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The Role of Oxygen Free Radicals in Postasphyxia Cerebral Hypoperfusion in Newborn Lambs

ADAM A. ROSENBERG, ELIZABETH MURDAUGH, AND CARL W. WHITE

Department of Pediatrics, Sections of Neonatology and Pulmonology, University of Colorado School of Medicine, Denver, Colorado 80262

ABSTRACT. Previous work in a neonatal lamb model has demonstrated abnormalities in cerebral blood flow (CBF) and oxygen consumption ($CMRO_2$) after asphyxia. Immediately after resuscitation, there was a marked increase in CBF and a significant decrease in $CMRO_2$ compared to control. During the late period after asphyxia (30 min to 4 h), both CBF and $CMRO_2$ were significantly depressed. The same postasphyxia model ($n = 16$) was used to examine the hypothesis that generation of oxygen free radicals during cerebral reperfusion may be involved in the genesis of late postasphyxia hypoperfusion and depressed $CMRO_2$. Before asphyxia, the animals were pretreated with either inactivated ($n = 8$) or active ($n = 8$) polyethylene glycol superoxide dismutase, 5000 U/kg, and polyethylene glycol catalase, 100 000 U/kg. CBF (radioactive microspheres) and arterial and venous (superior sagittal sinus) blood gases and O_2 contents were measured during control, and at 5 min, 1 h, 2 h, and 4 h postasphyxia (PA). In the active enzyme group, 5 min postasphyxia CBF was significantly increased compared to control: 211.5 ± 28.0 *versus* 78.6 ± 11.4 ml·100 g^{-1}·min^{-1}, ±SEM, $p < 0.005$. At 1 h (82.9 ± 17.6), 2 h (62.3 ± 5.5), and 4 h (78.9 ± 12.2) PA, CBF did not differ significantly from control. More importantly, $CMRO_2$ did not differ from control at any time PA. In the inactive enzyme group, both CBF and $CMRO_2$ were depressed at 1, 2, and 4 h PA. These findings are consistent with a conclusion that damage by oxygen free radicals during postasphyxia cerebral reperfusion is important to the genesis of late PA blood flow and O_2 metabolism abnormalities. To the extent that depressions in CBF and $CMRO_2$ result in ongoing brain injury, agents that ameliorate these abnormalities may improve neurologic outcome. (*Pediatr Res* 26:215–219, 1989)

Abbreviations

CBF, cerebral blood flow
$CMRO_2$ cerebral oxygen consumption
PA, postasphyxia
OFR, oxygen free radicals
PEG, polyethylene glycol
CaO_2, arterial oxygen content

Work in a newborn lamb PA model has demonstrated abnormalities in PA, CBF and $CMRO_2$ (1). In the immediate PA period, there was a state of reactive hyperemia. Despite this overperfusion, $CMRO_2$ was depressed. In the late period after asphyxia (30 min to 4 h) there was a significant depression of CBF and $CMRO_2$. To the extent that these abnormalities of CBF and $CMRO_2$ may contribute to brain injury, mechanisms involved in the genesis of cerebral hypoperfusion and decreased $CMRO_2$ become important.

The postischemic production of OFR (*e.g.* superoxide ion, hydroxyl radical, and singlet oxygen) during reperfusion has been recognized to be important in the pathogenesis of injury in a variety of organ systems (2). In brain, OFR have been implicated in vascular injury seen with both acute hypertension and in a fluid percussion injury model (3–6). Furthermore, lipid peroxidation (7) and production of the superoxide anion (8, 9) have been demonstrated in the brain after reperfusion from an ischemic insult. During ischemia, the brain concentration of hypoxanthine has been shown to increase (10–12) and the concentration of arachidonic acid to rise (13). When the tissue is reperfused, OFR can be generated from the oxidation of hypoxanthine (2) and as a by product of arachidonate metabolism through cyclooxygenase and lipoxygenase pathways (14, 15).

Using the newborn lamb PA model we sought to examine the hypothesis that generation of OFR during cerebral reperfusion plays a role in the genesis of late PA hypoperfusion and depressed $CMRO_2$. The role of the OFR was studied by treating two groups of lambs before asphyxia with active or inactivated PEG-superoxide dismutase and PEG-catalase.

MATERIALS AND METHODS

Surgical procedure. A total of 16 newborn lambs were operated on under pentobarbital anesthesia on d 1–3 of life. Polyvinyl chloride catheters (0.034 in ID × 0.054 in OD; Martech Medical Products, Lansdale, PA) were placed in the left ventricle via an axillary artery, the brachiocephalic artery via an axillary artery, the abdominal aorta via a femoral artery, the inferior vena cava via a femoral vein, and the posterior sagittal sinus proximal to the confluence of the veins. The sagittal sinus catheter was placed through a 1 inch in diameter burr hole in the midline proximal to the lambdoidal sutures. The catheters entering through the animal's extremities were protected in a pouch on the abdomen. The sagittal sinus catheter was cut, pinned, and sutured to the lamb's scalp. The animals were returned to their mothers and allowed a 24-h recovery period before study. At that time, all lambs were standing and feeding normally. Previous work has demonstrated this is an adequate time interval to eliminate any pentobarbital effect on CBF (16).

Physiologic measurements. CBF was measured using the reference organ radiolabeled microsphere technique as previously described (1, 16–18). Withdrawal of the reference organ through the brachiocephalic artery catheter was into a counting vial by a

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Correspondence and reprints Adam A. Rosenberg, M.D., Container B195, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262. Supported by NIH Grant R01-HL36301.

precalibrated pump (2.47 mL·min^{-1}; Harvard Apparatus, Dover, MA). After completion of the study, animals were killed with T-61 Euthanasia Solution (American Hoechst, Summerville, NJ), position of the catheters were checked, and the brain was removed. Brains were placed in formalin for 1 wk and then divided into regions as previously described (1). Regions examined were brainstem (medulla and pons), right and left cerebellum, midbrain/diencephalon, frontal lobes, temporal lobes, occipital lobes, and parietal lobes. The radioactivity in each sample was determined using a three-channel γ-counter (Tracor Analytic, Des Plaines, IL) and regional blood flows calculated as previously described (16). Whole brain flow was calculated using the sum of the radioactive counts and weights for all regions except brainstem and cerebellum. Adequate central mixing of microspheres using a left ventricle injection site has been confirmed in the newborn lamb (17). All reference blood samples and tissue samples (except white matter) contained more than 400 microspheres (19). White matter had 200 to 400 microspheres.

Blood samples for pH, P_{CO_2}, P_{O_2}, and O_2 content were withdrawn anaerobically into heparinized Natelson glass pipettes from the brachiocephalic artery and sagittal sinus catheters. P_{O_2}, P_{CO_2}, and pH were measured at 39.5°C using the radiometer BMS3 MK2 (Radiometer, Copenhagen, Denmark). Blood Hb concentration expressed at O_2 capacity and O_2 saturation were measured colorimetrically in duplicate by a hemoximeter (Radiometer), and O_2 content was calculated as the product of Hb and O_2 saturation. Blood pressure (referenced to the right atrium) and heart rate were continuously monitored in the abdominal aorta (Gould Instruments, Oxford, CA).

Experimental procedure. On the day of study, the animals were anesthetized with fentanyl (20 μg/kg loading dose followed by 10 μg/kg/min infusion), paralyzed with pancuronium (0.1 mg/kg), intubated and ventilated with an infant ventilator (Bird, Co., Palm Springs, CA) with a baseline gas mixture of 30–35% O_2 and 65–70% N_2 to provide a P_{aO_2} of 10.7–16.0 KPa, and a baseline ventilator rate of 25–35 breaths/min to provide a P_{aCO_2} of 4.4–5.1 KPa. Pancuronium and Fentanyl have been shown at the doses utilized in this study to have no effect on CBF and $CMRO_2$ (20, 21).

Measurements of CBF (radiolabeled microspheres), arterial and venous blood gases, and O_2 contents were made during the control period. Eight animals then received 100 000 U/kg of PEG-catalase and 5000 U/kg of PEG-superoxide dismutase intravenously, whereas eight received inactivated PEG enzymes. The enzyme doses were extrapolated from studies in rats using free and liposome entrapped enzymes to treat pulmonary and brain oxygen toxicity (22–24). The animals were then subjected to a gradual asphyxial insult by altering inspired gas concentrations and ventilator rate as previously described (1, 21). In a stepwise fashion over a 30-min period, P_{aO_2} was lowered to 2–2.66 KPa, C_{aO_2} was lowered to 0.67–1.12 mM, and P_{aCO_2} was increased to 8–9.33 KPa. Over the first 60 min of asphyxia, heart rate and blood pressure were stable, but during the final 10–15 min the lambs became bradycardic (heart rate <100 beats/min) and hypotensive (mean arterial blood pressure, MAP 20–35 mm Hg). The lambs were then returned to baseline ventilator settings and fractional inspired oxygen concentration. CBF (microspheres) arterial and venous blood gases, and O_2 contents were measured at 5 min, 1 h, 2 h, and 4 h after the termination of the insult. After the 4-h measurement, the animals were killed. Care and handling of animals was in accord with the published guidelines of the National Institutes of Health and has been approved by the Animal Care Committee of the University of Colorado.

Enzyme inactivation. PEG superoxide dismutase was irreversibly inactivated by its reaction with an excess of hydrogen peroxide that specifically oxidizes histidyl groups at the active site (25). After enzyme inactivation, excess H_2O_2 was removed by repeated dialysis. PEG-catalase was similarly inactivated in the presence of excess hydrogen peroxide and 3-amino-1,2,4-triazole that were then removed by dialysis (26, 27). After dialysis, each enzyme preparation was filtered (0.2 μ pore size) to assure sterility.

Data analysis. $CMRO_2$, cerebral O_2 delivery, and fractional O_2 extraction were calculated as previously described (16, 28). Comparisons were made for cerebral hemodynamic and other physiologic variables among control and the 4 PA determinations within each experimental group (active and inactive enzymes) using ANOVA with a repeated measures design. Regional flow data from the right and left sides of the brain were combined before analysis as no left-right differences were seen. Significance compared to control was determined at the $p < 0.05$ level. CBF and $CMRO_2$ data are also presented as percent of control measurements for both the active and inactive enzyme groups.

RESULTS

Changes in CBF over time are presented in Figure 1. The data are presented as percent of the control measurements. In both groups, CBF was markedly increased 5 min PA. In the active enzyme group CBF did not differ from control at 1 h, 2 h, and 4 h PA whereas CBF was significantly decreased at these times in the inactive enzyme group. Regional flow data demonstrated the same pattern of changes as seen in whole brain for both groups (Table 1). In the active enzyme group no significant decreases in flow were present 1, 2, and 4 h PA, whereas flow was decreased in all regions at 1, 2, and 4 h PA in the inactive enzyme group. Figure 2 presents changes in $CMRO_2$ over time in both groups with the data again presented as percent of the

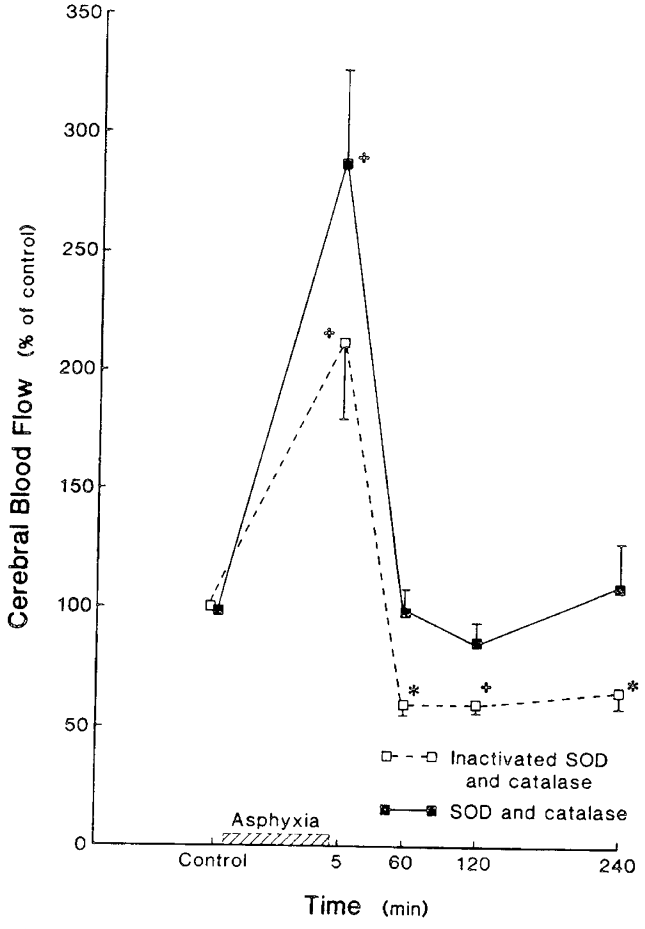

Fig. 1. CBF (% of control) versus time after asphyxia for both active (■) and inactive (□) enzyme groups. Postasphyxia values are mean ± SEM. Statistical analysis by repeated measures design ANOVA within each group. *$p < 0.01$ from control within group;† $p < 0.005$ from control within group.

Table 1. *Regional brain flood flows**

	Control	5 min PA	1 h PA	2 h PA	4 h PA
Inactive enzyme					
Brainstem	94.2 ± 12.0	266.2 ± 41.7†	54.4 ± 5.3‡	47.0 ± 1.4†	52.8 ± 4.7‡
Cerebellum	100.7 ± 11.8	263.5 ± 28.1†	70.0 ± 3.8‡	63.8 ± 1.8†	72.0 ± 7.7§
Midbrain/diencephalon	98.7 ± 11.6	272.5 ± 52.9†	58.4 ± 5.8‡	53.5 ± 2.3†	59.8 ± 5.4‡
Frontal cortex	86.7 ± 8.0	182.0 ± 20.4†	48.0 ± 2.7†	47.2 ± 2.5†	50.3 ± 3.5‡
Temporal cortex	66.7 ± 7.3	174.2 ± 23.1†	35.0 ± 1.9†	34.7 ± 2.0†	39.0 ± 3.1‡
Occipital cortex	92.3 ± 8.2	185.2 ± 19.2†	56.0 ± 3.6‡	55.2 ± 3.4‡	58.5 ± 4.0‡
Parietal cortex	89.8 ± 7.9	183.2 ± 20.4†	53.2 ± 3.6‡	52.5 ± 2.6†	57.0 ± 3.2‡
Active enzyme					
Brainstem	72.5 ± 12.2	291.3 ± 52.0†	102.9 ± 18.6	61.5 ± 7.4	84.3 ± 10.3
Cerebellum	85.6 ± 12.4	328.0 ± 41.5†	105.4 ± 15.2	90.8 ± 10.3	102.8 ± 10.9
Midbrain/diencephalon	79.4 ± 11.5	307.1 ± 58.2†	100.9 ± 15.6	72.8 ± 8.3	85.3 ± 16.6
Frontal cortex	77.5 ± 11.1	211.8 ± 27.6†	75.3 ± 14.6	61.5 ± 5.2	80.7 ± 8.7
Temporal cortex	58.6 ± 7.1	179.9 ± 27.8†	62.1 ± 15.7	48.9 ± 4.6	63.3 ± 7.4
Occipital cortex	85.2 ± 12.5	218.6 ± 30.3†	86.6 ± 18.3	69.4 ± 5.8	91.2 ± 12.0
Parietal cortex	81.3 ± 10.2	211.0 ± 27.5†	83.2 ± 17.4	65.5 ± 5.6	84.3 ± 11.0

* All values are mean ± SEM in ml·100 g^{-1}·min^{-1}.
† $p < 0.005$.
‡ $p < 0.01$.
§ $p < 0.05$.

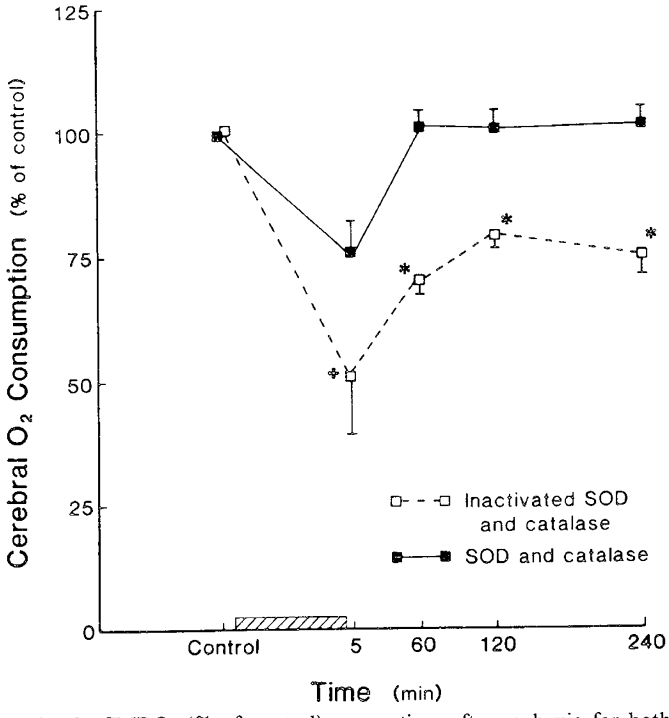

Fig. 2. $CMRO_2$ (% of control) versus time after asphyxia for both active (■) and inactive (□) enzyme groups. Postasphyxia values are mean ± SEM. Statistical analysis by repeated measures design ANOVA within each group. * $p < 0.05$ from control within group;† $p < 0.01$ from control within group.

control measurements. With inactive enzyme, $CMRO_2$ was depressed below control at all times PA, whereas $CMRO_2$ did not differ from control in the active enzyme group. Other cerebral hemodynamic variables for both the active and inactive enzyme groups are presented in Table 2. Cerebral O_2 delivery, the product of CBF × CaO_2, mirrors changes in CBF. In both groups, oxygen delivery is significantly increased compared to control 5 min PA. In the active enzyme group, oxygen delivery does not differ from control at 1, 2, and 4 h PA whereas significant decreases from control are present at these times in the inactive enzyme group. Fractional O_2 extraction, the relationship between O_2 uptake and delivery, is significantly decreased 5 min PA, and no different from control at 1, 2, and 4 h PA in both groups. Tables 3 and 4 present physiologic variables for the inactive and active enzyme groups. In each group, the only differences over time were an increase in Pao_2 5 min PA and a decrease in pH from control at 5 min, 1 h, and 2 h PA. In neither animal group did variables known to influence cerebral hemodynamic data; $Paco_2$, Cao_2, and mean arterial blood pressure change over time.

DISCUSSION

Although Myer's (29) elegant studies have detailed clinical and pathologic changes seen with neonatal asphyxia and emphasized the role of hypoxic ischemic insults in brain damage, little information is available in a neonatal model describing mechanisms of damage incurred during the PA period. The importance of postischemic events has been emphasized by work in several ischemia models. Neuronal damage in the neocortex and hippocampus of rats exposed to four-vessel occlusion worsened for hours to days after relatively brief forebrain ischemia (30). Furthermore, CNS damage has been minimized by posthypoxic ischemic pharmacologic interventions designed to improve CBF (31–33). The current study demonstrated that OFR scavenging enzymes prevent PA late hypoperfusion and depressed $CMRO_2$ in the newborn lamb. These findings are consistent with the hypothesis that damage by OFR during PA reperfusion may be important to the genesis of PA abnormalities in CBF and $CMRO_2$. The newborn lamb PA model has been used previously to study physiologic and metabolic changes after asphyxia (1, 21, 34). However, pathologic studies have not yet been performed to assess brain parenchymal or vascular injury in the model. Nonetheless, to the extent that depressions of CBF and $CMRO_2$ might contribute to and/or reflect PA brain injury, these findings may be important.

There are several mechanisms to consider that allow OFR scavengers to improve PA CBF and $CMRO_2$. The late hypoperfusion seen in the control lambs may be the result of cerebral edema impinging on capillaries impairing blood flow (35). OFR have been shown to increase endothelial permeability to macromolecules (36), however, previous work in the newborn lamb has failed to demonstrate cerebral edema during the period of late hypoperfusion (21). Alternatively, increased production of vasoconstrictor arachidonate metabolites (*e.g.* thromboxane A_2 or leukotrienes) potentiated by O_2 metabolites may be etiologic

Table 2. *Cerebral hemodynamic variables**

	Control	5 min PA	1 h PA	2 h PA	4 h PA
Inactive enzyme					
OD ($\mu M \cdot 100\ g^{-1} \cdot min^{-1}$)	611 ± 64	1359 ± 187†	380 ± 49†	384 ± 21†	394 ± 17‡
Fractional O_2 extraction	0.43 ± 0.04	0.07 ± 0.01†	0.51 ± 0.05	0.53 ± 0.03	0.48 ± 0.03
Active enzyme					
OD ($\mu M \cdot 100\ g^{-1} \cdot min^{-1}$)	600 ± 86	1650 ± 238†	667 ± 133	513 ± 52	596 ± 100
Fractional O_2 extraction	0.40 ± 0.05	0.13 ± 0.04‡	0.40 ± 0.06	0.42 ± 0.03	0.40 ± 0.05

* All values are mean ± SEM. OD, cerebral oxygen delivery.
† $p < 0.005$.
‡ $p < 0.01$.

Table 3. *Physiologic variables—inactivated SOD/CAT**

	Control	5 min PA	1 h PA	2 h PA	4 h PA
Pa_{O_2} (kPa)	15.3 ± 1.0	19.9 ± 0.8†	15.0 ± 1.1	14.4 ± 0.9	14.5 ± 0.7
Pa_{CO_2} (kPa)	4.47 ± 0.13	3.87 ± 0.15	4.20 ± 0.17	4.53 ± 0.16	4.40 ± 0.19
pH	7.47 ± 0.02	6.95 ± 0.03‡	7.16 ± 0.05†	7.26 ± 0.05†	7.35 ± 0.03
Ca_{O_2} (mM)	6.79 ± 0.67	7.28 ± 0.80	7.10 ± 0.63	7.28 ± 0.58	6.92 ± 0.49
MAP (mm Hg)	83 ± 7	98 ± 5	86 ± 3	83 ± 4	73 ± 2
HR (bpm^{-1})	235 ± 21	250 ± 0	275 ± 19	281 ± 22	250 ± 17

* All values are mean ± SEM. MAP, mean arterial blood pressure; HR, heart rate, 1 mm Hg, 0.1333 kPa.
† $p < 0.05$ from control.
‡ $p < 0.01$ from control.

Table 4. *Physiologic variables—SOD/CAT**

	Control	5 min PA	1 h PA	2 h PA	4 h PA
Pa_{O_2} (kPa)	15.6 ± 0.7	19.1 ± 0.8†	15.3 ± 0.6	14.5 ± 0.8	14.4 ± 0.8
Pa_{CO_2} (kPa)	4.67 ± 0.24	4.00 ± 0.33	4.48 ± 0.36	4.67 ± 0.20	4.75 ± 0.23
pH	7.43 ± 0.03	6.94 ± 0.05‡	7.18 ± 0.04†	7.26 ± 0.03†	7.30 ± 0.03
Ca_{O_2} (mM)	7.68 ± 0.22	7.68 ± 0.22	8.17 ± 0.27	8.26 ± 0.27	7.59 ± 0.31
MAP (mm Hg)	80 ± 5	88 ± 5	81 ± 5	79 ± 6	69 ± 5
HR (bpm^{-1})	250 ± 11	254 ± 15	276 ± 8	288 ± 9	293 ± 19

* All values are mean ± SEM. MAP, mean arterial blood pressure; HR, heart rate; 1 mm Hg, 0.1333 kPa.
† $p < 0.05$ from control.
‡ $p < 0.01$ from control.

to late hypoperfusion (37, 38). The other attractive explanation for decreased CBF at 1–4 h PA is that OFR damage cerebral vessels causing a vasoconstricted state. Vascular injury due to OFR has been seen in other brain injury models (3–6).

The decrease in $CMRO_2$ seen in the control lambs could be the result of impaired mitochondrial function as OFR have been shown to inhibit mitochondrial respiration (39). In the newborn lamb PA model, inhibition of mitochondrial respiration is present 5 min PA, but recovery of function toward baseline is seen by 2 h PA (34). Thus the improved $CMRO_2$ after treatment with superoxide dismutase and catalase at 5 min PA may be due to improved mitochondrial function, but another explanation must be sought for the increased $CMRO_2$ at 1–4 h PA. Alternatively, the higher $CMRO_2$ in the treated animals 1–4 h PA may simply be the result of the increased CBF. This concept would be supported by the normal relationship between CBF and $CMRO_2$, the fractional O_2 extraction, 1, 2, and 4 h PA in both active and inactive enzyme groups.

The data in the current study are consistent with work in other newborn as well as adult postischemia/asphyxia models. In adult dogs the combination of superoxide dismutase and deferoxamine (to decrease OFR production) improved post cardiac arrest CBF, $CMRO_2$ and recovery of somatosensory evoked potentials (40). In an exteriorized fetal sheep model, the combination of OFR scavengers and a calcium channel blocker also resulted in improved PA CBF and better recovery of somatosensory evoked potentials (41). However, in the latter study the role of of OFR scavengers alone in improved CBF and neurologic function cannot be confirmed because calcium channel blockers alone have been shown to improve postischemic CBF (31).

PEG superoxide dismutase and catalase were chosen for this study because the PEG linkage blocks renal clearance of the enzymes allowing active enzyme to circulate for long periods of time. Modification by PEG increases the circulating $t_{½}$ from 6 min to 30–40 h in the rat (42). The inert characteristics of PEG also reduce the immunogenicity of the enzymes and inhibits hydrolysis of protease sensitive proteins such as catalase (43). For these reasons a continuous infusion was not necessary. Furthermore, conjugation of the enzyme to PEG enhances enzyme uptake by cultured endothelial cells (44). The combination of the two enzymes was selected to scavenge both the superoxide ion as well as hydrogen peroxide. Both superoxide and hydrogen peroxide are implicated in the formation of the most reactive and toxic OFR, hydroxyl radical, through the Haber-Weiss reaction. It is the hydroxyl radical in particular that has been implicated as the damaging species in other brain injury models (3–6). Finally, it is worth noting that PEG has been shown itself to be a hydroxyl radical scavenger (22). For that reason, inactivated PEG enzymes were used in the control group. No independent effect of PEG was evident in the current study as the inactive enzyme group demonstrated similar PA changes in CBF and $CMRO_2$ as historic controls (1).

Another important issue is site of action of the superoxide dismutase and catalase and the source of production of OFR. Entry into the CNS of high mol wt enzymes would require a disruption in the blood brain barrier. Such may be the case PA.

Another possibility is that the enzymes act locally at the vessel wall. This seems likely because it has been demonstrated the cerebral vessel walls can produce OFR from arachidonate metabolism (45), and that the enzyme xanthine dehydrogenase is known to be present on the surface of brain endothelial cells (46). This enzyme is converted to xanthine oxidase during ischemia and with reperfusion metabolizes hypoxanthine and oxygen releasing OFR. It is also possible that the enzymes operate at non-central nervous system sites. Circulating xanthine oxidase and its substrate have been demonstrated in humans with adult respiratory distress syndrome (47). These circulating OFR could be the source of tissue injury with the PEG enzymes offering protection by scavenging these circulating OFR. The enzymes could also indirectly improve PA cerebral perfusion by preventing myocardial injury and improving PA cardiac output. This seems unlikely because previous work on this model (1) (Rosenberg AA, unpublished observations) did not demonstrate decreased cardiac output compared to control at 1, 2, and 4 h PA.

In conclusion, the present study has shown an improvement in postasphyxia CBF and $CMRO_2$ in the newborn lamb after treatment with OFR scavenging enzymes. To the extent that these abnormalities in CBF and $CMRO_2$ are important to the genesis of brain injury, agents that correct these abnormalities may be of use in this clinical setting.

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