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Resuscitation with 100% O_2 Increases Cerebral Injury in Hypoxemic Piglets

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ABSTRACT

Perinatal asphyxia is a major cause of immediate and postponed brain injury in the newborn. We hypothesized that resuscitation with 100% O_2 compared with ambient air is detrimental to the cerebral tissue. We assessed cerebral injury in newborn piglets that underwent global hypoxia and subsequent resuscitation with 21 or 100% O_2 by extracellular glycerol, matrix metalloproteinase (MMP) expression levels, and oxidative stress. Extracellular glycerol was sampled by cerebral microdialysis. MMP levels were analyzed in cerebral tissue by gelatin zymography, broad matrix degrading capacity, and real-time PCR. Total endogenous antioxidant capacity was measured by the oxygen radical absorbance capacity assay. Extracellular glycerol increased 50% after resuscitation with 100% O_2 compared with 21% O_2. Total MMP activity was doubled in resuscitated animals at endpoint compared with baseline ($p = 0.018$), and the MMP-2 activity was significantly increased in piglets that were resuscitated with 21% O_2 ($p = 0.003$) and 100% O_2 ($p = 0.001$) compared with baseline. MMP-2 mRNA level was 100% increased in piglets that were resuscitated with 100% O_2 as compared with 21% O_2 ($p < 0.05$). Oxygen radical absorbance capacity values in piglets that were resuscitated with 100% O_2 were considerably reduced compared with both baseline ($p = 0.001$) and piglets that were resuscitated with 21% O_2 ($p = 0.001$). In conclusion, our data show increased MMP-2 activity at both gene and protein levels, accompanied with cerebral leakage of glycerol, presumably triggered by augmented oxidative stress. Our findings suggest that resuscitation of asphyxiated piglets with 100% O_2 is detrimental to the piglet brain compared with resuscitation with 21% O_2. (***Pediatr Res* 56: 783–790, 2004**)

Abbreviations

ECM, extracellular matrix
MABP, mean arterial blood pressure
MMP, matrix metalloproteinase
ORAC, oxygen radical absorbance capacity
$Paco_2$, arterial carbon dioxide tension
PE, phycoerythrin
RFU, relative fluorogenic unit

Traditionally, asphyxiated newborn infants have been resuscitated using 100% oxygen in the delivery room (1). However, clinical trials have shown that room air is as efficient as pure oxygen in securing the survival of asphyxiated newborn infants (2–5). Therefore there is an ongoing debate whether to use ambient air or 100% O_2 in neonatal resuscitation (6–9).

Hyperoxia followed by reoxygenation is thought to increase the production of reactive oxygen species and disrupt the antioxidant mechanisms (10). Elevated oxidative stress can directly influence the cell cycle and additionally alter a number of significant cell functions, such as signal transduction, DNA and RNA synthesis, protein synthesis, and enzyme activation (11,12). Furthermore, hypoxic-ischemic injury and reactive oxygen species are found to trigger inflammation in the immature brain (13).

Interstitial glycerol is a sensitive and reliable marker of cell damage in experimental cerebral ischemia (14–16). Degradation of membrane phospholipids is a well-known phenomenon in acute brain injuries and is thought to underlie the disturbance of vital cellular membrane functions.

Matrix metalloproteinases (MMPs) are a family of >20 zinc- and calcium-dependent endopeptidases that are involved in the remodeling of the extracellular matrix (ECM) in a variety of physiologic and pathologic conditions (17). Most MMPs are secreted as proenzymes and are activated by cleav-

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age of their propeptide to lower molecular weight active forms (18). MMPs can degrade many components of the ECM, and enzyme activities are strictly regulated (19). Tissue inhibitors of MMP and interactions with surrounding ECM molecules tightly regulate proenzyme activation and enzyme activities. Uncontrolled expression of MMPs can result in nervous tissue injury and inflammation (20,21). Several studies have documented the up-regulation of MMPs and their activation in animal models of focal and global cerebral ischemia (22–27) and in humans (28). In the brain, MMP-2 and MMP-9 are synthesized primarily by reactive astrocytes and microglia (27,29). MMP-2 production and secretion are enhanced after prolonged hypoxia in cell culture, whereas reoxygenation further increases the level of MMP-2 (30). MMP-2 and -9 have been shown to degrade components of the cerebral basal lamina after ischemia (24), causing a disruption of the blood-brain barrier (31). In the pathologic condition of ischemia/reperfusion, digestion of the endothelial basal lamina is reported to occur as early as 2 h after ischemia (26).

In term newborn brain, cerebral oxygen demands are increased in areas of active neural development such as the basal ganglia and thalamus (32). Corpus striatum becomes selectively vulnerable in the hypoxic term piglet model. In a piglet model of hypoxic-ischemic brain injury in newborns, striatal neuron necrosis evolves rapidly (33). We have investigated changes in corpus striatum in a global hypoxia piglet model, imitating birth asphyxia, as in several of our earlier studies (34,35). Especially the cerebral ischemia after a hypoxia-induced myocardial failure is most harmful (36) because permitting the mean arterial blood pressure (MABP) to fall to 15 mm Hg also induces cerebral perfusion disturbances.

The main aim of this study was to explore whether resuscitation with 100% O_2 compared with ambient air increases the acute cerebral damage after global hypoxia in a piglet model. Birth asphyxia is often associated with hypercapnia, and P_{CO_2} influences cerebral blood flow (37,38). Therefore, we also investigated effects of different P_{CO_2} levels.

METHODS

Approval. The Norwegian Council for Animal Research approved the experimental protocol. The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals.

Surgical preparation and anesthesia

Sixty-nine Landrace piglets (12–36 h old) were transported (1 h) from a local farmer on the day of the experiment. Anesthesia was induced by Halothane 4% (Fluthane ZENECA); an ear vein was cannulated, halothane was disconnected, and the piglets were given pentobarbital sodium 20 mg/kg and Fentanyl 50 μg/kg intravenously as a bolus injection. Anesthesia was maintained by a continuous infusion of Fentanyl (50 $\mu g \cdot kg^{-1} \cdot h^{-1}$) and Midazolam (0.25 $mg \cdot kg^{-1} \cdot h^{-1}$; IVAC P2000 infusion pump). When necessary, a bolus of Fentanyl (10 μg) or Midazolam (1 mg) was added. A continuous i.v. infusion (saline 0.7% and glucose 1.25%, 20 $mL \cdot kg^{-1} \cdot h^{-1}$) was given throughout the experiment.

Tracheotomy was performed, and a pressure-controlled ventilator (Babylog 8000+; Drägerwerk, Lübeck, Germany) ventilated the piglets at a rate of 30 breaths/min. Normoventilation [arterial carbon dioxide tension ($Paco_2$) 4.5–6.0 kPa] and a tidal volume 10–15 mL/kg were achieved by adjusting the peak inspiratory pressure or ventilatory rate. During surgery, stabilization, and hypoxia, ventilatory rate was 30–40 respirations/min. Inspiratory time of 0.4 s and positive end-expiratory pressure of 4 cm H_2O were kept constant throughout the experiment. Inspired fraction of O_2 and end-tidal CO_2 were monitored continuously (Datex Normocap Oxy; Datex, Helsinki, Finland).

The right femoral artery was cannulated with polyethylene catheters (Portex PE-50, inner diameter 0.58 mm; Portex Ltd Hythe, Kent, UK). Rectal temperature was maintained between 38 and 40°C with a heating blanket and a radiant heating lamp. One hour of stabilization was allowed after surgery. At the end of the experiment, the piglets were given an overdose of 150 mg/kg pentobarbital intravenously.

Experimental protocol. Hypoxemia was achieved by ventilation with a gas mixture of 8% O_2 in N_2 (AGA, Oslo, Norway), until either MABP reached 15 mm Hg or base excess reached −20 mM. For imitating birth asphyxia, CO_2 was added during hypoxemia aiming at a $Paco_2$ of 8.0–9.5 kPa. Before start of resuscitation, the piglets were block randomized into six different groups by drawing lots. Resuscitation was performed with either ambient air (group A) or 100% O_2 (group B). Nine piglets, referred to as baseline piglets, were controls that went through surgery and 1 h of stabilization.

We divided each main group that was resuscitated with 100% (A) or 21% (B) oxygen in three subgroups ventilated with low (A1 and B 1), normal (A2 and B2), or high (A3 and B3) CO_2 level during resuscitation. The piglets in group 1 (A1, $n = 10$; B1, $n = 10$) were hyperventilated and had a low $Paco_2$ of 2.0–3.5 kPa. In group 2 (A2, $n = 10$; B2, $n = 10$), the piglets were resuscitated in a normal ventilatory modus ($Paco_2$: 4.5–6.0 kPa). The animals in group 3 (A3, $n = 10$; B3, $n = 10$) were normoventilated and had added CO_2 to reflect a condition of hypercapnia ($Paco_2$: 8.0–9.5 kPa). For hyperventilating the piglets (group 1), peak inspiratory pressure and ventilatory rate were elevated and adjusted after evaluating end-tidal CO_2 and blood gases. We resuscitated with ambient air or pure oxygen for 30 min in accordance with our previous studies. After resuscitation, the piglets were reoxygenated for 150 min by 21% O_2 and normal CO_2 (4.5–6.0 kPa), which was the same for all groups.

Hb with normal values was measured at baseline and after surgery (7.7 ± 0.18 g/dL; CO-OXIMETER 270; Instrumentation Laboratory, Lexington, MA). Temperature-corrected acid/base status and blood sugar were measured regularly with a Blood Gas Analyzer 860 (Ciba Corning Diagnostics, Midfield, MA), and the i.v. infusion was occasionally adjusted to keep s-glucose in the decided range (2–10 mM).

The head was fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). The skull was exposed, and a 2.0-mm hole was drilled through the skull 5.5 mm laterally and 10 mm anteriorly to the bregma in the right hemisphere. The dura was carefully pierced, and a microdialysis probe (CMA

10 20/04, outer diameter 500 μm, cut-off 20.000 D) was inserted stereotactically through the hole into the corpus striatum area (19–21 mm from the surface of the skull, depending on body weight). All CMA equipment was purchased from CMA/Microdialysis AB (Stockholm, Sweden). Microdialysis probes sampled extracellular glycerol throughout the experiment, and this was analyzed immediately (35). Because of contamination of the vials, microdialysis results were excluded in two animals (one in A2 and one in B1 resulting in $n = 29$ in both main groups). At the end of the experiment, the piglets were given an i.v. overdose of 150 mg/kg pentobarbital. The brains were removed immediately; corpus striatum was snap-frozen in liquid nitrogen and stored at −70°C.

Preparation of cerebral tissue extracts. For zymography and total MMP activity, tissue from corpus striatum from the left hemisphere was pulverized under liquid nitrogen, and proteins were extracted using ice-cold lysis buffer [Tris-HCl (pH 7.5) containing protease inhibitor cocktail without EDTA with 1% NP-40] at a ratio of 500 μL/50 mg wet weight tissue. Extracts were incubated on ice for 15 min and then centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatants were retained, and protein concentration of the samples was measured by the BCA method (Pierce, Cheshire, UK).

For the oxygen radical absorbance capacity (ORAC) assay, cerebral tissue was pulverized under liquid nitrogen, and 50 mg of frozen material was added to 1.0 mL of ice-cold 75 mM of K_2HPO_4/NaH_2PO_4 phosphate buffer (pH 7.0). The tissue samples were homogenized using a Polytron (PT 1200; Kinematica AG, Lucerne, Switzerland) at speed 1 for 30 s under nitrogen flush and centrifuged at 16,000 × *g* for 5 min at 4°C (Biofuge A; Hereaeus-Christ, Hanau, Germany). The pellet was resuspended in homogenization buffer and centrifuged as described above. The two supernatants were combined in a volumetric flask, filled up to 2.0 mL of total volume, and used for the ORAC assay.

Total MMP activity. Total MMP activity in tissue extracts was measured using a fluorogenic peptide substrate (cat. no. ES001; R&D Systems) according to the protocol recommended by the manufacturer. The substrate can be cleaved by MMP-1, -2, -7, -8, -9, -12, and -13. The term "total MMP activity" refers to the total activity of these enzymes and is measured by relative fluorogenic unit (RFU). Because of the limited amount of tissue, data from three animals were not obtained (two in group A and one in group B; A: $n = 28$; B: $n = 29$).

Gelatin zymography. Equal amounts (10 μg) of cerebral tissue protein extracts were loaded onto the electrophoretic gel and assayed for gelatinase activity using 10% SDS–polyacrylamide gel (containing 0.1% gelatin), with minor modifications according to the methods described elsewhere (39). Human MMP-2 and MMP-9 standards (CC073; Chemicon, Temecula, CA) were used. The gels were stained with Coomassie blue R-250 and subsequently destained before being scanned in an imager (Kodak Image Station 440CF). The images were analyzed using software from Total Lab v2.01. The results were calculated by densitometry, normalized to a sample used as an internal standard on every zymography run. When the gelatin gel was incubated with 200 μM of EDTA, no lysis zones were detected, demonstrating that the metal-dependent lysis zones were most likely the result of gelatinase activity (40). Cerebral MMP-9 expression could not be detected by gelatin zymography. Because of tissue limitations, three animals were excluded (one at baseline, one in A2, and one in B2).

Real-time quantitative reverse transcriptase–PCR. Total RNA was extracted from the tissue samples as described by the manufacturer, using Trizol (Invitrogen). The isolated RNA was treated with DNase (cat. No. M6101; Promega). Reverse transcription was performed according to the manufacturer's protocol (cat. no. N808-0234; Applied Biosystems) with 125 ng of RNA per 50 μL of reverse transcriptase reaction. Sequence-specific PCR primers and TaqMan probes for MMP-2 were designed using the Primer Express software version 1.5 (Applied Biosystems). Real-time quantitative reverse transcriptase–PCR assays for pig MMP-2 (forward primer: 5'-GTG GTG CGT GTG AAG TAT GG-3'; reverse primer: 5'-GCC ATC CTT GTC GAA GTT GT-3'; TaqMan probe: FAM 5'-AGC TGT TAT ACT CCT TGC CGT T-TAMRA-3') were designed using the Primer Express software version 1.5 (Applied Biosystems). The housekeeping gene 18S (Applied Biosystems) was included as an endogenous normalization control to adjust for unequal amounts of RNA. Quantification of mRNA was performed using the ABI Prism 7700 (Applied Biosystems). Each sample (each reaction: 2.5 μL of cDNA, total volume 25 μL) was run in triplicate. Cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Standard curves were run on the same plate, and the relative standard curve method was used to calculate the relative gene expression (Livak KJ; ABI Prism 7700 Sequence Detection System, User Bulletin 2, PE Applied Systems).

The predeveloped 18S assay from Applied Biosystems is designed for rodent and human. The probe therefore was tested by running a standard curve to verify that the predeveloped 18S assay was suitable for pig. The product was also run on a 1% agarose gel to make sure that there were no nonspecific products present.

ORAC assay. The ORAC assay was performed as described elsewhere (41) but with minor modifications. Measurements were performed on a Wallac 1420 Victor^2 96-well plate reader (Wallac Oy, Turku, Finland) with fluorescence filter. Excitation wavelength was 540 nm, and emission wavelength was 570 nm. The fluorescence probe was β-phycoerythrin (β-PE), and peroxyl radical generator was 2,2'-azobis (2-amidinopropane) dihydrochloride. 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E, was used as control standard. All reagents and samples were diluted with phosphate buffer (75 mM, pH 7.0), and the reaction was conducted at 37°C. Each sample was analyzed in duplicate at four concentrations using a "forward-then-reverse" ordering in each plate row. Trolox and blanks were applied at both ends of each row in the 96-well plate to improve reproducibility of the fluorescence measurements. Total volume in each well was 0.2 mL. The plate was preheated to 37°C, and to each well, 10 μL of tissue extracts, Trolox (20 μM, final concentration 1 μM) or blank (phosphate buffer), and 180 μL of β-PE (3.78 mg/L) were added. The plate was shaken for 30 s

(speed 3.3 mm orbital), and initial fluorescence was measured. The reaction was started by adding 10 μL of 2,2'-azobis (2-amidinopropane) dihydrochloride (80 mM) to each well and shaking the plate for 10 s. Measurements were taken every 3 min until the fluorescence was <5% of the initial reading. All fluorescent measurements were expressed relative to the initial reading. Results were calculated by using the area differences under the β-PE decay curves between the blank and a sample. The final results (ORAC value) were calculated by linear regression of areas *versus* sample concentration and expressed as micromol Trolox equivalents (TE) per gram of cerebral tissue (μmol TE/g). ORAC was measured in the normoventilated piglets A2 and B2 only. Because of loss of tissue, four piglets were excluded (two at baseline, one in group A2, and one in group B2).

Statistics. Statistical analysis was performed by SPSS11. For studying the relationship between glycerol, MMP-2 and total MMP-activity as dependent variables, and O_2 and CO_2 as independent variables, univariate ANOVA was used. For accommodating for multiple comparisons, the p values were adjusted according to Bonferroni. Comparing within two groups, independent t test was used. All values are given as mean ± SEM. A level of $p < 0.05$ was considered statistically significant.

RESULTS

Piglets. There were no significant differences between the groups with respect to the number of animals, body weight, age, sex, rectal temperature, cardiovascular, or biochemical variables at baseline. Furthermore, hypoxemia time, MABP, base excess, and pH were similar between groups at baseline, at the end of the insult, and at the end of the experiment (Tables 1 and 2). Extracellular glycerol and MMP-2 activity was not influenced by the P_{CO_2} level; hence, the subgroups were combined.

Microdialysis. Extracellular glycerol concentration in the piglets that were resuscitated with ambient air (group A, $n = 29$) and 100% O_2 (group B, $n = 29$) are shown in Figure 1. The values were 50% higher when resuscitation was done with 100% O_2 compared with ambient air at 90, 120, and 150 min after resuscitation ($p < 0.05$, independent sample t test at each time point).

Total MMP activity. Broad matrix degrading capacity was examined to study whether any differences in total MMP activity could be seen between the normal controls at baseline ($n = 9$) and the piglets at the end of the experiment [$n = 28$ (A) and $n = 29$ (B)]. Total MMP activity was 2-fold higher in resuscitated animals compared with controls (10169 ± 815 *versus* 5790 ± 1412 RFU; $p = 0.018$).

Gelatinolytic activity. Gelatin zymography was applied on cerebral extracts to determine the gelatinolytic activity of MMP-2 (Fig. 2). Gelatinolytic activity was detected at 68 kD, corresponding to MMP-2 proenzyme (42). MMP-2 activity was significantly higher in resuscitated piglets ($n = 58$) compared with baseline ($n = 8$). MMP-2 activity was also significantly higher in piglets that were resuscitated with 100% O_2 ($n = 29$) compared with ambient air ($n = 29$, $p = 0.001$).

mRNA expression of MMP-2. MMP-2 activity was not influenced by the P_{CO_2} level ($p = 0.4$; Fig. 3); hence, the mRNA and ORAC were analyzed in the normoventilated groups A2 and B2. To elucidate further the mechanism behind the augmented MMP activity, we measured the mRNA expression of MMP-2 by quantitative real-time PCR. As shown in Figure 4, mRNA expression of MMP-2 was higher in the normoventilated group that was resuscitated with 100% O_2 ($n = 9$) than in the normoventilated group that was resuscitated with ambient air ($n = 10$, $p < 0.05$).

ORAC

Total antioxidant capacity, denoted as ORAC values, was measured in cerebral tissue extracts from corpus striatum at baseline and in the normoventilated (A2 and B2) piglets (Fig. 5). ORAC was considerably lowered in the piglets that were resuscitated with 100% O_2 ($n = 9$) compared with baseline ($p = 0.001$), indicating that total antioxidant capacity was lowered during the experiment. By contrast, ORAC response was not decreased in piglets that were resuscitated with ambient air ($n = 9$) compared with baseline ($n = 7$). Piglets that were resuscitated with 100% O_2 had mean ORAC values that were 50% lower than the ones that were resuscitated with 21% O_2 ($p = 0.001$).

DISCUSSION

The findings of the current study indicate that resuscitation of piglets with 100% O_2 after global hypoxia significantly increases cerebral extracellular glycerol and MMP-2 levels. These changes were concomitant with a marked reduction of total endogenous antioxidant capacity assessed by ORAC. Furthermore, cerebral leakage of glycerol reflecting brain damage increased 50% more after resuscitation with 100% O_2 compared with resuscitation with ambient air. The glycerol data obtained at 90, 120, and 150 min after onset of resuscitation indicate that resuscitation with 100% O_2 adds further cerebral damage after global hypoxia. To our knowledge, it has not been previously reported that MMP-2 activity and MMP-2 mRNA in newborn animals are more up-regulated after resuscitation with 100% O_2 than with ambient air. In adult animals,

Table 1. *Weight and hypoxia time*

	Group A 21%			Group B 100% O_2		
	A1 ($n = 10$)	A2 ($n = 10$)	A3 ($n = 10$)	B1 ($n = 10$)	B2 ($n = 10$)	B3 ($n = 10$)
Weight (kg)	1.9 ± 88	1.6 ± 77	1.6 ± 89	1.8 ± 126	1.7 ± 99	1.8 ± 98
Hypoxemia time (min)	63 ± 6	75 ± 8	56 ± 8	61 ± 7	65 ± 5	67 ± 11

All values are mean ± SEM.

Table 2. *Physiological data at baseline, start of resuscitation (end of insult), 30 min of resuscitation, and end of experiment (endpoint)*

	Group A 21%			Group B 100% O_2		
	A1 (n = 10)	A2 (n = 10)	A3 (n = 10)	B1 (n = 10)	B2 (n = 10)	B3 (n = 10)
MABP (mm Hg)						
Baseline	72 ± 4	71 ± 6	74 ± 6	78 ± 3	74 ± 4	71 ± 4
Start of resuscitation	23 ± 3	21 ± 3	23 ± 4	20 ± 3	26 ± 5	20 ± 2
30 min of resuscitation	48 ± 5	43 ± 6	49 ± 7	58 ± 5	49 ± 5	44 ± 4
Endpoint	50 ± 4	46 ± 5	47 ± 4	51 ± 4	52 ± 5	53 ± 3
BE (mmol/L)						
Baseline	1 ± 2	4 ± 1	3 ± 2	1 ± 1	2 ± 1	4 ± 1
Start of resuscitation	−22 ± 1	−20 ± 1	−16 ± 2	−21 ± 1	−18 ± 1	−22 ± 1
30 min of resuscitation	−16 ± 1	−15 ± 1	−12 ± 2	−14 ± 1	−14 ± 1	−17 ± 2
Endpoint	−1 ± 1	−3 ± 2	−0 ± 1	−2 ± 2	−2 ± 1	−1 ± 1
pH						
Baseline	7.45 ± 0.01	7.43 ± 0.02	7.43 ± 0.01	7.44 ± 0.01	7.43 ± 0.02	7.45 ± 0.01
Start of resuscitation	6.92 ± 0.04	6.91 ± 0.01	7.10 ± 0.07	6.92 ± 0.01	6.96 ± 0.02	6.88 ± 0.03
30 min of resuscitation	7.30 ± 0.01	7.20 ± 0.01	7.10 ± 0.03	7.32 ± 0.03	7.20 ± 0.03	6.99 ± 0.10
Endpoint	7.39 ± 0.01	7.38 ± 0.02	7.41 ± 0.01	7.37 ± 0.02	7.39 ± 0.01	7.39 ± 0.01

All values are mean ± SEM. BE, base excess.

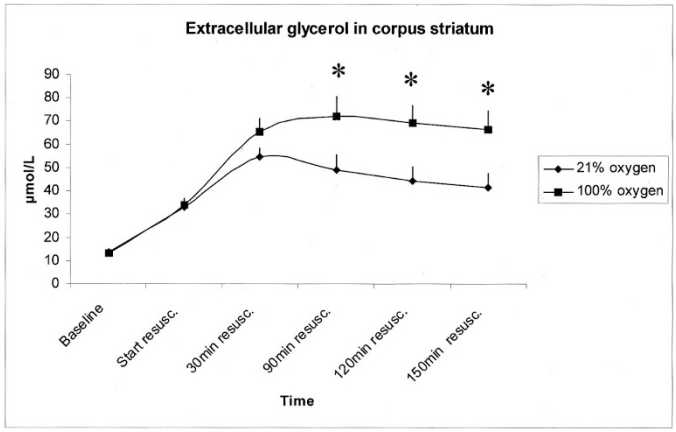

Figure 1. Microdialysate of extracellular fluid from corpus striatum during reoxygenation. Cerebral extracellular glycerol concentration was not significantly different in any of the groups (n = 29) at baseline (p = 0.5) or at start of resuscitation (p = 0.8). Glycerol level was significantly higher after resuscitation with 100% O_2 compared with 21% O_2 at 90, 120, and 150 min after resuscitation, suggesting an increased cellular damage after resuscitation with 100% O_2. Glycerol level was not significantly different related to P_{CO_2} level at 90, 120, or 150 min reoxygenation ($p > 0.05$). Results are presented as mean ± SEM; $^{**}p < 0.001$ 100% *vs.* 21% O_2.

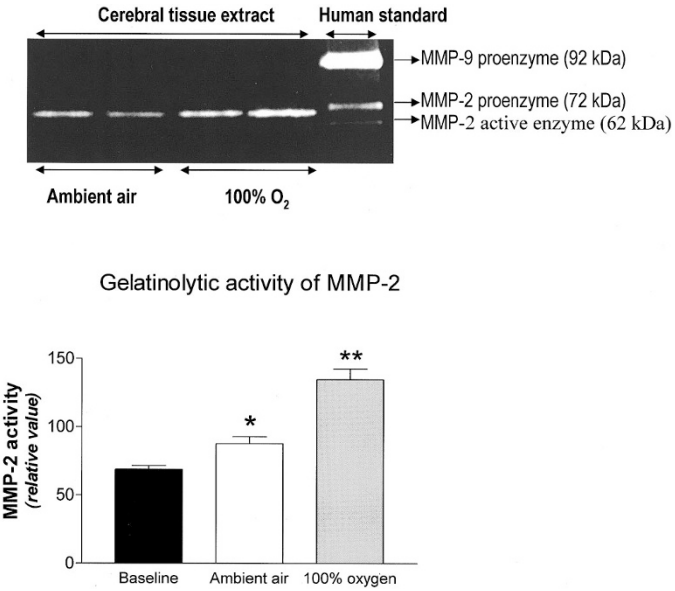

Figure 2. Gelatinolytic activity of MMP-2 measured by zymography in brain extracts. (*Top*) A representative zymogram demonstrating MMP-2 activity in brain extracts after resuscitation with 21% (n = 29) or 100% O_2 (n = 29), human standard to the right. (*Bottom*) MMP-2 activity compared with baseline (n = 8) was significantly increased in piglets that were resuscitated with 21% O_2 and 100% O_2. Results are presented as mean ± SEM; $^{*}p < 0.05$, 21% O_2 *vs* baseline; $^{**}p < 0.001$, 100% O_2 *vs* baseline.

abnormal MMP activity has been implicated in cerebral ischemia (43). MMP-2 and MMP-9 expression levels were significantly increased soon after the ischemic onset (23,24,44). MMP expression after hypoxia might contribute to debris digestion, edema formation, blood-brain barrier breakdown (22,23), and hemorrhagic transformation (25), although some beneficial contribution of MMPs in neuronal plasticity and tissue regeneration cannot be ruled out (45,46).

The applied ORAC assay provides significant information regarding the antioxidant capacity of various biologic tissue samples (41,47,48). We chose this assay because it directly measures a broad spectrum of different types of antioxidant activities over a given time span, representing a relevant *in vivo* situation (41). In normoventilated piglets that were resuscitated with ambient air, we did not observe a significant change of the ORAC. In contrast, a marked reduction (50%) of ORAC was found in piglets that were resuscitated with 100% O_2 compared with the ones that were resuscitated with ambient air and baseline. This indicates that these piglets have less total antioxidant capacity left in cerebral tissue. Free radicals have previously been reported to induce gene expression of several MMPs (49). That MMP-2 mRNA level was significantly higher in piglets that were resuscitated with 100% O_2 compared with 21% O_2 suggests that higher levels of free radicals not only could be responsible for posttranslational modifications of MMPs but also could affect their transcriptional regulation. It is interesting that these changes occur early after reoxygenation, placing MMPs upstream in the pathologic cascade of events that may lead to more conspicuous brain damage across time. In this context, further studies should be

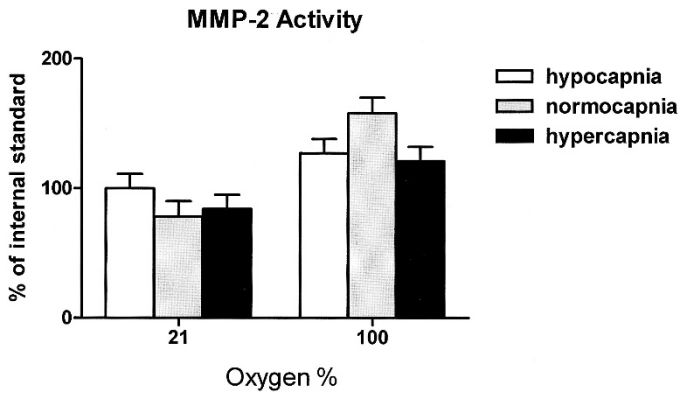

Figure 3. MMP-2 zymography. MMP-2 activity was measured in cerebral tissue extracts at baseline and endpoint of the experiment. MMP-2 activity at baseline ($n = 8$) was 69 ± 3% of baseline. There were significant differences between MMP-2 activity and different oxygen concentrations ($p < 0.001$), but there were no significant differences between P_{CO_2} levels and MMP-2 activity ($p = 0.4$). Hypocapnia = low P_{CO_2} = 2.0–3.5 kPa ($n = 10$); normocapnia = normal P_{CO_2} = 4.5–6.0 kPa ($n = 9$); hypercapnia = high P_{CO_2} = 8.0–9.5 kPa ($n = 10$). All values are given as mean ± SEM.

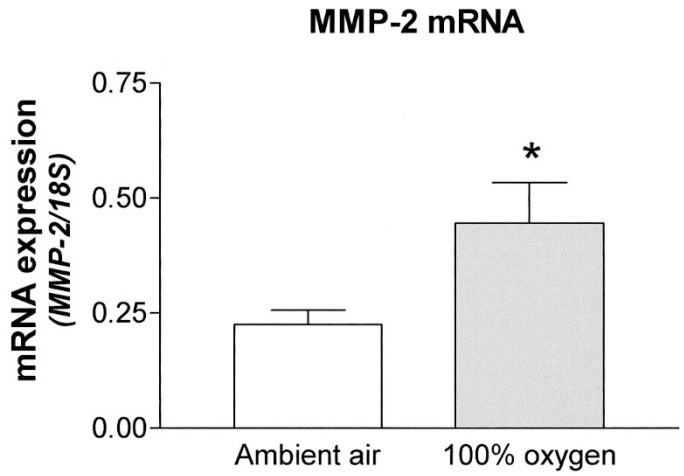

Figure 4. Cerebral mRNA expression of MMP-2 in normoventilated piglets that were resuscitated with ambient air ($n = 10$) or 100% O_2 ($n = 9$). mRNA MMP-2 levels were significantly increased in the piglets that were resuscitated with 100% O_2 compared with 21% O_2. Results are presented as mean ± SEM; $^*p < 0.05$ 100% *vs* 21% O_2.

undertaken to investigate the pathologic and histologic long-term consequences for the brain after resuscitation with 100% O_2.

In our experimental protocol, we did mRNA expression profiling of MMP-2 3 h after hypoxia. Because of our experimental protocol, we unfortunately could not determine the exact time course of MMP-2 mRNA expression during the experiment. However, our ORAC analysis after 3 h revealed that an oxidative stress had occurred during 3 h after hypoxia, causing a significant drop in endogenous antioxidant capacity. This drop was more significant in the 100% group than in the ambient air group. The generation of reactive free radicals is an acute event (30–60 min after hypoxia) (50). Furthermore, we know that activation of different genes, including MMP-2, can be driven by free radicals (51), and it is most likely that a maximum mRNA expression occurs later than 60 min as a result of a gene transcription and processing of mRNA. It could

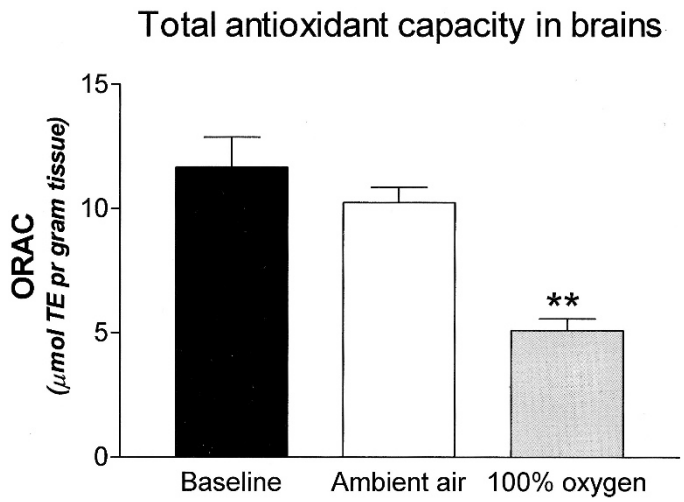

Figure 5. Cerebral endogenous antioxidant capacity measured as ORAC after resuscitation with 21% O_2 ($n = 9$) or 100% O_2 ($n = 9$) in normoventilated piglets. The results are expressed as μmol TE per gram wet weight of tissue samples. The mean ORAC value of tissue from piglets that were resuscitated with ambient air was not significantly different from baseline ($n = 7$). Comparing piglets that were resuscitated with 100% O_2 with baseline and with ambient air, there was a significant lowering of the ORAC value. Results are presented as mean ± SEM; $^{**}p < 0.001$ *vs* both baseline and ambient air.

be claimed that the peak MMP-2 transcript level might be reached between 2 and 3 h after hypoxia or even later (*e.g.* 6–12 h) and that an underestimation of mRNA expression especially in the ambient air group had occurred 3 h after hypoxia. However, our data do not support this. First, our protein expression study of MMP-2 3 h after hypoxia demonstrates a significantly increased MMP-2 level in the 100% oxygen group, compared with the ambient group. Second, significantly higher glycerol level in corpus striatum was found in the 100% group, compared with the ambient air group. These data suggest that more brain damage has occurred in the piglets that were resuscitated with 100% O_2, which at least partly could be due to augmented MMP-2, and we suggest that the elevated generation of free radicals is the driving force in these adverse processes observed in the 100% oxygen group in comparison with the ambient group.

With our outcome variables, there were no significant differences comparing different P_{CO_2} levels during resuscitation. This is in contrast to a study that showed that different CO_2 levels influence cerebral outcome after hypoxia-ischemia (52). In a different model, piglets that were younger than 4 d had a low CO_2 reactivity and did not fully autoregulate in response to MABP changes during the first days of life. At 4 d of age, adult responses have developed in the piglets (53). This may explain the lack of significant differences between the CO_2 groups in our study based on piglets <36 h of age.

One limitation of the study is that the piglets were observed for only 2.5 h after resuscitation. A 24- or 48-h follow-up of the piglets for histologic examination may have been valuable. To investigate neuronal damage in the most vulnerable areas of the piglet brain—basal ganglia and thalamus—it would be necessary to do histologic analysis at least 8 h after resuscitation. However, histologic analysis in other cerebral regions of hypoxic piglets have not demonstrated significant differences

between 21 and 100% oxygen even 4 d after hypoxia (54). Despite this, our study indicates the importance of adjusting down the oxygen level during resuscitation after global hypoxia. In most clinical situations, infants are coupled to pulse oximeter, and oxygen may be lowered when saturation reaches levels that are too high. Thus, the current accepted recommendation to use 100% oxygen in the resuscitation of asphyxiated newborn infants should be discussed and investigated further.

In conclusion, the current study documented a good correspondence between the increased levels of MMP-2 and glycerol, a marker of tissue injury, in a piglet model of global hypoxemia and subsequent resuscitation with ambient air or 100% O_2. Cerebral glycerol values were 50% increased and MMP-2 levels were considerably up-regulated in the piglets that were resuscitated with 100% O_2 compared with ambient air.

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