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Cerebral Inflammatory Response After Fetal Asphyxia and Hyperoxic Resuscitation in Newborn Sheep

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ABSTRACT: Resuscitation with pure oxygen at birth after fetal asphyxia may aggravate brain damage by inducing pro-inflammation. The toll-like receptors (TLRs) may serve a pro-inflammatory role in hyperoxemia during ischemia-reperfusion. Sixteen near-term fetal sheep (132-136 d) were subjected to 10 min of cord occlusion, delivery and mechanical ventilation with 100% O_2 ($n = 8$), or 21% O_2 ($n = 8$) for 30 min followed by normoxemia for 90 min. Eight sheep fetuses were delivered immediately with inspired O_2 targeted at normoxemia for 120 min (controls). Levels and distributions of mRNAs for IL-1β, TNF-α, IL-12p40, IL-18, IL-6, IL-10, IFN-γ, TLR-2, -3 and -4 in cerebral tissue at 2 h after birth were evaluated with real-time polymerase chain reaction (PCR) and *in situ* hybridization. Expressions of IL-1β, IL-12p40, TLR-2, and TLR-4 were increased in cortex/subcortex after resuscitation with 100% O_2 compared with 21% O_2 (all $p < 0.05$) and to controls (all $p < 0.05$). Increased cellular expression of IL-1β was localized to subpmeningeal cortical layers and to sub-cortical white matter. Hyperoxic resuscitation at birth following fetal asphyxia induces a cerebral pro-inflammatory response with an up-regulation of TLR-2 and -4. These may be early events leading to increased tissue damage after exposure to hyperoxemia at birth. **(*Pediatr Res* 62: 71–77, 2007)**

Fetal asphyxia remains an important cause of brain damage with subsequent neuro-developmental impairment. Resuscitation after asphyxia is normally instituted immediately after birth with the aim to restore perfusion and oxygenation of tissues and thus takes place during the transition from the fetal to the newborn state. This transition is characterized by a gradual increase from the normally lower oxygen saturation of the feto-placental circulation to that of the newborn after established breathing. An increasing number of *in vitro* and *in vivo* studies indicate that exposure to pure oxygen after preceding ischemia aggravates tissue damage (1,2). The newborn infant, normally exposed to a gradual increase in oxygen exposure after birth, may be especially vulnerable to hyperoxemia. In support of this, clinical studies have shown that resuscitation with 100% oxygen instead of room air in newborns increases neonatal mortality, increases oxidative stress at 4 wk after birth and increases myocardial and kidney injury (3–5).

Exposure of the immature brain to hyperoxia triggers apoptosis associated with oxidative stress and down-regulation of cell-survival related pathways (6). Increased levels of oxygen induce inflammation with increased levels of cytokines, which are known to be associated with neuro-degeneration. Increased levels of IL-1β and IL-18 have both been causally linked to hyperoxia-induced cell death in the immature rat brain (7).

The essential role of toll-like receptors (TLRs) in the response of innate immunity to infectious ligands has been extensively studied (8). In this context, the TLR system plays a constitutive part in cytokine response and signaling to adaptive immunity. Although less known, the TLR system may be important in tissue response to ischemia and reperfusion without microbial involvement. Endogenous ligands released from damaged tissue have been shown to activate the TLR system with a subsequent increase in inflammatory response. The Toll/IL-1 pathway, specifically the TLR-2 receptor has been implicated in the pro-inflammatory response to necrotic cell death (9). It is not known how hyperoxia during reperfusion may modify the TLR-system after an ischemic insult.

The translation of experimental evidence for damaging effects of oxygen exposure to clinical guidelines for newborn infants requires the use of experimental models, which resemble the sequence of asphyxia and resuscitation present in the human newborn. We have previously described postnatal cerebral metabolism after fetal asphyxia in a sheep model involving the transition from fetal to neonatal life (10). This model mimics important aspects relevant for the study of oxygen exposure following birth. We used this sheep model of fetal asphyxia induced by cord occlusion *in utero* followed by resuscitation instituted after birth followed by postnatal pressure-controlled ventilation and care.

We hypothesized that resuscitation with pure oxygen after asphyxia would increase mediators of inflammatory response in the newborn brain. Additionally we evaluated changes in the TLR-system in association with hyperoxic or normoxic resuscitation after fetal asphyxia.

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Abbreviations: MABP, mean arterial blood pressure; **TLR,** toll-like receptor

MATERIALS AND METHODS

Animals. Observations were made in 24 near-term fetal sheep of mixed breed and gender at mean (range) gestational age of 135 (133-137) d (term = 145 d) and with a mean (SD) weight of 3.4 (0.6) kg. The gestational age was calculated from the conception date. Sixteen sheep fetuses were subjected to fetal cord occlusion, and eight fetal sheep served as sham controls. The study was approved by the Animal Ethics Research Committee, Lund University.

Experimental protocol. The pregnant ewes were intubated after tiopenthal induction and cesarean section was performed during isoflurane anesthesia supplemented by opiate infusion. The ewes were operated on lying in a dorsal position. After a lower midline incision, the fetal head and neck were carefully extracted through a small incision of the uterus. A rubber glove was placed over the head to preclude fetal breathing. Catheters were placed in the axillary artery and in the jugular vein and used for baseline blood sampling. An endotracheal tube was inserted by tracheostomy, excess of lung fluid was allowed to drain passively into a closed system, there after the endotracheal tube was clamped. With the fetus remaining in the uterus, the umbilical cord was gently extracted through the incision of the uterus and ligated. After ligation of the umbilical cord, an additional catheter was positioned in the fetal aorta *via* the umbilical artery and used for continuous monitoring of fetal mean arterial blood pressure (MABP). At 8 minutes after cord ligation the fetuses were delivered, weighed, sedated (see below), and placed on a heated open incubator.

The eight control sheep fetuses were prepared *in utero* with catheters inserted in the axillary artery and jugular vein and tracheostomy as above. Thereafter these lambs were immediately delivered, weighed, sedated (see below), and connected to pressure-controlled ventilation. The umbilical artery was cannulated and the tip of the catheter placed in the aorta for continuous measurement of blood pressure.

Pressure-regulated ventilation was performed using the ventilator Servo 900 C (Siemens-Elema, Solna, Sweden) with initial settings of inspiratory pressure of 25 cm H_2O and post end expiratory pressure of 4 cm H_2O. Thereafter, inspiratory pressure was adjusted to achieve a targeted minute volume of 1.5 L/min and a $PaCO_2$ of 4.5-6 kPa.

In the sixteen sheep subjected to cord occlusion pressure-regulated ventilation was initiated at 10 min after start of cord occlusion. These sheep were randomized to receive an inhaled gas mixture with an oxygen gas mixture of either 21% ($n = 8$) or 100% ($n = 8$) during the first 30 min of ventilation. During the following 90 min inspired oxygen gas mixture was adjusted to achieve normoxemia targeting a Pao_2 of 7–9 kPa and an oxygen-saturation between 90–95% as determined by continuous tail pulse-oximetry (Nellcor N-395, Nellcor Puritan Bennet Inc., Pleasanton, California, USA). The eight control sheep were connected to pressure-regulated ventilation immediately after birth with pressure-settings as described above. Inspired oxygen gas mixture in control sheep was adjusted to achieve normoxemia as defined above.

Sedation and analgesia after delivery were obtained with an initial bolus dose of 20 μg/kg Fentanyl administered i.v. followed by a continuous i.v. infusion of 10 μg/kg/h. Fentanyl was dissolved in 5% Glucose solution in a concentration allowing for an infusion rate of 3 mL/kg/h. The lambs were cared for on a heated open incubator (Dräger, Lubeck, Germany) maintaining a body temperature of 38.5°C. Lamb temperature was monitored *via* a temperature probe positioned in the rectum. Mean arterial blood pressure from the aorta *via* the umbilical catheter, and heart frequency derived from blood pressure oscillations were registered continuously.

Arterial blood-samples (0.5 mL) for blood-gases, Hb and arterial oxygen saturation were obtained from the axillary artery catheter at baseline, end of cord occlusion, at 10, 20, 30, 60, 90, and 120 min after start of ventilation and analyzed on the ABL 700 (Radiometer, Copenhagen, Denmark). The ABL was calibrated for measurements of ovine Hb and measurements were adjusted for actual rectal temperature. Lactate concentration in arterial blood was measured using an amperometric method based on lactate oxidase enzymatic conversion, Lactate Pro (Arkray Inc., Kyoto, Japan).

At 120 min after delivery the sheep were killed by an i.v. injection of potassium chloride. The brain was immediately removed from the scull and separated in the midline. One hemisphere was placed *en bloc* on dry ice for later cryo-section. From the other hemisphere tissue sections were taken from the temporo-parietal cortex including sub-cortical white matter and from the thalamus and frozen on dry ice. Tissue was subsequently stored at −80°C. Tissue sections were cut on a cryostat, thaw-mounted on to silanized slides, and stored at −80°C. Thawing of tissue and slides was avoided to ensure best possible tissue integrity.

RNA Probes and* in situ *hybridization. For the ovine IL-1β and IL-12p40 mRNAs, DNA probes were used corresponding to nucleotides 47-577 (Gene Bank accession number NM_001009465) and nucleotides 120-536 (Gene Bank accession number *NM_001009438*), respectively. The ovine TLR-2, TLR-3, and TLR-4 mRNA probes were synthesized corresponding to nucleotides 1-439 (Gene Bank accession number AY957613), nucleotides 2-327 (Gene Bank accession number AY957614) and nucleotides 1-409 (Gene Bank accession number AY957615), respectively.

DNA templates were generated by PCR from a plasmid containing the gene encoding for IL-1β (kindly provided by Dr. Suhas Kallapur) or from cDNAs by using gene specific primers including modified T3 and T7 extension for subsequent cRNA transcription.

Complementary RNA (cRNA) probes were transcribed from 40 ng of gel-purified DNA template using [α^{35}S]UTP (1300 Ci/mmol; Dupont, NEN, Boston, MA) and either T3 or T7 RNA polymerase according to the manufacturers instruction (Ambion MAXIscript, Huntington, Cambridgeshire, UK) to generate sense and anti-sense probes, respectively.

Tissue sections were fixed, dehydrated and delipidated as previously described (11). Sections were hybridized (18-24 h, 55°C) with 1 × 10 E6 cpm of denatured [35S] cRNA probe per 50 μL hybridization buffer. Following washes to remove excess probe, slides were apposed to Kodak Hyperfilm Biomax MR for 3 d, then coated with nuclear track emulsion (NTB, Kodak). After a 4-wk exposure at 4°C, slides were developed in Dektol (Kodak), fixed and counter stained with a Giemsa stain.

Real-time PCR. Total RNA was extracted from frozen tissue using Trizol® (GIBCO BRL) according to the manufacturer's instructions. Proteoglycan and polysaccharide contaminations were removed by adding isopropanol followed by salt precipitation with 0.8 mM sodium citrate and 1.2 mM sodium chloride. Only RNA samples with visible 18S/28S bands as determined by electrophoresis were included for further analysis.

For cDNA synthesis RNA (0.4 μg) was reverse-transcribed in a final volume of 20 μL using reverse transcription reagents (Applied Biosystems, Roche, Nutley, New Jersey) according to the manufacturer's protocols. The samples were stored at −20°C until further use.

Gene transcripts were quantified using real-time PCR on ABI PRISM® 7000 sequence detection system (Applied Biosystems). Primers and probes were ordered from Assays by-Design ™ (Applied Biosystems). Each primer pair was located on different exons of the investigated gene to avoid amplification of genomic DNA (Table 1), with the exception of the primers for TNF-α. cDNA used to analyze levels of TNF-α was therefore treated with DNase according to the protocol supplied by the manufacturer (Promega, SDS, Falkenberg, Sweden). Exon-exon boundaries for the ovine homologues of the investigated genes were determined by inter-species analysis based on at least three different species using information on sequence and exon-exon boundaries on http://www.ensembl.org/index.html. TaqMan MGB probes for the respective genes were labelled with fluorogenic dye, 6-carboxyfluorescein (FAM) and quenched with a nonfluorescent quencher. PCR reactions were assayed in a 25 μL final volume containing final concentrations: 1 × TaqMan Universal Master Mix (Applied Biosystems), 1 × Assaymix (Applied Biosystems) and 1.6 μL of 20 ng/μL of a DNA aliquot. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. Two negative controls, with vehicle, were included for every probe that was run at one occasion. The assay was tested for amplification in genomic DNA using the same amount of a RNA extraction as template. Each reaction was assayed in duplicate. A calibration curve, obtained by serial 4-fold dilutions of the template DNA (0.08-80 ng), was used to quantify each sample. The quantitative value of each sample was normalized to the corresponding value of beta-actin and results expressed as relative values.

Micrograph and figure preparation. Micrographs were prepared using a microscope (Olympus BX51) equipped for dark-field and bright-field microscopy with a digital camera (Olympus U-PMTVC). Captured images were assembled electronically using Adobe Photoshop 5.0.

Statistics. Statistical analysis was performed using SPSS v 14.0. Differences between groups were assessed using ANOVA with *post hoc* Bonferroni correction for multiple comparisons. *P*-values of <0.05 were considered significant.

RESULTS

Physiologic variables. Mean values of MABP are given in Table 2. There were no differences between the groups at baseline or at the end of the study protocol. Sheep fetuses subjected to cord occlusion developed severe arterial hypotension with a mean (SEM) MABP of 11 (4) mm Hg at 10 min. At 10 min after start of ventilation MABP was higher in sheep subjected to cord occlusion (both in the 21 and 100% O_2 group) than in controls.

Table 1. *Accession numbers, forward and reverse primer sequences, probe sequences, and exon boundaries for ovine transcripts analyzed with real-time PCR*

Transcript	Accession number	Primers, forward and reverse	Probe	Exons
Ov. β-actin		Forward: GCTCTTCCAGCCGTCCTT Reverse: TGAAGGTGGTCTCGTGAATGC	CCTGGGTATGGAGTCCTG	2–3
Ov. IL-1β		Forward: GCTCTCCACCTCCTCTCACA Reverse: CCTCTCCTTGTACGAAGCTCATG	CAGAACACCACTTCTCG	1–2
Ov. IL-6		Forward: ACATCGTCGACAAAATCTCTGCAA Reverse: GCCAGTGTCTCCTTGCTGTTT	CACACTCGTCATTCTTC	3–4
Ov. IL-10		Forward: TGGAGCAGGTGAAGAGAGTCT Reverse: AAACTCACTCATGGCTTTGTAGACA	CCCTCTCTTGGAGCATAT	4–5
Ov. IL-12p40	NM_001009438	Forward: ATCGTGGCCATATGGGAACTG Reverse: CTCCAGGAGCATTAGGATACCAATC	CTACAACATAAACATTTTT	1–2
Ov. IL-18	NM_001009263	Forward: CCTGTCTTTGAGGATATGCCTGATT Reverse: GGTTACAGCCAGACCTCTAGTGA	ACTGTTCAGATAATGC	1–2
Ov. TNF-α		Forward: CCCAGGGCTCCAGAAGTTG Reverse: GCAACCAGGAGGAAGGAGAA	CTGGTGCCTCAGCCTC	
Ov. IFN-γ	X52640	Forward: GGGTGGGCCTCTTTTCTCA Reverse: GTAGAAGGAGACAATTTGGCTCTGA	TCACTCTCCTCTTTCC	2–3

Table 2. *Mean arterial blood pressure (MABP) during baseline, fetal cord occlusion and postnatal ventilation with 21 or 100% O_2*

		Cord occlusion		After start of ventilation			
	Group	Baseline	10 min	10 min	30 min	60 min	120 min
MABP, mm Hg	100 % O2	54 ± 6	11 ± 4	68 ± 7*	55 ± 5	50 ± 4	48 ± 5
	21 % O_2	52 ± 5	11 ± 4	72 ± 6*	53 ± 6	48 ± 3	50 ± 4
	Control	53 ± 5		56 ± 6	49 ± 5	49 ± 3	46 ± 5

Results are presented as mean ± SEM; Groups: 100 % O_2 ($n = 8$), 21 % O_2 ($n = 8$), Control ($n = 8$). Significant difference between either 100 % O_2 group or 21 % O_2 group and control group: * = $p < 0.05$.

Mean values of arterial blood-gases, Hb and lactate measurements of the respective groups are given in Table 3. Fetal sheep subjected to cord occlusion developed a combined metabolic and respiratory acidosis with an increase in lactate concentration at the end of cord occlusion. The combined acidosis and increase in lactate normalized gradually with no remaining differences between sheep subjected to cord occlusion and controls at 2h after start of ventilation. Ventilation

Table 3. *Arterial blood-gas variables, hemoglobin, and lactate concentrations at baseline, fetal cord occlusion, and postnatal ventilation with 21 or 100% O_2*

		Cord occlusion		After start of ventilation			
	Group	Baseline	10 min	10 min	30 min	60 min	120 min
pH	100 % O_2	7.21 ± 0.02	6.91 ± 0.01	7.20 ± 0.06**	7.30 ± 0.04*	7.35 ± 0.03	7.40 ± 0.03
	21 % O_2	7.21 ± 0.01	6.88 ± 0.02	7.22 ± 0.05**	7.30 ± 0.04*	7.31 ± 0.04	7.38 ± 0.02
	Control	7.24 ± 0.03		7.41 ± 0.04	7.41 ± 0.02	7.44 ± 0.02	7.40 ± 0.02
$PaCO_2$, kPa	100 % O_2	9.9 ± 0.3	17.6 ± 0.6	7.2 ± 1.3*	6.1 ± 0.5	5.9 ± 0.6	5.2 ± 0.6
	21 % O_2	9.4 ± 0.2	18.2 ± 0.5	6.8 ± 0.9*	6.0 ± 0.5	6.4 ± 0.6	5.3 ± 0.2
	Control	9.9 ± 0.7		5.2 ± 0.5	5.5 ± 0.4	5.1 ± 0.4	5.0 ± 0.2
PaO_2, kPa	100 % O_2	2.2 ± 0.2	0.3 ± 0.1	50.0 ± 7.1**††	47.1 ± 5.6**††	6.7 ± 1.0	5.9 ± 0.3
	21 % O_2	2.4 ± 0.2	0.4 ± 0.1	9.2 ± 1.0	6.0 ± 0.7	5.6 ± 0.5	6.0 ± 0.6
	Control	2.1 ± 0.2		10.9 ± 1.9	5.4 ± 0.3	6.4 ± 0.3	7.5 ± 0.4
BE, mmol/l	100 % O_2	−1.0 ± 1.1	−13.1 ± 1.8	−8.3 ± 2.2**	−4.3 ± 1.5*	−1.5 ± 1.1	−1.3 ± 0.6
	21 % O_2	−1.5 ± 0.9	−13.9 ± 0.6	−8.6 ± 1.1**	−5.4 ± 1.0*	−2.9 ± 1.1	−0.7 ± 0.8
	Control	1.3 ± 1.2		−0.1 ± 1.2	0.1 ± 1.1	2.0 ± 1.0	−0.9 ± 1.3
Hb, g/l	100 % O_2	112 ± 5	121 ± 5	112 ± 4	119 ± 3	121 ± 5	122 ± 5
	21 % O_2	113 ± 7	127 ± 8	117 ± 6	125 ± 1	124 ± 8	127 ± 8
	Control	113 ± 6		113 ± 6	123 ± 5	122 ± 6	121 ± 7
aSat, %	100 % O_2	45.5 ± 6.0	8.4 ± 0.2	99.5 ± 1.3**	100 ± 0.2**	94 ± 1.5	94 ± 1.2
	21 % O_2	50.2 ± 5.0	8.6 ± 0.5	93.8 ± 2.0	92.5 ± 2.1	91.2 ± 2.2	94 ± 1.6
	Control	43.8 ± 5.3		96.8 ± 0.8	92.5 ± 1.4	95 ± 0.7	96 ± 0.5
Lactate, mmol/l	100 % O_2	4.0 ± 0.7	9.7 ± 0.9		6.7 ± 1.0*		6.2 ± 0.8
	21 % O_2	4.7 ± 0.5	10.4 ± 0.5		7.6 ± 1.0*		5.8 ± 0.6
	Control	4.3 ± 0.4			4.3 ± 0.9		5.8 ± 0.9

Results are presented as mean ± SEM; Groups: 100% O_2 ($n = 8$), 21 % O_2 ($n = 8$), Control ($n = 8$). Hb, hemoglobin. Difference between either 100% O_2 group or 21% O_2 group and control group: * $p < 0.05$; ** $p < 0.01$. Difference between 100% O_2 group and 21% O_2 group: † $p < 0.05$; †† $p < 0.01$.

with 100% O_2 caused an arterial hyperoxemia with mean values of Pao_2 around 50 kPa at 10, 20, and 30 min, respectively. The rate whereby pH, BE and lactate levels were restituted during postnatal ventilation did not differ between sheep ventilated with 100 or 21% O_2.

Expression of cytokines. Levels of expression of cytokines in the respective groups as determined by real-time PCR are given in Fig. 1. Quantitative expression of IL-1β was increased in cortex/subcortex in the 100% O_2 group compared with the 21% O_2 group and to controls, $p = 0.018$ and $p = 0.02$, respectively. No significant differences were present in thalamus. There was no significant difference between the 21% O_2 group and controls. *In situ* hybridization revealed increased expression of IL-1β in meninges, in superficial cortical layers, in white matter, and in the choroid plexus. The sub-meningeal cortical expression of IL-1β was localized to relatively large rounded cells with a macrophage appearance, which were isolectin positive (Fig. 2). Expression of IL-1β in white matter was localized to smaller scattered cells with a microglial appearance. Representative illustrations are given in Fig. 3 and 4.

Quantitative expression of IL-12p40 was increased in cortex/subcortex in the 100% O_2 group compared with the 21% O_2 group and to controls, $p = 0.021$ and $p = 0.032$, respectively. No differences were present in thalamus. There was no significant difference between the 21% O_2 group and controls. *In situ* hybridization for IL-12p40 showed an unspecific weak hybridization, which was undistinguishable from background signal.

Expression of TNF-α and IL-18 as determined by real-time PCR did not differ between groups, neither in cortex/subcortex nor in the thalamus. Expressions of IL-6, IL-10, and IFN-γ were frequently below the detection level with no differences between groups.

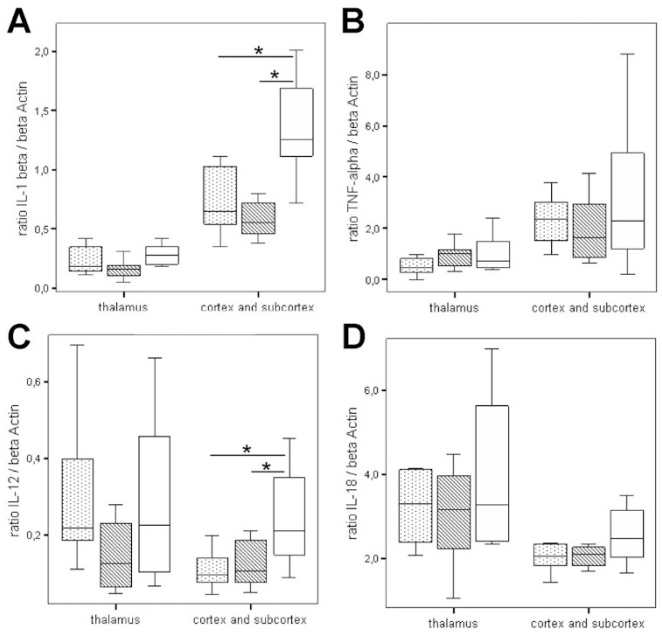

Figure 1. Median and interquartile ranges of expression of mRNA for (*A*) IL-1β, (*B*) TNF-α, (*C*) IL-12p40, and (*D*) IL-18 in cerebral regions in sheep at 2 h after birth. Control □ ($n = 8$); Asphyxia + 21% O_2 ▨ ($n = 8$); Asphyxia + 100% O_2 □ ($n = 8$). *$p < 0.05$.

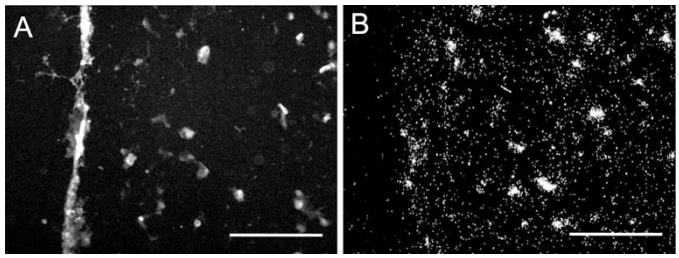

Figure 2. Sections of sub-meningeal cortex in sheep at 2 h after birth following asphyxia + 100 O_2 (30 min) stained with isolectin (*A*) and dark-view of *in situ* hybridization for IL-1β (*B*). Bars = 100 μm.

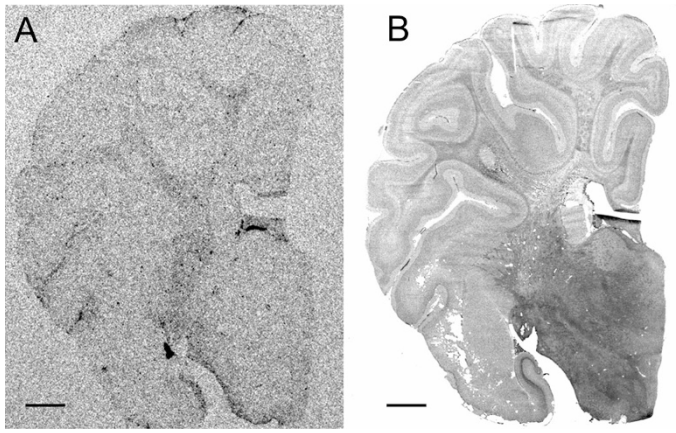

Figure 3. Coronal section of right cerebral hemisphere in sheep at 2 h after birth following asphyxia + 100 O_2 (30 min). (*A*) Overview of *in situ* hybridization for IL-1β. Increased expression of IL-1β in choroid plexus, meninges and submeningeal cortical layer, and white matter. (*B*) Hematoxylin-eosin stain of adjacent section. Bars = 5 mm.

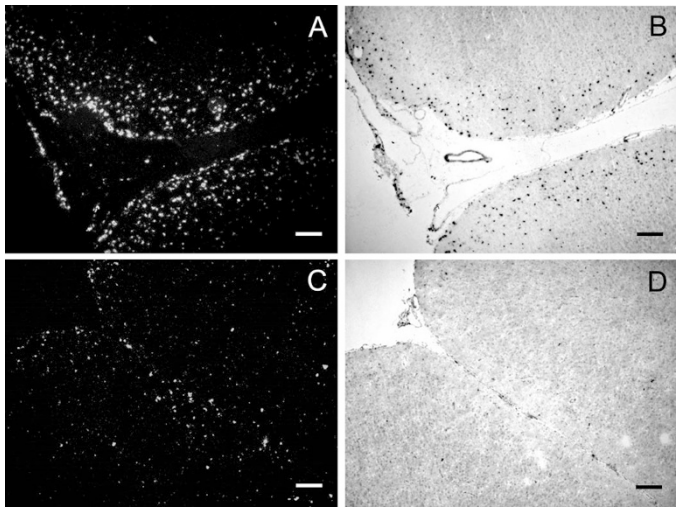

Figure 4. *In situ* hybridization for IL-1β in cerebral cortex in sheep at 2 h after birth. (*A*) (dark-field) and (*B*) (bright-field) illustrate representative cortical section of a newborn sheep subjected to asphyxia + 100% O_2 (30 min). Expression of IL-1β is shown in meninges and in rounded cells located in superficial cortical layer. (*C*) (dark-field) and (*D*) (bright-field) illustrate corresponding cortical region in a control sheep. Bars = 200 μm.

Expression of toll-like receptors. Levels of expression of TLR-2, -3, and -4 in thalamus and cortex /subcortex as determined by real-time PCR are given in Fig. 5. Levels of

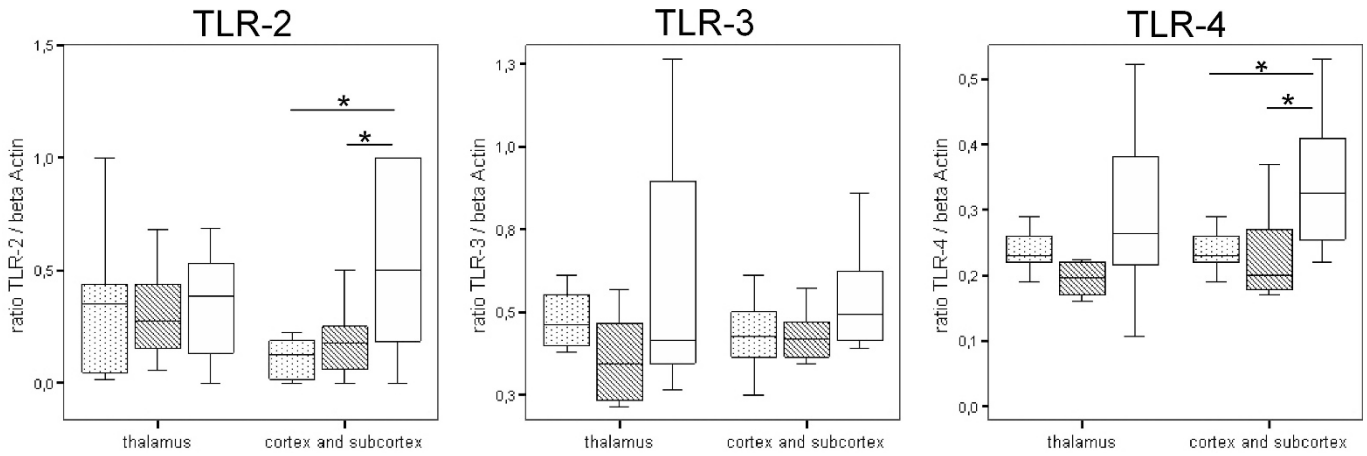

Figure 5. Median and interquartile ranges of expression of mRNA for TLR-2, TLR-3 and TLR-4 from cerebral regions in sheep at 2 h after birth. Control □ ($n = 8$); asphyxia + 21% O_2 ▨ ($n = 8$); Asphyxia + 100% O_2 □ ($n = 8$). *$p < 0.05$.

TLR-2 and TLR-4 were increased in cortex/subcortex in the 100% O_2 group as compared the 21% O_2 group and to controls, $p = 0.028$, $p = 0.04$ and $p = 0.02$, $p = 0.015$, respectively. Levels of TLR-3 did not differ significantly between groups. *In situ* hybridization for TLR-2 and -4 was weak and nondistinguishable from background signal. Expression of TLR-3 was present in the epithelial cells of choroids plexus and was present in all groups. There were no differences between the 21% O_2 group and controls.

DISCUSSION

The main finding of this study was an increase in cerebral gene expression of the inflammatory mediators IL-1β and IL-12p40 following ventilation with 100% oxygen compared with room air after fetal asphyxia. In addition we found an increased gene expression of TLR-2 and TLR-4 in the same cerebral region. Animals ventilated with room air after fetal asphyxia did not differ from controls.

Previous studies investigating effects of resuscitation with 21% *versus* 100% O_2 have mainly used animals with an established postnatal transition before being subjected to hypoxia and resuscitation (2). Our results were obtained in a sheep model, which aimed to mimic the resuscitation of newborn infants after birth asphyxia. Fetal asphyxia was induced by total cord occlusion, which caused severe combined metabolic and respiratory acidosis and arterial hypotension. We have previously shown that this type of asphyctic insult is associated with a gradual decrease in cerebral blood flow with resulting cerebral ischemia in fetal sheep of the same gestational age (12).

Ventilation of newborn sheep with 100% O_2 during 30 min caused arterial hyperoxemia with an arterial Pao_2 of around 50 kPa as opposed to normoxemia in the group ventilated with room air. Of note, the rate of restitution of metabolic acidosis and lactic acid levels up to 2 h of postnatal age did not differ according to inhaled gas mixture during the first 30 min.

The advantage of the applied experimental sheep model is the coverage of the transition from the normally low fetal oxygenation state to that of the ventilated newborn thereby strengthening the study of effects of hyperoxemia in the newborn. Limitations include that the gestational age of the sheep corresponds to a brain maturity, which is beyond that of the newborn infant at term age. Secondly, the experimental protocol was terminated at 2h postnatal age limiting observations to a short time frame.

Our primary hypothesis was that ventilation with 100% O_2 causing hyperoxemia would induce increased gene expression of inflammatory mediators in the brain. This was confirmed by increased levels of IL-1β and IL-12p40. An increased expression of IL-1β in the cerebral cortical region at 2h after exposure to hyperoxemia is consistant with previous study on P6 mice subjected to hyperoxia (7). In that study increased cerebral expression of IL-1β and of IL-18 was detected at 2 h after hyperoxemia with a peak of apoptotic neuronal cell death at 12 and 24 h. Importantly, both IL-1β and IL-18 were shown to be causally involved in cell death. We found a trend toward an increase in expression of IL-18 ($p = 0.077$) at 2h in the group subjected to hyperoxic ventilation after fetal asphyxia compared with the control group.

Cerebral gene expression levels of IL-1β have been shown to increase early in response to both ischemia and induced inflammation (13,14). IL-1β is a well-known mediator of neuronal loss and administration of the antagonist IL-1ra has documented protective effects on cellular damage (15). Interestingly, our finding of increased expression of IL-1β was restricted to the group subjected to asphyxia and 100% oxygen with no apparent difference between controls and asphyxia sheep ventilated with room air. Thus, hyperoxemia during reperfusion after ischemia was associated with the observed increase in IL-1β. The effect of hyperoxemia without preceding asphyxia cannot be determined from the present study.

In situ hybridization showed an increased number of IL-1β-positive macrophage-like cells in the superficial cortical layer. In addition, IL-1β expression was increased in subcortical white matter in cells of microglial appearance as well as in endothelial structures. The observed distribution suggests an activated passage of immune cells over the blood-brain barrier as well as an activation of resident microglia within the brain. This pattern of immune activation is similar to that resulting from peripheral lipopolysaccharide (LPS) adminis-

tration in adult rats (16). Sub-septic doses of LPS induced increased expression of IL-1β at 2 h after administration in meninges, choroid plexus, and circum-ventricular organs whereas higher doses of LPS induced widespread increased expression of IL-1β within the brain parenchyma.

The present study showed an increased cerebral expression of mRNA for IL-12p40 in sheep ventilated with 100% oxygen after fetal asphyxia. The pro-inflammatory cytokine IL-12 induces IFN-γ production by T cells and natural killer cells and promotes a generation of Th1 cells (17). Mice subjected to hyperoxia exhibit an early increase in IL-12 protein in broncheo-alveolar lavage fluid followed by IFN-γ related lung tissue damage (18). IL-12 is produced by microglia after stimulation by bacterial and viral antigens and increased in the brain after experimentally induced encephalitis (19,20). We speculate that increase in cerebral expression of IL-12p40 at 2h after hyperoxic ventilation is an early upstream event participating in subsequent activation of the adaptive immune system. Gene expression of IFN-γ was undetectable in the present study which was expected due to the short time elapsed after hyperoxic exposure.

The exact mechanisms whereby hyperoxemia and oxidative stress induce inflammation are unknown. A basal mechanism whereby pro-inflammation is initiated is by activation of the membrane-bound TLR family. We found increased levels in gene expression of TLR-2 and -4 together with increased levels of pro-inflammatory mediators in the group subjected to hyperoxia after asphyxia. The role of TLRs on astrocytes and microglia for the response to pathogen-associated molecular patterns is established (21). Less is known concerning the role of TLRs in response to brain ischemia or oxidative stress. A recent study has shown increased levels of TLR-4 in myocardial tissue after ischemia and reperfusion (22). Toll-like receptor-4 appeared to be mechanistically linked to tissue damage as TLR-4 deficient mice exhibited significantly reduced myocardial damage. Function of TLR-4 is linked to several elements associated with the production of reactive oxygen species such as neutrophil invasion in damaged tissue and up-regulated expression of cyclooxygenase (23). Toll-like receptors may play an essential role in generation of tissue damage in situations characterized by oxidative stress. A recent study in hypoxic mice showed that resuscitation with 100% by contrast to 21% O_2 activated NF-κB, a transcription factor for cytokines that is activated *via* TLRs (24). Several endogenous ligands with the ability to activate both TLR-2 and TLR-4 have been characterized. Substances released from damaged cells or extra-cellular matrix such as heat shock proteins and hyaluran have been shown to activate TLR-2 and TLR-4 (25,26). The specific ligands responsible for activating TLRs during oxidative stress in the ischemia-reperfusion sequence remain to be determined.

In summary, ventilation with 100% O_2 during 30 min after fetal asphyxia in newborn sheep caused significant hyperoxemia. This was associated with an activation of pro-inflammation in the brain at 2 h after birth. This indicates that cerebral damage may be aggravated by the uncontrolled use of oxygen during resuscitation of the newborn after fetal asphyxia. It remains to be shown how a shorter duration or lower level of hyperoxemia would affect induction of cerebral pro-inflammation. We propose that the TLR system has an important regulatory function in cerebral response to hyperoxemia during reperfusion after ischemia. The relationship between components of innate immunity and pro-inflammatory response in the context of ischemia-reperfusion and oxidative stress will be studied further.

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