.1</td>
<td>4.8 ± 0.9</td>
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<td>10.4 ± 3.0*</td>
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<td>5.6 ± 1.4</td>
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<td>NLA-40</td>
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<td>10.7 ± 2.5*</td>
<td>5.6 ± 1.4</td>
<td>5.8 ± 1.9</td>
<td>4.8 ± 3.9</td>
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<tr>
<td>Po₂ (kPa)</td>
<td>16.3 ± 4.8</td>
<td>20.0 ± 7.3</td>
<td>22.5 ± 9.6</td>
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<td>15.4 ± 0.9</td>
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<td>14.2 ± 2.6</td>
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<td>11.8 ± 1.2</td>
<td>16.2 ± 2.6</td>
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<td>NLA-40</td>
<td>13.2 ± 3.7</td>
<td>17.2 ± 9.8</td>
<td>21.8 ± 7.2</td>
<td>12.3 ± 3.2</td>
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<td>BE</td>
<td>−3.9 ± 3.8</td>
<td>−19.7 ± 4.6*</td>
<td>−12.1 ± 1.3*</td>
<td>−9.6 ± 4.2*</td>
<td>−12.6 ± 5.7*</td>
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<td>−18.7 ± 2.7*</td>
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<td></td>
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<td>−18.9 ± 2.1*</td>
<td>−16.2 ± 2.5*</td>
<td>−12.7 ± 3.7*</td>
<td>−9.3 ± 2.0*</td>
</tr>
</tbody>
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* p < 0.05 vs pre-HI values.
Figure 1. Mean values ± SEM of plasma concentrations of non-protein-bound iron of the CONT, NLA-10, and NLA-40 groups at the various time points. *p < 0.05 vs CONT; f p < pre-HI.

from the pre-HI values. In the CONT group α-tocopherol showed a significant and consistent decrease during the post-HI period compared with pre-HI values (pre-HI: 3.4 ± 0.5 μM; 120 min post-HI: 2.3 ± 1.2 μM (p = 0.05), and 180 min post-HI: 1.9 ± 0.3 μM, p < 0.05). In the NLA groups, however, α-tocopherol values remained stable. Sulfhydryl groups showed no significant changes or differences within groups during the various time points or among groups respectively, although the CONT group showed a transient (nonsignificant) drop from pre-HI (278 ± 23 μM) to 15 min post-HI (239 ± 21 μM).

Uric acid, allantoin, and its ratio (allantoin/uric acid), and retinol (not shown) remained stable in all groups during the study period. Respective mean values (±SEM) of the allantoin/uric acid-ratio ranged from 2.4 ± 0.4 to 3.2 ± 0.2 in the CONT group; from 3.5 ± 1.0 to 6.2 ± 0.5 in the NLA-10 group; and from 2.7 ± 0.4 to 5.1 ± 1.2 in the NLA-40 group. Respective ranges of retinol were: CONT group: 0.72 ± 0.19 to 0.85 ± 0.20 μM; NLA-10 group: 0.55 ± 0.05 to 0.79 ± 0.17; NLA-40 group: 0.83 ± 0.11 to 1.11 ± 0.18.

Lipid peroxidation. Figure 3 summarizes the pattern of malondialdehyde of the three groups (mean values ± SEM). Malondialdehyde did not differ significantly during the post-HI period compared with pre-HI values in any group. However, values tended to increase in the CONT group but tended to be lower in both NLA groups, in particular in the NLA-40 group. At 120 min post-HI, malondialdehyde was significantly lower in both NLA groups (NLA-10: 0.46 ± 0.03 μM, p < 0.05; NLA-40: 0.44 ± 0.01 μM, p < 0.01) compared with the CONT group (0.61 ± 0.04 μM).

DISCUSSION

Ventilation with 6–8% of oxygen and subsequent withdrawal of blood caused severe metabolic acidosis (as indicated by the grossly abnormal blood gases determined immediately after completion of HI), severe hypotension, and hypofusion of the brain, making it very likely that hypoxia and ischemia of the brain took place during the actual HI period. The present study indicates that post-HI reperfusion and reoxygenation in our newborn lambs induced an increased prooxidative activity by liberation of iron. This was most obvious in the CONT group, where non-protein-bound iron showed the highest values. These values in the CONT group remained significantly higher compared with pre-HI values and the NLA groups, which showed a decrease to pre-HI values from 120 min post-HI onward. In the CONT group, there was also a reduction of the antioxidative capacity. In particular, we found a consistent increased oxidation of ascorbic acid, which is a sensitive indicator of oxidative stress (28), and a steady decrease in the antioxidant α-tocopherol in the CONT group.
Our finding that in most of the newborn lambs low concentrations of non-protein-bound iron were already available during the first 24 h of life. In addition, the post-HI period. Inhibition of nitric oxide production with a competitive analog of L-arginine, the precursor of nitric oxide, NLA, mitigated these adverse effects on prooxidative activity and the consumption of the antioxidants ascorbic acid and α-tocopherol of the newborn lamb. This attenuating effect of NLA was not dose-dependent, as indicated by the similar results obtained in low and high dose NLA groups. Although malondialdehyde did not significantly change in the post-HI period compared with pre-HI values in any group, it was higher in the CONT group compared with the NLA groups (120 min post-HI, p < 0.05 vs CONT). However, it is important to realize that malondialdehyde is a rather unstable marker of lipid peroxidation in the in vivo situation. Moreover, it measures only lipid peroxidation, whereas proteins and DNA are more often the targets of oxidative damage than are lipids. It is also important to realize that lipid peroxidation often occurs late in the oxidative injury process (26).

In 1991 Beckman (6) highlighted the fact that post-HI reperfusion and reoxygenation induced the production of large amounts of superoxide, hydrogen peroxide, and nitric oxide in neonatal brain tissue and in the cerebral microcirculation. Although these substances are relatively poorly reactive free radical species themselves, superoxide and nitric oxide are able to form peroxynitrite, which can decompose to form the powerful and cytotoxic oxidants hydroxyl and nitrogen dioxide. These oxidants are highly diffusible and can easily cross the blood-brain barrier to exert their destructive action on brain tissue itself (6, 28). Peroxynitrite itself is able to initiate lipid peroxidation and react directly with sulfhydryl groups at physiologic pH values (29). Moreover, superoxide and hydrogen peroxide can be converted into the hydroxyl radical by transition metal ions, in particular non-protein-bound iron, by the so-called superoxide-driven Fenton reaction (4). Siesjo (30) showed that lowering of the plasma pH, as occurs during and after ischemia, enables transferrin to liberate its iron-inducing free radical production, whereas a recent study from our group (31) showed that 12 out of 15 severely birth-asphyxiated term neonates had substantial concentrations of non-protein-bound iron in their plasma during the first 24 h of life. In addition, nitric oxide also reacts with transition metals (e.g. iron), releasing them from their binding proteins (32). It is conceivable that the above mentioned cascade may have contributed to the excessive production of non-protein-bound iron which further increased lipid peroxidation. This suggestion is strongly supported by our finding that inhibition of nitric oxide synthesis with NLA had an attenuating effect on non-protein-bound iron levels measured in blood effluent from the brain and preserved antioxidative capacity by preventing oxidation of ascorbic acid and consumption of α-tocopherol. In this respect it is important to stress the findings of a recent in vitro study of Van Der Vliet et al. (28). They reported that peroxynitrite formation in body fluids is likely to cause antioxidant depletion and oxidative damage, but that peroxynitrite leads in particular to rapid peroxidation of ascorbic acid and to a lesser extent to a decrease in α-tocopherol. This is in line with the results of the present study. The mechanism of the reaction of ascorbic acid with peroxynitrite is not yet clear, but it has been suggested that it reacts with hydroxyl and/or nitrogen dioxide, the decomposition products of peroxynitrite, rather than with peroxynitrite itself (28). Although α-tocopherol was significantly decreased at 180 min post-HI, it has been reported that this antioxidant may be recycled by ascorbic acid, which prevents further decreases (33, 34). The remarkably steady sulfhydryl levels (although a rather impressive, albeit insignificant, decrease of sulfhydryl levels was found in the CONT group at 15 min post-HI compared with pre-HI levels) may be explained by the addition of quite large amounts of bicarbonate in the immediate post-HI period to treat metabolic acidosis and to restore arterial pH. Bicarbonate protects sulfhydryl from oxidation (35) and will also scavenge peroxyl radicals (28).

Our finding that in most of the newborn lambs low concentrations of non-protein-bound iron were already available during the baseline condition was remarkable because this is usually not the case in healthy adult animals and adult humans (36, 37). It confirms the results of recent studies in apparently healthy human (preterm) newborns, in which substantial levels of non-protein-bound iron are measured during the early neonatal period (37). Although not very likely, we cannot exclude the possibility that the experimental instrumentation caused an oxidative stress leading to liberation of protein bound iron.

Finally a short comment should be made on the two different NLA-regimes: one group received low dose NLA (10 mg/kg), the other group received high dose NLA (40 mg/kg). We aimed to determine whether or not partial rather than total nitric oxide synthesis inhibition had a more favorable effect on the redox status of blood effluent from the brain after severe hypoxia and ischemia. As already suggested by earlier studies (15–17), we speculated that our low dose NLA regimen could prevent excessive production of reactive oxygen species and at the same time allowed a sufficient blood supply to critical regions of the brain, whereas the high dose regimen prevented adequate brain perfusion by total inhibition of nitric oxide production with consequent constriction of the cerebral vascular bed, eventually leading to more production of reactive oxygen species. In a previous report we commented on the pattern of electrical cortical brain activity of the studied animals during the post-HI period. Only in the low dose NLA group electrical cortical brain activity recovered to pre-HI values, whereas it remained significantly lower compared with pre-HI values in
the CONT group and the high dose NLA group (38). In the present study, however, we did not find consistent differences between the low and high dose NLA groups with respect to the redox status of blood effluent from the brain.

In summary, after a hypoxic and ischemic insult in newborn lambs substantial levels of non-protein-bound iron were measured. Moreover, antioxidative capacity was decreased in this post-HI period as indicated by a decrease in AA/DHA ratio, indicating oxidation of ascorbic acid and decreased α-tocopherol levels. The abnormal production of non-protein-bound iron was reduced, and the decrease in AA/DHA ratio and α-tocopherol levels was prevented in the NLA-treated lambs. This suggests that inhibition of nitric oxide may have a beneficial effect on the excessive formation of reactive oxygen upon reperfusion and reoxygenation after hypoxia and ischemia. The present study, however, did not support earlier findings suggesting that partial rather than complete inhibition of nitric oxide production was superior, because we did not find a difference in results with respect to the redox status between high dose and low dose NLA treatment.

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REFERENCES