

Brain Expression of Inducible Cyclooxygenase 2 Messenger RNA in Rats Undergoing Cardiopulmonary Bypass

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Background: We hypothesized that systemic proinflammatory cytokines or endotoxemia, or both, associated with cardiopulmonary bypass (CPB) would increase expression of inducible cyclooxygenase (COX-2) or inducible nitric oxide synthase (iNOS) messenger RNA (mRNA), or both, in brain.

Methods: Isoflurane-anesthetized Sprague-Dawley rats were randomly selected for CPB (n = 6) or sham surgery (n = 6). All animals underwent tracheotomy and controlled ventilation, arterial and venous pressure monitoring, insertion of a jugular venous outflow catheter, insertion of a subclavian arterial inflow catheter, systemic anticoagulation (500 U/kg heparin) and, except during CPB, servoregulation of pericranial temperature at 37.5°C. Animals selected for CPB underwent 1 h of CPB at 165 ml · kg⁻¹ · min⁻¹ (31.8 ± 0.2°C), whereas animals having sham surgery underwent no intervention during this interval. Thereafter, all animals were given protamine and remained anesthetized for 4 more h. Brain and liver COX-2 and iNOS mRNA expression were determined by a ribonuclease protection assay with ribosomal L32 mRNA as a loading control. Arterial blood was analyzed for interleukin 1β, interleukin 6, and endotoxin concentrations.

Results: Endotoxin concentrations did not increase above baseline values in either group. At 4 h after the CPB interval, interleukin 6 concentrations were significantly greater in CPB animals (101 ± 45 pg/ml) versus sham animals (44 ± 17 pg/ml) (P = 0.025). Brain COX-2 expression was significantly greater in CPB animals (0.36 ± 0.11) versus shams (0.19 ± 0.08) (P = 0.013). Brain COX-2 expression correlated with interleukin 6 concentration 4 h after CPB (r = 0.91; P = 5 × 10⁻⁵). In brain, iNOS mRNA was not detected in any animal. Cardiopulmonary bypass animals had only trace COX-2 and iNOS mRNA induction in liver.

Conclusions: Cardiopulmonary bypass was associated with increased systemic interleukin 6 concentrations and increased brain COX-2 expression.

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CARDIAC surgery conducted with cardiopulmonary bypass (CPB) is associated with increased systemic concentrations of proinflammatory cytokines including interleukins 6 and 8 (IL-6 and IL-8),¹⁻⁶ tumor necrosis factor α,^{3,6-8} and, in a few studies, interleukin 1β (IL-1β).⁵ Endotoxemia is also commonplace during CPB.^{2,7-11} Prior studies have focused on the roles of proinflammatory cytokines and endotoxin in adverse cardiopulmonary outcomes after cardiac surgery^{2,8,10,12-16} but have not fully considered potential central nervous system sequelae.

In animal sepsis and inflammation models, systemic proinflammatory mediators result in extensive changes in brain gene expression,¹⁷⁻²⁷ neurochemistry,^{28,29} neuroendocrine status,^{28,29} thermoregulation,^{21,22,29} and cognition²⁸ and result in behavioral alterations. Both systemic endotoxin^{17-21,23,24} and proinflammatory cytokines^{22,24} trigger expression of inducible cyclooxygenase (COX-2)²¹⁻²⁴ and inducible nitric oxide synthase (iNOS)¹⁷⁻²⁰ in the cerebral vasculature^{19,21,22,24} or associated perivascular microglia.^{20,23,24} Cerebrovascular COX-2³⁰⁻³² and iNOS^{32,33} expression result in cerebral vasodilation^{30,32,33} and increased blood-brain barrier permeability.^{31,33} Expression of these genes also modulates the hypothalamic-pituitary-adrenal response to systemic inflammation (fever, adrenocorticotropic hormone and cortisol secretion).^{19,26,27}

Based on the collective clinical and animal literature, we hypothesized that there would be an association between systemic proinflammatory mediators occurring with CPB (e.g., cytokines or endotoxin) and expression of COX-2 or iNOS messenger RNA (mRNA), or both, within the brain. Although the organ of interest was brain, we measured COX-2 and iNOS expression in liver as well. We selected liver as a comparison organ because it receives portal venous blood (potentially high in endotoxin) and because it is rich in macrophages (Kupffer cells), making it highly responsive to inflammatory stimuli.

Materials and Methods

Experimental protocols were approved by the Animal Care and Use Committee of the University of Iowa in

accordance with the "Guide for the Care and Use of Laboratory Animals," revised in 1996. All surgery was performed under sterile conditions, with particular attention given to avoiding environmental endotoxin contamination. Using computer-generated random numbers and block design, animals were preassigned to one of two groups: surgical shams ($n = 6$), or CPB ($n = 6$).

Basic Preparation

Anesthesia was induced in nonfasting male Sprague-Dawley rats (weight, 268–355 g) (Harlan, Indianapolis, IN) by inhalation of isoflurane in room air. *Via* a midline neck incision, the trachea was cannulated with a 14-gauge intravenous cannula. Animals were ventilated (tidal volume ≈ 15 ml/kg; rate ≈ 40 breaths/min) with ≥ 1 minimum anesthetic concentration (MAC) of isoflurane (1.5–2.0%) in oxygen. Exhaled gas was monitored continuously with a calibrated anesthetic agent analyzer (Datex; Puritan-Bennet, Helsinki, Finland). A calibrated 22-gauge needle thermistor (Model 552; Yellow Springs Instruments, Yellow Springs, OH) was placed percutaneously into the right temporalis muscle to measure pericranial temperature, which was servoregulated at 37.5°C (Model 73A; Yellow Springs Instruments) using surface warming and cooling.

Via a neck incision, the right external jugular vein was isolated. To serve as a CPB venous outflow cannula, a multiple-orifice catheter (Model PE-200; Intramedic/Becton Dickinson, Sparkes, MD) (OD = 2.0 mm, ID = 1.5 mm, length = 6.5 cm) was inserted into the right external jugular vein, and the tip advanced into the right atrium. Heparinized normal saline, 20 U/ml, was administered at 1 ml/h through this cannula until animals were given large-dose systemic heparin, later in the experiment. *Via* an axillary incision, the right brachial-subclavian artery was isolated. To serve as a CPB arterial inflow cannula, a 20-gauge intravenous catheter with a tapered 22-gauge tip (OD = 1.1 mm, ID = 0.8 mm, length = 3 cm) was inserted into the brachial artery, and its position adjusted to obtain a nondamped arterial pressure waveform. *Via* a left groin incision, the femoral artery and vein were cannulated with saline-filled (heparin, 5 U/ml) polyethylene catheters (Model PE-50, Intramedic Becton Dickinson, Sparkes, MD). The femoral arterial catheter was used to record mean arterial pressure and for blood sampling. The femoral venous catheter was used to administer medications and to measure venous pressure. Animals in both groups were then given 500 U/kg heparin and 0.5 mg/kg pancuronium intravenously. The heparin dose was based on pilot studies ($n = 5$), wherein 500 U/kg heparin increased whole-blood activated clotting time (Hemotec Incorporated, Englewood, CO) from a baseline of 60 ± 10 to 376 ± 108 s. Five minutes after heparin administration, arterial blood was collected for baseline measurement of systemic endotoxin and cytokine concentrations. At the same time, baseline values

for mean arterial pressure, venous pressure, pericranial temperature, arterial blood gases (pH, arterial oxygen tension, arterial carbon dioxide tension) (IL1306; Instrumentation Laboratory, Lexington, MA), hemoglobin (OSM3 with rat coefficients; Radiometer, Copenhagen, Denmark), and whole-blood glucose concentration (Model 27; Yellow Springs Instruments) were recorded. Thereafter, animals were managed according to group-specific protocols.

Surgical Sham Protocol

Surgical shams underwent no additional procedures. For the remainder of the experiment (5 h in total), sodium bicarbonate and 10% dextrose in water were given as needed to maintain base excess of -4 mEq/l or greater and glucose concentration of 100 mg/dl or greater, respectively. No blood or blood products were administered at any time. To compensate for repeated blood collection, a modified hetastarch solution was given as needed to maintain venous pressure at baseline values. One hour after baseline measurements, all measurements were repeated. Thereafter, to reverse heparin anticoagulation, 7 mg/kg protamine was administered intravenously. Subclavian artery and jugular venous catheters were flushed with heparinized saline and capped for the remainder of the experiment. Surgical shams remained anesthetized and monitored for another 4 h, at which time a final set of measurements and an activated clotting time were obtained.

Cardiopulmonary Bypass Protocol

After baseline measurements, CPB animals had the jugular and subclavian catheters connected to the CPB circuit and underwent CPB for 1 h. Cardiopulmonary bypass flow rate was maintained constant at $165 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. Normal cardiac output in anesthetized rats is approximately $200 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$.³⁴ Complete, or near-complete, CPB was indicated by the absence or near-absence (≤ 5 mmHg) of arterial pulsation measured from the femoral artery catheter. The oxygenator was ventilated with oxygen containing 1.5–2.0% isoflurane. Pulmonary ventilation was continued during CPB with isoflurane, 1.5%, in oxygen at a reduced rate and tidal volume (10 ml/kg, 20 breaths/min). Mean arterial pressure, venous pressure, pericranial temperature, blood gas values, and arterial hemoglobin and glucose concentrations were measured every 15 min during CPB. Arterial blood gases were measured at electrode temperatures of 37°C and were not temperature corrected (α -stat method). During CPB, a modified hetastarch mixture was given to maintain venous reservoir volume as needed. For the remainder of the experiment, sodium bicarbonate and 10% dextrose in water were given as needed to maintain base excess of -4 mEq/l or greater and glucose concentration of 100 mg/dl or greater, respectively. During the first 30 min of CPB, animals were allowed to cool pas-

sively to 32°C. This was performed to approximate clinical practice. Rewarming was initiated at 30 min of CPB. After 1 h of CPB, but before separation from CPB and protamine, all measurements (endotoxin, cytokines, systemic variables) were repeated. Blood was also collected for activated clotting time measurement. Normal ventilation was restored, and animals were separated from CPB without any inotropic or vasopressor support. Thereafter, to reverse heparin anticoagulation, 7 mg/kg protamine was administered intravenously. After separation from CPB, subclavian and jugular catheters were flushed with heparinized saline and capped. Hemodynamics, temperature, and blood chemistries were measured at least hourly after CPB. Perfusate remaining in the CPB circuit was centrifuged to obtain buffy coat-free erythrocytes. After CPB, these erythrocytes were given to maintain a hemoglobin concentration of 12 g/dl or greater. A modified hetastarch solution was also given to maintain intravascular volume and maintain mean arterial pressure of 80 mmHg or greater. CPB animals remained anesthetized and monitored for 4 h after separation from CPB, at which time a final set of measurements (endotoxin, cytokines, systemic variables, activated clotting time) were obtained.

All CPB circuit components were sterile for each use. The circuit consisted of a venous reservoir (20-ml syringe), a peristaltic pump (Masterflex Model 7523-10; Cole-Parmer, Vernon Hills, IL), and a neonatal hollow-fiber oxygenator with the heat exchanger removed (Micro; Cobe Cardiovascular Incorporated, Arvada, CO), connected by Tygon tubing (size 16; Saint Gobain Plastics, Akron, OH). Except for the oxygenator, all circuit components were new for each use. The oxygenator was reused to reduce cost. After each use, the oxygenator was rinsed with tap water for 2 h, followed by a flush of 4 l sterile water, air dried overnight, and then ethylene oxide gas-sterilized. Before each experiment, the circuit was primed with 50 ml Plasmalyte A (Baxter Healthcare Corporation, Deerfield, IL), circulated for 30 min at 150 ml/min through a 0.2- μ m filter. Thereafter, the circuit was drained, refilled with Plasmalyte A, and filtered again. Following this, the filter was removed and the circuit was drained and refilled with 50 ml of a heparinized (5 U/ml) modified hetastarch solution. The modified commercial hetastarch solution had the following composition: 4.8% high-molecular-weight hydroxyethyl starch, Na^+ = 141 mEq/l, Cl^- = 137 mEq/l, K^+ = 3.2 mEq/l, HCO_3^- = 18 mEq/l, Ca^{2+} = 5.4 mEq/l, Mg^{2+} = 4.1 mEq/l, glucose = 200 mg/dl.

Approximately 15 min before CPB, 25–30 ml of the modified hetastarch was removed from the circuit and replaced with 25–30 ml of fresh (< 5 min from collection) whole heparinized (10 U/ml) rat blood, collected from two isoflurane-anesthetized donor rats. The final priming mixture of blood and modified hetastarch had a hemoglobin concentration ranging between 7.0 and

8.3 g/dl. Immediately before CPB, a sample of the blood and hetastarch priming mixture was obtained for measurement of hemoglobin concentration, as well as endotoxin and cytokine concentrations. Circuit warming was achieved by use of heat tape, which was wrapped around the oxygenator and reservoir.

End of Experiment

In both groups, after obtaining the final set of systemic measurements (4 h after protamine administration), animals were killed by an intravenous overdose of 390 mg/kg pentobarbital and 50 mg/kg phenytoin (Euthasol®; Delmarva Laboratories, Midlothian, VA). Brain and liver were rapidly removed and frozen on dry ice. Tissue remained frozen at -70°C until assayed for mRNA expression.

Positive Control Animals

To serve as positive control animals for iNOS and COX-2 gene induction, two additional rats were assigned to be given intravenous lipopolysaccharide (LPS), the active moiety of endotoxin. Anesthesia, tracheotomy, mechanical ventilation, temperature monitoring, and femoral arterial and venous catheterization were performed as previously described. These animals did not undergo jugular or brachial arterial cannulation. Animals were given a 2-mg/kg intravenous bolus of LPS (*Escherichia coli* 055:B5; Sigma Chemical Company, St. Louis, MO), which has been shown to induce both COX-2 and iNOS^{18,24} mRNA in rat brain and iNOS mRNA in rat liver.¹⁸ Peak responses to systemic LPS occur 2–6 h after administration. After LPS administration the animals remained anesthetized, ventilated, and monitored for 4 h. Thereafter, they were killed and tissue harvested as previously described.

Endotoxin Assay

Endotoxin was assayed using the limulus amoebocyte lysate method with chromogenic substrate (QCL-100; BioWhittaker, Inc., Walkersville, MD). All tubes used for collection, processing, and assay of endotoxin were endotoxin free. Arterial blood samples of 300 μ l were centrifuged immediately after collection, and plasma was immediately stored at -70°C until assay. All endotoxin assays were run in duplicate. To inactivate nonspecific inhibitors of the limulus amoebocyte lysate reaction, 250 μ l endotoxin-free water was added to 50- μ l samples of thawed plasma and the mixture incubated at 60°C for 30 min. Fifty microliters of limulus amoebocyte lysate was added to 50 μ l of the diluted, heat-treated sample. The mixture was incubated at 37°C for 10 min. Thereafter, 100 μ l of chromogenic substrate was added, and the mixture was vortexed and incubated at 37°C for 30 min. The reaction was stopped with 800 μ l of stop solution provided by the manufacturer. Absorbance (optical density) was measured at 410 nm using a spectrophotome-

ter. To correct for nonspecific absorbance, for each sample a "blank" was prepared exactly as just described, except 50 μ l of endotoxin-free water was substituted for 50 μ l limulus amebocyte lysate. Sample absorbance was calculated as the mean of two sample measurements minus the absorbance of the corresponding sample blank. Standard absorption curves (410 nm) were prepared with known concentrations of standard endotoxin supplied with the kit. The endotoxin standard has a potency of approximately 1 endotoxin activity unit (EU) per 100 pg of endotoxin (0.01 EU/ml \approx 1 pg/ml). Control assays indicated that neither heparin (\leq 10 IU/ml) nor hetastarch had any effect on the endotoxin assay. The minimum detection level was 0.01 EU/ml.

Cytokine Assays

We decided to measure IL-1 β and IL-6. Although an increase in IL-1 β has been detected in only a few CPB studies, IL-1 β has been shown to induce both COX-2^{22,24} and iNOS mRNA in brain.³⁵ Interleukin 6 was selected because it is uniformly reported to be increased after CPB and because systemic IL-6 has been shown to mediate a host of central nervous system responses.

Arterial blood samples of 400 μ l were collected into tubes containing trisodium EDTA, with a final EDTA concentration of 1.5 mg/ml. Blood samples were centrifuged immediately after collection, and plasma was immediately stored at -70°C until assay. Assays were performed using commercial rat-specific ELISA kits (BioSource International Inc., Camarillo, CA) in exact accordance with manufacturer's instructions. To eliminate interassay variation, cytokine analyses were performed "in batch" in such a manner that, for each cytokine, all samples were assayed on a single ELISA plate. For IL-1 β the minimal detection level was 3 pg/ml, with an intraassay coefficient of variation of 8%. For IL-6 the minimal detection level was 8 pg/ml, with an intraassay coefficient of variation of 4%. Any individual measured value for cytokine concentration that was less than the minimal detection level for the assay was recorded as zero.

Ribonuclease Protection Assays

Ribonuclease protection assays to quantitate of COX-2, iNOS, and RPL32-4A ("L32") mRNA were performed as previously described.^{36,37} The L32 mRNA codes for a constitutively expressed ribosomal protein, which is not affected by inflammatory stimuli and thereby serves as an internal loading control. Accordingly, COX-2 and iNOS mRNA expression was quantitated relative to L32 mRNA expression. Murine complementary DNA fragments for COX-2, iNOS, and L32 in pGEM plasmids were generous gifts of Iain L. Campbell, Ph.D. (The Scripps Research Institute, La Jolla, CA). The expected protected fragment sizes are 300 base pairs (bp) for COX-2, 275 bp for iNOS, and 80 bp for L32. The ability of these probes to detect rat COX-2 and iNOS transcripts was previously estab-

lished using an LPS- and cytokine-activated rat alveolar cell line, NR8383 (American Type Culture Collection, Manassas, VA).

Liver and brain were thawed on ice and homogenized in ice-cold RNA-STAT 60 (Tel-Test Incorporated, Friendswood, TX). Total RNA was isolated following the manufacturer's instructions. For the synthesis of a ³²P-radio-labeled antisense RNA probe, equimolar mixtures of linear COX-2, iNOS, and L32 templates were used. Hybridization reactions were performed overnight at 56°C. After ribonuclease digestion, RNA duplexes were isolated by electrophoresis in a standard sequencing gel of 7.5% acrylamide, 12 M urea, and 0.5% Tris-boric acid-EDTA. Dried gels were placed on BioMax MR film (Eastman Kodak Company, Rochester NY) and were exposed at -70°C . Band intensity was quantitated by densitometry. To eliminate interassay variation, these assays were performed in batch in such a manner that each assay, which incorporated all samples, was performed on a single gel. In our laboratory, the intraassay coefficient of variation of COX-2/L32 and iNOS/L32 ratios ranges between 2 and 12%.

Statistical Analysis

Data are reported as mean \pm SD. Between-group comparisons of arterial IL-6 concentration and brain COX-2 mRNA expression were performed using Student *t* test with separate variances (SYSTAT version 9.0 for Windows; SPSS, Chicago, IL). The correlation between IL-6 and brain COX-2 mRNA was assessed using the Pearson correlation. *P* values were interpreted using the Hochberg method to correct for multiple comparisons. The overall α for the three statistical comparisons was less than 0.05.

Results

Systemic Variables

Systemic variables are summarized in table 1. At baseline, sham and CPB groups were equivalent in all measured variables. During the CPB interval, CPB animals had lesser values for mean arterial pressure, hemoglobin concentration, and pericranial temperature than sham animals, which is consistent with clinical CPB. There were no hypotensive reactions to protamine in any animal. In the 4 h after protamine administration, sham and CPB animals did not differ with respect to mean arterial pressure, venous pressure, pericranial temperature, arterial pH, partial pressure of oxygen (P_{O₂}), partial pressure of carbon dioxide (P_{CO₂}), or blood glucose. The CPB animals tended to be given more bicarbonate (3.4 \pm 0.9 vs. 1.3 \pm 0.3 ml), glucose (4.3 \pm 4.3 vs. 0.2 \pm 0.4 ml), modified hetastarch (1.3 \pm 1.3 vs. 0.8 \pm 1.6 ml), and packed erythrocytes (4.7 \pm 1.3 vs. 0 \pm 0 ml) than sham animals. Although not intended, CPB animals had greater final (4-h) values for hemoglobin than did sham animals.

Table 1. Systemic Variables

Variable	Group	Prime	Baseline	CPB Interval				Post-CPB		
				15 min	30 min	45 min	60 min	0.5 h	1 h	4 h
Mean arterial pressure (mmHg)	Sham	—	107 ± 13	107 ± 15	105 ± 15	107 ± 10	111 ± 15	103 ± 16	105 ± 16	99 ± 12
	CPB	—	98 ± 8	72 ± 22	73 ± 21	66 ± 18	71 ± 16	110 ± 8	111 ± 13	88 ± 11
Venous pressure (mmHg)	Sham	—	5 ± 1	5 ± 2	5 ± 1	4 ± 2	5 ± 1	4 ± 1	5 ± 2	4 ± 1
	CPB	—	5 ± 0	5 ± 1	5 ± 1	5 ± 1	5 ± 2	5 ± 0	5 ± 1	6 ± 1
Pericranial temperature (°C)	Sham	—	37.6 ± 0.5	38.0 ± 0.1	37.5 ± 0.2	37.8 ± 0.2	37.9 ± 0.4	37.6 ± 0.4	37.9 ± 0.3	37.6 ± 0.4
	CPB	—	37.7 ± 0.4	31.8 ± 0.2	31.8 ± 0.2	33.4 ± 0.6	34.5 ± 0.9	37.4 ± 0.7	37.5 ± 0.2	37.6 ± 0.3
Hemoglobin (g/dl)	Sham	—	14.4 ± 0.3	—	—	—	13.6 ± 0.7	—	11.7 ± 1.0	11.4 ± 1.8
	CPB	7.7 ± 0.5	13.2 ± 0.8	7.3 ± 0.5	7.3 ± 0.5	7.3 ± 0.8	8.2 ± 0.7	10.4 ± 2.6	13.4 ± 0.9	14.1 ± 1.4
Glucose (mg/dl)	Sham	—	163 ± 43	—	—	—	144 ± 37	—	139 ± 31	149 ± 54
	CPB	—	151 ± 68	151 ± 59	159 ± 68	168 ± 65	176 ± 68	124 ± 50	120 ± 25	129 ± 25
pHa	Sham	—	7.38 ± 0.07	—	—	—	7.40 ± 0.06	—	7.39 ± 0.05	7.42 ± 0.03
	CPB	—	7.33 ± 0.06	7.40 ± 0.03	7.37 ± 0.03	7.39 ± 0.02	7.42 ± 0.04	7.38 ± 0.04	7.37 ± 0.03	7.41 ± 0.05
Paco ₂ (mmHg)	Sham	—	40 ± 7	—	—	—	37 ± 7	—	30 ± 3	37 ± 2
	CPB	—	44 ± 7	30 ± 4	32 ± 4	36 ± 6	39 ± 5	35 ± 3	38 ± 7	40 ± 4
Pao ₂ (mmHg)	Sham	—	494 ± 42	—	—	—	471 ± 40	—	457 ± 37	515 ± 31
	CPB	—	475 ± 45	502 ± 51	505 ± 53	487 ± 41	543 ± 67	438 ± 52	421 ± 44	472 ± 105
Endotoxin (EU/ml)	Sham	—	0.01 ± 0.02	—	—	—	0.02 ± 0.03	—	—	0.01 ± 0.02
	CPB	0.03 ± 0.03	0.01 ± 0.02	—	—	—	0.03 ± 0.05	—	—	0.03 ± 0.04
Interleukin 1β (pg/ml)	Sham	—	57 ± 34	—	—	—	49 ± 22*	—	—	81 ± 81*
	CPB	44 ± 25	65 ± 62	—	—	—	60 ± 70	—	—	51 ± 28
Interleukin 6 (pg/ml)	Sham	—	8 ± 14	—	—	—	11 ± 11	—	—	44 ± 17
	CPB	0 ± 0	7 ± 8	—	—	—	15 ± 12	—	—	101 ± 45†
Activated clotting time (s)	Sham	—	—	—	—	—	—	—	—	101 ± 21
	CPB	—	—	—	—	—	999 ± 0	—	—	140 ± 25

Values are mean ± SD. Except where noted, n = 6 (* n = 5).

† Interleukin 6 greater in cardiopulmonary bypass (CPB) group versus, sham, P = 0.025.

pHa = arterial pH; Paco₂ = arterial carbon dioxide tension; Pao₂ = arterial oxygen tension.

Before CPB, the CPB priming mixture contained IL-1β, IL-6, and endotoxin at concentrations that were not greater than baseline *in vivo* values. Therefore, CPB animals were not given large doses of exogenous endotoxin, IL-1β, or IL-6. At the end of the CPB interval (before protamine administration), systemic IL-1β, IL-6, and endotoxin concentrations did not differ from baseline values in either group, nor did they differ between groups. Four hours after the CPB interval, IL-1β and endotoxin concentrations did not differ from baseline values, nor did they differ between groups. In contrast, at 4 h after the CPB interval, systemic IL-6 concentrations were greater than baseline in both groups and were significantly greater in CPB animals than in sham animals (101 ± 45 vs. 44 ± 17 pg/ml, respectively; P = 0.025).

Gene Expression

Gene expression is summarized in table 2. In brain, COX-2 mRNA expression was significantly greater in CPB animals versus sham animals; COX-2/L32 ratios were 0.36 ± 0.11 versus 0.19 ± 0.11, respectively (P = 0.013). As shown in figure 1, there was a significant correlation between brain COX-2 mRNA expression and systemic IL-6 concentrations measured 4 h after the CPB interval (r = 0.91; P = 5 × 10⁻⁵). There was no discernible correlation between systemic endotoxin concentra-

tion and either systemic IL-6 concentration or brain COX-2 mRNA expression. Brain iNOS mRNA expression was not detected in any sham or CPB animal.

In liver, CPB animals exhibited extremely weak COX-2 mRNA expression (COX-2/L32 ratio = 0.06 ± 0.04), which was discernibly greater than that of sham animals, which showed no liver COX-2 mRNA expression at all (COX-2/L32 ratio = 0 ± 0). Likewise, CPB animals also exhibited extremely weak liver iNOS mRNA expression (iNOS/L32 ratio = 0.06 ± 0.06), which was, again, discernibly greater than that of sham animals, which

Table 2. Gene Expression

mRNA	Group	Brain	Liver
COX-2/L32	Sham	0.19 ± 0.08	0 ± 0
	CPB	0.36 ± 0.11*	0.06 ± 0.04
iNOS/L32	Sham	0 ± 0	0 ± 0
	CPB	0 ± 0	0.06 ± 0.06

Values are mean ± SD; n = 6.

* COX-2/L32 ratio greater in cardiopulmonary bypass (CPB) group versus Sham; P = 0.013.

COX-2 and iNOS mRNA expression is quantitated relative to L32 mRNA expression, which serves as an internal loading control. Positive controls (n = 2) received intravenous lipopolysaccharide (LPS). Gene expression ratios were as follows: COX-2 in brain (0.98 and 1.04); COX-2 in liver (0 and 0.87); iNOS in brain (0 and 0); iNOS in liver (15.42 and 11.23).

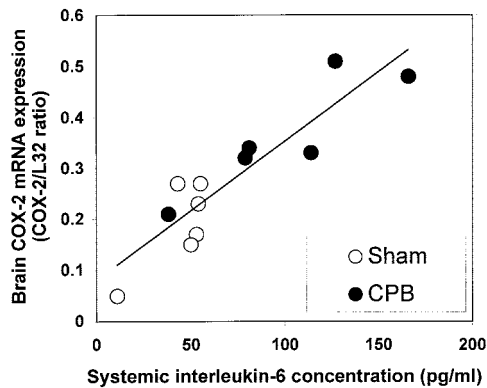


Fig. 1. Correlation between systemic interleukin 6 concentration 4 h after the cardiopulmonary bypass (CPB) interval and brain cyclooxygenase 2 messenger RNA (COX-2 mRNA) expression. $r = 0.91$; $P = 5 \times 10^{-5}$.

showed no liver iNOS mRNA expression at all (iNOS/L32 ratio = 0 ± 0).

In the positive control animals (LPS animals), there was robust brain COX-2 mRNA expression (COX-2/L32 ratios of 0.98 and 1.04 respectively) and robust liver iNOS expression (iNOS/L32 ratios of 15.42 and 11.23 respectively). In these two animals, liver COX-2/L32 ratios were 0 and 0.87, and contrary to expectation, brain iNOS mRNA was not detected.

Discussion

Overview of Findings and Clinical Implications

Neurologic and neurocognitive abnormalities are common after cardiac surgery and CPB and are associated with greater intensive care unit and hospital length of stay, greater mortality, greater need for rehabilitative care,³⁸ and long-term cognitive dysfunction.³⁹ Despite intensive investigation, the mechanisms underlying neurocognitive abnormalities after CPB remain incompletely understood and therapies intended to prevent these complications have often failed.⁴⁰ In part, progress has been hampered because of the lack of a suitable survival animal model of CPB.⁴⁰ Because (1) brain physiology and pathophysiology are well characterized in the rat, (2) rats can be readily separated from CPB and survive for extended periods,⁴¹ and (3) preliminary results indicate that neurologic dysfunction after CPB is present in rats,⁴¹ this model holds promise for characterization of CPB-associated brain injury.

In the current experiment, systemic concentrations of proinflammatory cytokines and endotoxin in CPB animals were generally less than expected. In CPB animals, systemic IL-1 β and endotoxin concentrations were not significantly greater than baseline values at any time, nor did they differ from values obtained from sham animals. The vast majority of CPB studies have not observed increases in IL-1 β . In contrast, endotoxemia (generally in the range of 20–40 pg/ml) is commonly observed.^{2,7–11}

Therefore, in CPB animals the absence of a detectable IL-1 β response was expected, but the absence of endotoxemia was not. Interleukin 6 concentrations did not differ between groups after the 1-h CPB interval but were significantly greater in CPB animals *versus* sham animals 4 h later. This is consistent with prior studies which showed that IL-6 concentrations do not increase during CPB but, instead, peak 2–6 h afterward.^{1,5–7,12,13,42} In the current experiment, IL-6 concentrations after CPB (101 ± 45 pg/ml) were twofold to fourfold less than generally reported after human cardiac surgery and CPB.^{1,2,4–6,12,42} Despite relatively low levels of systemic proinflammatory mediators, brain COX-2 mRNA expression in CPB animals was twofold greater than in sham animals and was approximately one third as great as that of rats given a potent stimulus for brain COX-2 induction (LPS). There was a significant correlation between systemic IL-6 concentrations and brain COX-2 expression, which suggests an interaction between systemic and brain inflammatory responses to CPB.

We propose that systemic IL-6 or brain COX-2 induction, or both, could be responsible for some of the commonly observed abnormalities in cerebral physiology after CPB, such as fever,^{14,43} blood-brain barrier dysfunction,^{44,45} and, perhaps, acute abnormalities in neurologic status or cognition as well.⁴⁶ We base this proposal on the following observations. In animals, systemic IL-6 results in fever,⁴⁷ increased blood-brain barrier permeability,⁴⁸ and altered neurotransmitter balance⁴⁹ and behavior.⁵⁰ In mice, IL-6 mediates the constellation of “sickness behaviors” (fever, anorexia, cachexia, and lethargy) that occur in response to noninfectious tissue injury and inflammation.⁵¹ In animals, cerebrovascular COX-2 induction has been shown to mediate neuroendocrine (cortisol) and thermoregulatory (fever) responses to systemic proinflammatory mediators^{26,27} and to result in increased blood-brain barrier permeability³¹ and cerebral vasodilation.^{30,32} In humans, administration of IL-6 results in fever, headache, myalgia, malaise, nausea,^{52,53} cortisol secretion,⁵⁴ and, at high doses, transient neurologic abnormalities.⁵² In the setting of cardiac surgery, IL-6 concentration after CPB has been shown to correlate with both postoperative fever¹³ and S-100 β concentration after CPB.¹ S-100 β is a glial protein that gains access to the systemic circulation when blood-brain barrier permeability is increased.⁵⁵ The association between IL-6 and S-100 β suggests that IL-6 might increase blood-brain barrier permeability directly or, perhaps, by inducing cerebrovascular COX-2.

Features and Limitations of This Experiment

The current experiment is largely exploratory and clearly is not definitive. We did not demonstrate an association between brain COX-2 induction and any neurologic or neurophysiologic abnormality. Nevertheless, the experiment does make three new observations: (1)

like humans, rats exhibit a systemic inflammatory response (albeit mild) after clinically analogous CPB, (2) brain inflammatory gene induction occurs after CPB, and (3) there may be an interaction between these two responses.

COX-2 mRNA is constitutively expressed in some neurons in the amygdala, hippocampus, hypothalamus, and neocortex.^{21,23,24} Therefore, some "background" COX-2 mRNA expression in sham animals was expected. In rats, brain COX-2 mRNA has been shown to be most strongly induced by systemic endotoxin, but systemic IL-1 β , tumor necrosis factor α , and even noninfectious peripheral tissue inflammation can, to lesser extent, also induce cerebrovascular COX-2 mRNA.²⁴ Given the short half-lives of IL-1 and endotoxin (minutes), it is possible that transient increases of these proinflammatory mediators could have occurred in the current experiment without detection.

Although there was a significant correlation between systemic IL-6 concentration and brain COX-2 mRNA expression, such an association does not necessarily imply causation. Indeed, prior rat studies indicate that IL-6 *alone* does *not* induce brain COX-2.^{24,25} This suggests either (1) that the process (or mediator) which led to increased systemic IL-6 production in CPB animals proportionally coinduced brain COX-2 or (2) that increased brain COX-2 mRNA expression after CPB is augmented by systemic IL-6. Support for the latter possibility comes from experiments showing activation of the hypothalamic-pituitary-adrenal axis and that induction of brain c-fos mRNA in response to IL-1 β or LPS varies proportionally with plasma IL-6 level.⁵⁶ Therefore, in some circumstances, systemic IL-6 modulates the central nervous system effects of other proinflammatory stimuli.^{57,58}

The CPB animals differed from sham animals in that they had 1 h of mild systemic hypotension, anemia, and hypothermia. This was performed to reproduce conditions common during clinical CPB. Although mean arterial pressure during CPB was greater than the lower limit of cerebral autoregulation in rats (50–60 mmHg),⁵⁹ both hemodilution⁶⁰ and hypothermia⁶¹ can impair cerebral autoregulatory responses. Both animal⁶² and human⁶³ studies have indicated that global cerebral oxygenation is adequately maintained under clinical CPB conditions. Nevertheless, we cannot rule out the possibility that CPB animals may have had some degree of mild cerebral ischemia during CPB. Transient cerebral ischemia, even when insufficient to result in neuronal necrosis, can rapidly increase brain COX-2 mRNA expression.⁶⁴ Therefore, in the current experiment, it is possible that brain COX-2 induction might have been triggered by ischemia, with subsequent modulation by systemic IL-6. However, even if brain COX-2 induction were to be triggered on an ischemic rather than on an inflammatory basis, this does not alter the potential central nervous system sequelae of brain COX-2 induction.

Systemic IL-6 peaks 2–6 h after all forms of surgery and roughly corresponds to the overall magnitude of tissue injury.⁶⁵ In the setting of cardiac surgery, the heart^{3,42,66} and, perhaps, the lungs¹⁵ are major contributors to the increase of IL-6 after CPB, producing IL-6 in response to temporary ischemia. In the current experiment, the heart was continuously perfused and beating, hypotension during CPB was moderate, and we observed no signs of myocardial dysfunction whatsoever after CPB. Although the lungs of CPB animals had low pulmonary artery flow for 1 h and, in rats, ischemia of this duration can result in lung injury,⁶⁷ there was no evidence of overt pulmonary dysfunction after CPB. Therefore, in the current experiment, neither heart nor lung would seem likely as a major source of IL-6 production. This may in part explain why IL-6 levels after CPB were less than generally observed in clinical studies.

Greater IL-6 levels have been observed in patients who were given allogenic red blood cells during or shortly after CPB *versus* patients who were not.⁶⁸ Because all CPB animals were exposed to donor red cells, whereas sham animals were not, greater IL-6 levels in CPB animals could have been on this basis. However, in separate pilot experiments, rats undergoing exchange transfusions with the CPB priming mixture (95 ml/kg whole heparinized rat blood and hetastarch; $n = 6$) did not differ from sham animals ($n = 6$) in systemic IL-6 levels or brain COX-2 mRNA expression 4 h later (unpublished data available on request from Brad Hindman, M.D., Associate Professor of Anesthesia, University of Iowa, College of Medicine, Iowa City, Iowa, August, 1999).

Both animal⁶⁹ and human⁷⁰ studies have indicated that splanchnic perfusion is often unfavorably affected by CPB, and indications of gut mucosal ischemia are commonly present. Probably on this basis, CPB increases gut permeability,^{9,16} allowing endotoxin from resident flora access to the portal and then systemic circulation.⁹ Therefore, it is possible that gut-associated macrophages, producing IL-6 in response to translocated endotoxin, could have been the source of increased IL-6 in CPB animals.⁷¹ Although we did not observe a significant increase in systemic endotoxin in CPB animals, the liver has a huge capacity to clear endotoxin originating from the portal circulation.⁷² We observed an extremely small increase in COX-2 and iNOS mRNA expression in the livers of CPB animals, approximately 200 and 14 times less than the respective responses to intravenous LPS. This barely detectable upregulation of liver iNOS and COX-2 expression argues against a significant amount of endotoxin being present in the portal vein of CPB animals and thereby argues against transient undetected systemic endotoxemia as the primary cause of brain COX-2 induction. The apparent absence of endotoxemia in the current experiment suggests that splanchnic perfusion in this rat CPB model is relatively well maintained. Because liver macrophages (Kupffer cells) typically ex-

hibit a robust inflammatory response to systemic inflammatory stimuli, the near-absence of liver iNOS and COX-2 induction is consistent with the possibility that brain COX-2 mRNA induction was facilitated but not directly triggered by systemic IL-6.

In brain, iNOS mRNA was not detected in either sham animals or CPB animals. Unexpectedly, we also did not detect brain iNOS mRNA in our two "positive control animals" that were given LPS. The dose of LPS given to our two positive control animals has been shown to result in brain iNOS mRNA induction in prior studies.¹⁷⁻²⁰ Although brain iNOS was not detected, animals being given LPS did have a strong liver iNOS mRNA signal. Therefore, the iNOS probe used in the current experiment appropriately binds and protects rat iNOS mRNA during the ribonuclease digestion phase of the assay. Prior studies demonstrating brain iNOS induction after LPS used either *in situ* hybridization^{19,20} or polymerase chain reaction gene amplification techniques.^{17,18} Both techniques are extremely sensitive. Furthermore, these studies show that endotoxin-mediated brain iNOS mRNA induction, although pronounced, is limited to discrete anatomic regions and cell populations. Because we measured whole-brain iNOS mRNA expression, it is possible that iNOS induction in a relatively small number of cells would go undetected with our methodology. An alternative explanation for the absence of brain iNOS mRNA expression, even in the positive control animals, may relate to our use of isoflurane anesthesia. All studies demonstrating brain iNOS induction after LPS administration were performed in animals that were not anesthetized.¹⁷⁻²⁰ In a murine macrophage-like cell line, clinically relevant concentrations (1 minimum anesthetic concentration) of isoflurane have been shown to completely inhibit LPS-induced iNOS mRNA expression.⁷³ Therefore, in the current experiment, isoflurane anesthesia may have reduced or prevented brain iNOS mRNA expression in response to LPS or CPB-associated proinflammatory stimuli, or both. In summary, we found that four hours after CPB, brain COX-2 mRNA expression was significantly greater in CPB rats *versus* sham animals and correlated with arterial IL-6 concentration.

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