Ischemia-reperfusion rapidly increases COX-2 expression in piglet cerebral arteries

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Domoki, Ferenc, Roland Veltkamp, Nishadi Thrikawala, Greg Robins, Ferenc Bari, Thomas M. Louis, and David W. Busija. Ischemia-reperfusion rapidly increases COX-2 expression in piglet cerebral arteries. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1207–H1214, 1999.—In the newborn, cyclooxygenase (COX)-derived products play an important role in the cerebrovascular dysfunction after ischemia-reperfusion (I/R). We examined effects of I/R on expression of COX-1 and COX-2 isoforms in large cerebral arteries of anesthetized piglets. The circle of Willis, the basilar, and the middle cerebral arteries were collected from piglets at 0.5–12 h after global ischemia (2.5–10 min, n = 50), hypoxia (n = 3), or hypercapnia (n = 2) and from time-control (n = 19) or untreated animals (n = 7). Tissues were analyzed for COX-1 and COX-2 mRNA and protein using RNase protection assay and immunoblot analysis, respectively. Ischemia increased COX-2 mRNA by 30 min, and maximal levels were reached at 2 h. Hypoxia or hypercapnia had minimal effects on COX-2 mRNA. COX-2 protein levels were also consistently elevated by 8 h after I/R. Increases in COX-2 mRNA or protein were not influenced by pretreatment with either indomethacin (5 mg/kg iv, n = 5) or nitro-L-arginine methyl ester (15 mg/kg iv, n = 7). COX-1 mRNA levels were low in time controls, and ischemic stress had no significant effect on COX-1 expression. Thus ischemic stress leads to relatively rapid, selective induction of COX-2 in cerebral arteries.

prostaglandin H synthase; messenger ribonucleic acid; ribonuclease protection assay; immunoblot analysis; cerebrovascular responsiveness

cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS), is the rate-limiting enzyme in the metabolism of arachidonic acid (AA) into prostanooids (prostaglandins and thromboxanes). COX-derived metabolites, such as prostanoioids and superoxide anion, are important vasoactive stimuli in the cerebral circulation (35). At least two different isoforms of COX (COX-1 and COX-2) have been characterized so far. The isoenzymes have similar catalytic activity, but they differ in molecular weights and pharmacological properties as well as tissue and cellular distribution (13). Originally COX-1 was considered the constitutively expressed isoform, and COX-2 was designated as the inducible isoform. However, in the brain and cerebral blood vessels of newborn pigs and rats, COX-2 but not COX-1 has been identified as the the major constitutively expressed isoform, and constitutive COX-2 expression appears to be developmentally and functionally regulated (11, 14, 31, 33).

Little is known about the regulation of COX-2 expression in cerebral blood vessels. COX-2 but not COX-1 mRNA levels were shown to increase after cerebral ischemia in various brain areas of newborn pigs, adult rats, and gerbils (12, 29, 30, 34). In piglet cerebral arteries, our previous immunohistochemical study demonstrated rapid increases in COX-2 but not COX-1 immunoreactivity, especially in the endothelial cells (8). However, specific information concerning details of this response is unknown. Because the COX-2 gene is an immediate-early gene, the induction of COX-2 mRNA does not require de novo protein synthesis but is dependent on the activation of preexisting transcription factors (18). Nuclear factor-κB (NF-κB) p65 has been shown to participate in the induction of COX-2 in human endothelial cell cultures subjected to hypoxia (37). COX itself may influence the COX-2 gene expression, because induction of COX-2 mRNA after I/R was prevented in the cerebral cortex and hippocampus of piglets pretreated with indomethacin (12). Nitric oxide (NO) may also modulate vascular COX-2 expression by inhibiting or facilitating COX activity (10, 28).

In the present study our goal was to assess the regulation of COX-1 and COX-2 gene expression in cerebral arteries after total cerebral ischemia in newborn pigs. Our specific aims were to determine 1) if COX-2, but not COX-1, mRNA and protein levels are upregulated in the cerebral arteries after global cerebral ischemia and to describe the time course of the response; 2) how the duration of ischemia affects the COX-2 mRNA response; 3) how the effect of ischemia compares with isolated hypoxia or hypercapnia; 4) if NF-κB could be associated in the mechanism of increased COX-2 expression; 5) the effect of COX inhibition by indomethacin, and 6) the effect of NOS inhibition by nitro-L-arginine methyl ester (L-NAME) on induction of COX-2 mRNA and protein levels.

MATERIALS AND METHODS

Animals

In these experiments newborn piglets of either sex (1–7 days old, 1–2 kg body wt) were used. All procedures were approved by the Institutional Animal Care and Use Committee. The animals were anesthetized with thiopental sodium...
(30–40 mg/kg ip) followed by intravenous injection of α-chloralose (75 mg/kg). Supplemenal doses of α-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. The pigs were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. For example, in the time-control group (n = 18) the values were a pH of 7.46 ± 0.02, P\textsubscript{CO\textsubscript{2}} of 31.2 ± 2.2 mmHg, and P\textsubscript{O\textsubscript{2}} of 88.6 ± 3.9 mmHg. Body temperature was maintained at 37–38 °C by a water-circulating heating pad.

The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19-mm diameter) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic. The closed window was filled with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O\textsubscript{2} and 6.5% CO\textsubscript{2} in Balance N\textsubscript{2} to give a pH of 7.33, a P\textsubscript{CO\textsubscript{2}} of 46 mmHg, and a P\textsubscript{O\textsubscript{2}} of 43 mmHg. The aCSF consisted of the following (in mmol/l): 132 NaCl, 2.9 KCl, 1.2 CaCl\textsubscript{2}, 1.4 MgCl\textsubscript{2}, 24.6 NaHCO\textsubscript{3}, 6.7 urea, and 3.7 glucose. Pial arterioles were visualized using a microscope (Wild M36, Switzerland) equipped with a video camera (Panasonic, Japan).

Cerebral Ischemia

To induce global cerebral ischemia, a 3-mm hole was drilled by an electric drill with a toothless bit, and the dura was exposed. A hollow brass bolt was inserted in the left frontal cranium rostral to the cranial window and secured in place with cyanoacrylate ester and dental acrylic. Cerebral ischemia was produced by infusion of aCSF to raise intracranial pressure (ICP) above arterial pressure. Ischemia was verified by the cessation of blood flow in the observed vessels. Previously, we have shown using microspheres that CBF is virtually zero in all brain areas examined during the ischemic period (7). Venous blood was withdrawn as necessary to maintain mean arterial blood pressure near normal values. At the end of the ischemic period the infusion tube was ally zero in all brain areas examined during the ischemic period. Venous blood was withdrawn as necessary to maintain arterial blood pressure and to administer drugs and fluids, respectively. The pigs were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20 breaths/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. For example, in the time-control group (n = 18) the values were a pH of 7.46 ± 0.02, P\textsubscript{CO\textsubscript{2}} of 31.2 ± 2.2 mmHg, and P\textsubscript{O\textsubscript{2}} of 88.6 ± 3.9 mmHg. Body temperature was maintained at 37–38 °C by a water-circulating heating pad.

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Tissue Preparation

The animals were killed while anesthetized with an intravenous bolus of KCl at the end of the experiments. In addition, several naive, nontreated animals (n = 7) were killed with an overdose of thiopental sodium (150 mg/kg ip). The brains were quickly removed, and the basilar artery, the circle of Willis, and branches of the middle cerebral arteries were harvested and immediately frozen by immersion in 2-methylbutane at 70°C. Samples were stored at −70°C. From the frozen cerebral arteries, protein was extracted in boiling lysis buffer (10 mmol/l Tris and 1% SDS). The samples were sonicated, heated at 95°C for 5 min, and centrifuged for 5 min at 12,000 rpm at 4°C. An aliquot of the supernatant was transferred to phase-lock gel tubes (5 Prime, Boulder, CO), and then, 100 µl of 2 mol/l sodium acetate were added to each sample, followed by the addition of 1 ml of H\textsubscript{2}O-saturated phenol. After we thoroughly mixed the samples, 300 µl of chloroform-isomyl alcohol (49:1) were added, and then the solution was incubated on ice for 10 min before centrifugation at 6,000 rpm for 5 min. The supernatants were transferred to fresh tubes. After the addition of 1 ml of isopropanol, the samples were incubated in room temperature for 30 min and centrifuged at 12,000 rpm for 25 min. The resulting pellets were washed with 100% ethanol and redissolved in ultra pure water before storing at −60°C. The integrity of RNA was determined by resolving in a 1% agarose gel, and RNA aliquots were quantified using a spectrophotometer.

Ribonuclease Protection Assays (RPAs) were performed with 25 µg of total RNA from each sample using RPA II kits (Ambion, Austin, TX). γ-32P-labeled sense and antisense probes were generated for COX-1 and COX-2 using Maxi-Script kits (Ambion) after linearizing pCSMs containing fragments of cDNA for porcine COX-1 and COX-2, respectively. We used antisense probes for glyceraldehyde-phosphate dehydrogenase as a housekeeping gene in some RPAs.

RPA blots were scanned using a Hewlett-Packard Desk Scan II. The density of individual bands were then determined using NIH Image analysis software (version 1.55).

Immunoblot Analysis

From the frozen cerebral arteries, protein was extracted in boiling lysis buffer (10 mmol/l Tris and 1% SDS). The samples were sonicated, heated at 95°C for 5 min, and centrifuged for 5 min at 12,000 rpm at 4°C. An aliquot of the supernatant was removed for protein concentration determination. After the addition of an equal volume of sample buffer (100 mmol/l Tris, pH 6.8; 42% glycerol; 5% bromophenol blue; and 1% SDS) to each sample, the protein was separated on a 4–20% gradient mini gel (Bio-Rad, NY) and transferred to nitrocellulose. After nonspecific protein binding was blocked by incubating the blot in 5% milk, primary antibody was applied, followed by horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, Jackson Immunoresearch Labs, PA). The COX-2 murine polyclonal antibody and COX-1 ovine polyclonal antibody (Cayman Chemical, Ann Arbor, MI) were used at 1:1,000; secondary antibody was used at 1:10,000. Molecular weight markers and purified COX (Cayman) were included on
each blot. Electrochemiluminescence (ECL, NEN Life Science Products, Boston, MA) was used to visualize the bands.

Immunohistochemistry

In these experiments, time-control and ischemia animals were operated in pairs, and every procedure was carried out simultaneously. At 8 h after 10 min of ischemia, the piglets were perfused transcardially with ice-cold saline, and then the cerebral arteries were quickly removed and immersion fixed in 10% buffered Formalin overnight. After dehydration, the vessels were embedded in paraffin as a cluster and then sectioned (6 µm) to provide random arrays of arteries and arterioles cut in different planes. The sections were deparaffinized in xylene, rehydrated in alcohol, and washed three times in 10 mmol/l phosphate buffer containing 0.9% saline, pH 7.4 PBS for 10 min each time. All washes and solutions were made in PBS unless otherwise stated. After being washed, the sections were blocked in 10% normal goat serum (NGS); 0.1% Tween 20 for 4 h. COX-2-specific polyconal antibody (Dr. A. W. Ford-Hutchinson, Merck Frost, Center for Therapeutic Research, Pointe Claire, Quebec, Canada) was diluted 1:2,000, and incubated with the sections overnight at room temperature. After sections were rinsed in 2% NGS, endogenous peroxidase was blocked by incubating the sections in 3% H2O2-10% methanol for 30 min. After sections were washed, they were incubated with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) diluted 1:1,000 in 2% NGS for 2 h. Subsequently, sections were washed and reacted with Vector ABC reagent (Vector) for 30 min, washed again, and reacted with diaminobenzidine. Stained sections were mounted on slides, dried, and sealed with a 1% cover glass. Specificity of the staining was established previously (11). Sections were visualized and photographed with a Zeiss Axioskop microscope (Jena, Germany). Figures were created with Adobe Photoshop (San J ose, CA) software from original 35-mm slides and were printed with a Fuji Pictograph 3000 digital printer (Encino, CA). Scanning contrast and intensity were altered only to replicate the original images. The color images were converted to gray scale using filters as necessary, formatted to plate form, and labeled.

Electrophoretic Mobility Shift Assay

In these experiments, time control and ischemia animals were operated in pairs, and every procedure was carried out simultaneously on each pair. At 1 h after 10 min of ischemia, the cerebral arteries were collected and nuclear protein was extracted immediately. The cerebral arteries were homogenized in 800 µl of ice-cold extraction cell lysis buffer [buffer A: 10 mmol/l HEPES (pH = 7.8), 1.5 mmol/l MgCl2, 10.0 mmol/l KCl, 1.0 mmol/l dithiothreitol, 1.0 µmol/l phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin] using a Dounce homogenizer. The homogenates were centrifuged at 14,000 rpm for 15 min, the supernatant containing the nuclear protein was removed, and an aliquot was used for protein concentration determina-

tion using the Bradford method. Double-stranded NF-κB binding consensus DNA probes were synthesized by annealing sense and antisense oligonucleotides, and each was end labeled with 50 µCi of [γ-32P]ATP/20 pmol using T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden) and then isolated using a CENTRI·SEP column (Princeton Separations, Adelphi, NJ). DNA probe (20,000 counts/min) and 1.0 µg of bulk carrier poly(dl-dC) DNA was incubated with 2.5 µg of nuclear protein extract at room temperature for 30 min. Antibody supershift was performed with polyclonal rabbit antibody raised against human amino-terminal NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA), and as a control for this procedure we used anti-α-tubulin antibodies (Sigma). Blocking with 400-fold excess of cold DNA or substitution of 5.0 µg of bovine serum albumin for nuclear protein was also performed as a control. The protein-DNA product was then run in a 5% nondenaturing polyacrylamide gel and subjected to autoradiography.

Drugs

The drugs used in this study were indomethacin (Merck) and L-NAME (Sigma).

Statistics

Data are means ± SE. Data were analyzed using one-way ANOVA, followed by pairwise comparisons using the Student-Newman-Keuls test where appropriate.

RESULTS

RNase Protection Assay for COX mRNA Levels

COX-1 mRNA response to cerebral ischemia. In the piglet cerebral arteries the mRNA for COX-1 was barely detectable even with long exposure times. Cerebral ischemia did not induce expression of COX-1; mRNA levels were very low and essentially unchanged after 1, 2, and 8 h of ischemic stress (Fig. 1).

COX-2 mRNA response to cerebral ischemia. Ten minutes of global cerebral ischemia increased the expression of COX-2 mRNA in cerebral arteries (Fig. 2). The increase in mRNA levels was detected as early as 30 min after ischemic stress (Fig. 2, top), and mRNA levels were noticeably higher compared with time-matched controls for at least as long as 12 h in the postischemic period (Fig. 2, bottom). However, the maximum of the mRNA signal was observed at 2 h after ischemia.

![Fig. 1. Effect of ischemia-reperfusion (I/R) on cyclooxygenase (COX)-1 mRNA levels in cerebral arteries. RNase protection assay (RPA) showed little detectable COX-1 mRNA. Note that exposure time is substantially longer than for COX-2 RPAs. Lanes 11, 12, 18, and 2 represent samples taken at 1, 2, 8, and 10 min of global cerebral ischemia (I); lane C8 represents an 8-h time control. In the last 2 lanes only the COX-2 single-stranded antisense probe was loaded in absence (−) and presence (+) of RNase treatment. In the other lanes double-stranded protected COX-1 fragments can be observed.](image-url)
The COX-2 mRNA response was similar in the different segments of large cerebral arteries. Thus the circle of Willis, middle cerebral arteries, and basilar artery showed similar increases in COX-2 mRNA abundance (Fig. 3).

The enhanced expression of COX-2 mRNA was not sensitive to the duration of global ischemia. Cerebral ischemia of 2.5, 5, and 10 min produced similar increases in the maximal COX-2 message at 2 h after the ischemia (Fig. 4A). Hybridizing procedure using the sense probe failed to produce any bands on the gel (Fig. 4B).

COX-2 mRNA response to hypoxia and hypercapnia. Severe hypoxia (15 min) or hypercapnia (15 min) resulted only in a minor increase in COX-2 mRNA levels compared with nontreated controls, in contrast to the robust increase usually seen with ischemia (blot not shown). The slight increase in COX-2 mRNA compared with nontreated animals was not different between the piglets subjected to hypoxia and those exposed to hypercapnia.

Effect of indomethacin and L-NAME pretreatment on induction of COX-2 mRNA by ischemia. The doses of L-NAME and indomethacin used in this study are sufficient to inhibit NOS and COX activity, respectively (2, 12, 35). L-NAME significantly increased mean arterial blood pressure from 76 ± 5 to 118 ± 8 mmHg (n = 9). L-NAME or indomethacin did not change the expression of COX-2 mRNA in time-control animals. In animals treated with indomethacin or L-NAME 20 min before cerebral ischemia, we observed a trend of increased expression of COX-2 mRNA (Figs. 5 and 6, respectively). However, in the quantitative analysis of the blots, although there was a 30–40% increase in the mRNA in pretreated animals compared with ischemia alone, the ANOVA failed to produce significant differences among the ischemia groups. Nevertheless, there were significant increases in COX-2 mRNA in all

Fig. 2. Effect of I/R on COX-2 mRNA in cerebral arteries. Lanes 1.5, 1, 2, 4, 8, and 12 represent samples taken at 30 min and 1, 2, 4, 8, and 12 h, respectively after 10 min of global cerebral ischemia. Lanes C2, C4, C8, and C12 represent 2-, 4-, 8-, and 12-h time controls, respectively. In contrast with COX-1 (Fig. 1), the RPA reveals a tremendous increase in COX-2 mRNA. Increased abundance of COX-2 mRNA was detected by 0.5 h in postischemic period (top) and peaked at 2 h consistently. Message was elevated compared with time controls at all time points studied, at least as long as 12 h (bottom).

Fig. 3. Effect of I/R on COX-2 mRNA in different segments of cerebral arteries. Middle cerebral arteries (MCA), circle of Willis (CW), and basilar artery (BA) all showed similar increases in COX-2 mRNA at 2 h after ischemia.

Fig. 4. Effect of the duration of ischemia on COX-2 mRNA in cerebral arteries. A: at 2 h after 2.5 (2.5 ± 8), 5 (5 ± 8), and 10 (10 ± 8) min of cerebral ischemia, COX-2 RPA demonstrates significant elevation in mRNA levels compared with 2-h time-control (C) and nontreated (NT) animals. However, there is no duration-dependent difference among mRNA levels in ischemic animals. B: RPA confirms indifference between 2.5 and 10 min of ischemic stress regarding induction of COX-2 mRNA. To show specificity of assay, extracted RNA hybridized with sense strand (B, right) of COX-2 riboprobe produced no bands on film.

Fig. 5. Effect of indomethacin pretreatment (Indo) on induction of COX-2 mRNA by I/R in cerebral arteries. On this (and Fig. 7) RPA, glyceraldehyde-phosphate dehydrogenase (GAPDH) was also used as a housekeeping message as a control for equal loading of the lanes. Indo did not induce COX-2 mRNA in 2-h time control (lane C2) and did not inhibit induction of COX-2 mRNA at 2 h after ischemia (lanes 12). All indomethacin-pretreated animals showed large increases in COX-2 mRNA levels at 2 h after I/R.
ischemia groups compared with nontreated or time-control animals (Fig. 7).

**Immunoblot (Western) Analysis for COX Protein Levels**

The polyclonal COX-2 antibody reacted selectively with the piglet COX-2 as indicated by the appearance of a single immunoreactive band having the same electrophoretic mobility of the standard, migrating at 70 kDa. Omission of the primary antibody resulted in a lack of immunoreactive band. Previously, we had found that preadsorption of the primary antibody with COX-2 completely eliminated this immunoreactive band. In 8-h time-control animals, COX-2 protein levels were almost undetectable. In contrast, in animals subjected to cerebral ischemia, after 8 h significant increases in COX-2 protein levels were observed (Fig. 8). In indomethacin- or L-NAME-pretreated animals the increases in COX-2 protein levels were essentially similar to those of ischemia alone (data not shown). COX-1 antibody failed to produce any substantial bands on the Western blots (data not shown).

**COX-2 Immunoreactivity in Cerebral Arteries After I/R**

Substitution of the COX-2 antibody with preimmune serum resulted in minimal immunostaining of the cerebral arteries (Fig. 9). These results are representative of all vessels examined. COX-2 immunoreactivity was enhanced in cerebral arteries at 8 h after I/R compared with time controls. Especially strong labeling was observed in the endothelial cell layer in postischemic arteries (Fig. 10). Adventitia and vascular smooth muscle also stained heavily, confirming the results from our previous study (7). Preliminary in situ hybridization studies also suggested similar distribution of increased COX-2 mRNA expression in the cerebral arteries (results not shown).

**Effect of Cerebral Ischemia on NF-κB Nuclear Translocation in Cerebral Arteries**

Electrophoretic mobility shift assay (EMSA) was employed on nuclear protein extracted from cerebral arteries from paired ischemia and time-control animals. EMSA demonstrated increased binding of nuclear protein to a generic consensus NF-κB oligonucleotide sequence at 1 h after I/R compared with time-matched paired controls. Furthermore, anti-NF-κB p65 but not anti-α-tubulin supershifted the NF-κB oligonucleotide-nuclear protein complex showing specificity of the assay (Fig. 10).

**DISCUSSION**

There are five major findings from this study. First, in piglet cerebral arteries, similarly to the cortex and other brain areas, COX-2 but not COX-1 is the major COX isoform under basal conditions. Second, global cerebral ischemia selectively induces COX-2 but not COX-1 mRNA and protein levels. The endothelial cells appear to be the predominant cell type affected by I/R, but to a lesser extent adventitia and vascular smooth
muscle cells are also involved. Third, although the COX-2 mRNA levels were elevated at all time points studied, the peak mRNA levels were consistently detected at 2 h after ischemic stress. Fourth, the duration of ischemic stress from 2.5 to 10 min appeared not to have a major effect on the magnitude of increased COX-2 mRNA levels. Finally, inhibiting COX with indomethacin or NOS with L-NAME did not significantly affect the increase of COX-2 mRNA or protein levels after global cerebral ischemia.

The mechanism of arterial COX-2 induction by cerebral ischemia is unknown. The COX-2 gene is an immediate-early gene that has several potential upstream response elements, including NF-κB binding regions. NF-κB is known to be activated by oxygen radicals (17), making NF-κB a possible candidate to link I/R to altered COX-2 expression. NF-κB has been shown to participate in the induction of COX-2 by hypoxia in human umbilical vascular endothelial cells (HUVEC) (37). Also in HUVEC, cooperation between the transcriptional activator high-mobility-group protein I(Y) and NF-κB has been reported to completely induce COX-2 after hypoxia (16). We showed that nuclear protein binding to NF-κB consensus sequence increases within an hour after I/R. In this study we demonstrated the rapid nuclear translocation of NF-κB p65 in cerebral arteries after I/R. However, this finding is only a necessary but not sufficient requirement to establish the role of NF-κB in the vascular induction of COX-2. Clearly, further studies are needed to delineate the role of NF-κB and probably other transcription factors in the regulation of COX-2 expression after I/R.

We found that severe hypoxia or hypercapnia per se was not sufficient to elicit major changes in cerebral arterial COX-2 expression. This is in accordance with the results of a previous study in which asphyxia (anoxia + hypercapnia) also failed to increase COX-2 levels in the brain regions studied (12). In contrast, relatively short (2.5 min) ischemia elicited the increase in COX-2 message. We conclude that the stoppage of blood flow and/or the subsequent reperfusion injury, rather than reoxygenation only, plays an important role in the initiation of COX-2 gene transcription.

Indomethacin or L-NAME pretreatment did not significantly influence the COX-2 induction in the cerebral arteries. This finding is in contrast with those of a previous study in which indomethacin, but not the nNOS inhibitor 7-nitroindazol, prevented the induction of COX-2 in cerebral cortex and hippocampus of piglets.

[Fig. 9. Effect of I/R on COX-2 immunoreactivity in cerebral arteries. Top: vessel incubated with preimmune (PI) serum (×40). Middle and bottom: vessels from 8-h time control (C8) or 8 h after I/R (I8) incubated with polyclonal COX-2 antibody (×20, inset ×40). Note heavy labeling of COX-2 immunopositive endothelial cells in arteries at 8 h after ischemic stress.]

[Fig. 10. Effect of I/R on nuclear translocation of NF-κB in cerebral arteries. Figure was constructed from 2 blots showing results of 2 paired experiments. Data should be interpreted only by comparing within paired samples. Electrophoretic mobility shift assay shows increased binding of NF-κB-binding DNA to nuclear protein extracted from cerebral arteries 1 h after I/R (I1) compared with paired time controls (C). Addition of anti-NF-κB antibody resulted in a supershift (SS NF-κB) of the band, whereas addition of anti-α-tubulin antibody (α-tubulin) did not supershift bands, verifying specificity of assay.]
after I/R (12). The apparent difference in the results is yet unexplained but suggests differences in the mechanism of COX-2 induction in blood vessels compared with neural tissues. One such difference could be the interaction of the endothelial cells with platelets and neutrophils. Unlike neurons and glial cells, neutrophils possess various COX-independent radical-producing systems. The most important of such enzymes may be NADPH oxidase, which is responsible for the majority of superoxide anions produced by neutrophils. Prostacyclin and nitric oxide (NO) have been shown to inhibit platelet and neutrophil adhesion and aggregation (39). Furthermore, NO has been reported to inhibit NADPH oxidase (9). NO has been shown to prevent the adhesion of white blood cells to cerebrovascular endothelium after asphyxia in newborn piglets, indicating a potentially protecting antiinflammatory effect after I/R in cerebral blood vessels (15). Thus pretreatment with L-NAME or especially indomethacin may not decrease the oxygen radical exposure to the cerebral arteries in contrast to the brain parenchyma.

The COX system may play a central role in the pathophysiology of neuronal injury after ischemic stress. Cerebral I/R results in decreased vascular reactivity to specific stimuli as well as altered function of the blood-brain barrier (BBB). During hypoxia and total brain ischemia substantial amounts of AA are released from cellular membranes (1), but the lack of molecular oxygen inhibits COX activity. In the early reperfusion period, both substrates become available, resulting in a tremendous increase in prostaglandin and superoxide anion levels. In fact, most of the superoxide anion produced in the cortex is dependent on COX activity after global ischemia in piglets (2). After I/R, many endothelium/prostanoid-dependent (23–25) vascular responses such as vasodilation to hypercapnia or arterial hypotension are eliminated (6, 21, 20). For instance, at 1 h after 10 min of global ischemia, hypercapnia fails to dilate cerebral arterioles; however, the vascular responsiveness is restored in 2–4 h (6). Also, vascular dilation to prostacyclin is diminished after I/R, whereas the vasodilation to PGE₂ remains intact (5, 19). However, after ischemic stress the piglet brain is capable of producing prostaglandins from exogenous AA, indicating that COX is not inhibited in the posts ischemic period as early as 1 h after hypoxic/ischemic stress (22). I/R also increases blood to brain transport of sodium and albumin and increases endothelial vesicle formation, indicating the impaired function of the BBB (4, 26). The vascular responsiveness to several stimuli, as well as the integrity of the BBB after I/R, can be preserved by pretreatment with oxygen radical scavengers or indomethacin, indicating the involvement of COX (4, 40).

Our present results demonstrate a rapid and substantial induction of the COX-2 enzyme in the cerebral arteries at 8 h after ischemic stress. The beneficial or harmful effects of the induction of COX-2 after I/R have yet to be elucidated. Cerebral I/R occurs commonly in the newborn. The initial damage is often followed by epileptic seizures, apnea, and intracranial hemorrhage occurring usually at 8–10 h after I/R. Thus, in our present study, these complications develop after the vascular induction of COX-2. Because these secondary insults also elicit the activation of COX (3, 30, 32), they may result in enhanced radical and prostanoid production, further compromising the already challenged cerebrovascular functions, resulting in severe neurological damage. Also, the induction of COX-2 in transient focal (29) or global (27) brain ischemia has been reported to increase infarct size as well as hippocampal CA1 neuronal death in rats, respectively. However, delayed induction of COX-2 may also participate in the reparative processes in both the brain and cerebral blood vessels (36, 38).

In summary, piglet cerebral arteries express COX-2 under normal conditions. The COX-2 expression has been demonstrated to increase tremendously at both the mRNA and the protein levels after ischemic stress. In contrast with neural tissue, the vascular induction of COX-2 cannot be inhibited by indomethacin. The acute upregulation of COX-2 may result in 1) increased oxidative stress caused by superoxide production, altering cerebrovascular responsiveness, and 2) increased production of vasoconstrictor and/or inflammatory prostanoids, both leading to reduced cerebral blood flow and further neurological damage. Importantly, the time course of COX-2 induction may offer a therapeutic window for treatments, preventing the deleterious secondary insults after hypoxic/ischemic events in the newborn.

We gratefully thank Drs. K. Peri and S. Chenboth for generously providing the COX-1 and COX-2 probes for the RNase protection assay.

This research was supported by National Heart, Lung, and Blood Institute Grants HL-30260, HL-46558, and HL-50587 and in part by the Hungarian Science Foundation (T-026295 OTKA).

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Received 3 February 1999; accepted in final form 18 May 1999.

REFERENCES

7. Beasley, T. C., F. Bari, C. Thore, N. Thrikawala, T. M. Louis, and D. W. Busija. Cerebral ischemia/reperfusion increases endothelial nitric oxide synthase levels by an indomethacin-