Indomethacin, Prostacyclin, and Heparin Improve Postischemic Cerebral Blood Flow Without Affecting Early Postischemic Granulocyte Accumulation

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Six anesthetized dogs treated with indomethacin, prostacyclin (PGI₂), and heparin were compared with 7 anesthetized controls (ischemia without treatment) to determine whether cyclooxygenase inhibition would lead to enhanced granulocyte accumulation because of preferential formation of lipoxygenase products. Cortical somatosensory evoked response, [¹⁴C]iodoantipyrine autoradiographic blood flow, and [¹¹¹]In-labelled granulocyte accumulation were compared 4 hours after a 60-minute exposure to multifocal brain ischemia. Treatment with indomethacin, PGI₂, and heparin eliminated neuron-disabling brain blood flows without altering early postischemic granulocyte accumulation. Granulocyte accumulation after 4 hours of reperfusion was not significantly different in control and treated dogs. The final amplitude of the cortical somatosensory evoked response in the treated group averaged 38.0 ± 13.6% (mean ± SEM) of the corresponding baseline value compared with 21.0 ± 4.6% in the control group, but this difference was not significant. (Stroke 1987;18:634–637)

Recent experiments in brain ischemia have demonstrated the formation of both cyclooxygenase- and lipoxygenase-derived metabolites of arachidonic acid during early postischemic reperfusion.¹-³ We have demonstrated the accumulation of granulocytes in ischemic areas following incremental air embolism. In the same model, we have shown that indomethacin, prostacyclin, and heparin improved the outcome of neuronal function and blood flow, acting presumably by reducing the production of thromboxane and prostacyclin, with replacement of prostacyclin to achieve reduced platelet aggregation and vasodilatation.⁴-⁶ Previous work indicated that heparin is necessary for enhanced recovery as well.³ Higgs et al. however, have demonstrated that indomethacin may increase leukocyte accumulation in models of inflammation and have postulated that this may be due to increased metabolism of arachidonic acid via lipoxygenase when the cyclooxygenase pathway is blocked.⁶ We expected, therefore, that our therapeutic combination might produce increased granulocyte accumulation after ischemia. If this were true, it would indirectly suggest that leukotrienes are mediators of leukocyte chemotaxis after ischemia and that inhibition of lipoxygenase might improve the outcome after ischemia by reducing leukocyte-induced damage.⁸-¹⁰

As a test of this hypothesis, we compared the cortical somatosensory evoked response (CSER), brain blood flow, and accumulation of [¹¹¹]In-labelled leukocytes in dogs subjected to 1 hour of multifocal ischemia induced by air embolism and treated with prostacyclin, heparin, and indomethacin with untreated ischemic animals.

Materials and Methods

The techniques for incremental air embolism, as well as the collection, labelling, and detection of leukocytes in brain tissue, have been described in detail.¹⁵ For this experiment, 21 conditioned, male mongrel dogs (Canis familiaris) weighing 9–15 kg were premedicated with 1.1 mg/kg xylazine and 0.05 mg/kg atropine s.c.; surgical anesthesia was induced with 80 mg/kg α-chloralose and maintained with doses of 20 mg/kg as needed. The dogs were intubated and mechanically ventilated. Femoral arterial and venous catheters were inserted for monitoring of blood pressure, administration of drugs, and intermittent monitoring of blood gases, pH, and hematocrit. The right internal carotid artery was catheterized for administration of air, and electrodes were mounted in the skull for recording the response evoked by left median nerve stimulation, using a Nicolet CA-1000 evoked response system.

These experiments were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Resources, National Research Council, Department of
Health, Education, and Welfare publication no. (NIH) 78-23.

Prior to ischemia, 102 ml of blood was collected from a femoral artery catheter and placed in 18 ml of anticoagulant citrate dextrose solution (ACD-Formula A, Fenwall Laboratories, Deerfield, Ill.). One hundred milliliters of Ringer's lactate solution was injected immediately after blood sampling. A Beckman JE-6B elutriator was used to collect a sample of granulocytes with 95% purity and viability as assessed by microscopy and trypan blue exclusion.

The granulocyte suspension was incubated with 1 mCi of $^{111}$Inoxine and then centrifuged and washed to assure that the radioactivity was present only in the granulocytes and was not bound to plasma protein. The cells were then resuspended in saline and reinfused 5 minutes prior to ischemia.

Five CSERs were measured prior to ischemia, and the average P1–N1 amplitude of these 5 responses was calculated as a baseline. Ischemia began with the infusion of 50 μl of air into the right internal carotid artery. CSERs were obtained every 2 minutes during the next 60 minutes, and repeated doses of 20-50 μl of air were used according to a predetermined algorithm to maintain the P1–N1 amplitude of the CSER at 10-20% of baseline. After ischemia, dogs received either no therapy (control) or 4 mg/kg indomethacin (Merck Sharp & Dohme, West Point, Penn.) i.v. in Tris buffer, 300 IU/kg heparin i.v., and a continuous infusion of prostacyclin (PGI2, Upjohn Co., Kalamazoo, Mich.) in Tris buffer at a rate of 90–140 ng/kg/min. CSER was measured every 10 minutes for 4 hours following ischemia. Recovery was calculated as percent of the baseline amplitude.

At the conclusion of the 4-hour recovery period, 60 μCi/kg of $^{14}$C]iodoantipyrine was infused over 1 minute, and arterial blood samples were collected from a previously placed femoral shunt for later calculation of cerebral blood flow by autoradiography. The dog was killed rapidly with KCl injected into the right ventricle, and the brain was removed and frozen at −50 to −60°C in Freon suspended over liquid nitrogen. Later, the brain was divided coronally into 3 segments, each containing symmetrical portions of the right and left hemispheres, and termed anterior (containing the head of the caudate nucleus), middle (containing the thalamus), and posterior (containing the posterior portion of the lateral ventricle and the adjacent hippocampal formation). Twenty-micron sections were cut from each segment for autoradiography, and samples of the cortex were excised from the superolateral right and left hemispheres of each segment. The cortical samples were weighed and counted without further preparation to determine the $^{111}$In activity in counts per minute per gram in each, and a right (injured) minus left (noninjured) difference was calculated as an expression of leukocyte accumulation.

Eight dogs were excluded from the therapy comparison. Two dogs in the control group had more than 70% of the readings during ischemia <10% of baseline CSER and were excluded from the therapy comparison but were included in the correlation of leukocyte accumulation and severity of ischemia. Another dog was excluded because of insufficient ischemia (fewer than 60% of readings were <20% of baseline). One dog in the treatment group had profound hypotension during PGI2 infusion despite the infusion of only 50 ng/kg/min and volume expansion with Ringer's lactate. One dog in the treatment group suffered an extubation accident during the recovery phase, and a single dog in each group experienced significant hemorrhage from the carotid catheter. An unexplained electrical artifact in 1 treatment dog made interpretation of the CSER impossible.

Single comparisons between the control and treatment groups were made using Wilcoxon's rank sum test, and estimates of the Type II error were based on the F distribution. We considered results significant at p < 0.05.

### Results

Hematocrit, pH, PaCO2, PaO2, rectal temperature, and mean arterial pressure were not different in the control and treatment groups during the baseline, ischemia, and therapy sampling times. We attempted to ensure that the duration and severity of ischemia in both groups was similar by comparing the amount of air injected during the ischemic period (0.22 ± 0.11 ml, control; 0.15 ± 0.07 ml, treated), the proportion of CSER readings <10% of baseline P1–N1 amplitude during ischemia (32.9 ± 16.5%, control; 35.5 ± 18.5%, treated) and the proportion of CSER readings <20% of baseline (71.7 ± 11.9%, control; 71.2 ± 14.6%, treated) in both groups. There were no significant differences between the groups for any index. Treatment with indomethacin, PGI2, and heparin did not produce significant recovery of the CSER amplitude 4 hours after ischemia when compared with control (38.0 ± 13.6% vs. 21.0 ± 4.6%, mean ± SEM). However, this negative conclusion is not statistically powerful, having a Type II error probability of <0.07.

The percent of injected granulocyte suspension $^{111}$In activity that was not in cells was 1.7 ± 1.0% for all 13 experimental dogs. The $t_0$ for clearance of labelled cells from the blood was determined in 7 animals, and did not differ from published values. $^{111}$In activity after 4 hours of posts ischemic reperfusion were the same in both groups. The values were (mean ± SEM) 3,298 ± 763 cpn/g in the control group and 3,807 ± 665 in the treated group. Based on the sample size and SD of our samples, a 50% enhancement of granulocyte accumulation could have been shown with a power of 0.50.

Our method of preparing sections for autoradiography yielded sections that allowed identification of 9 gray matter and 5 white matter structures for each dog. Blood flows were calculated for each area and averaged for the gray and white matter in each hemisphere. Average flows were not different in the two groups, probably because of the multifocal nature of the insult. The number of dogs with very low flows (<15 ml/100
g/min in gray and <6 ml/100 g/min in white matter) differed markedly, however. None of the treated dogs had any areas that fell below these neuron-disabling flows while 2 of the 7 controls had one or more areas with very low flows.

Two control dogs were not considered in the therapy comparison because they were severely ischemic and would have biased the control group toward a more severe insult. When the leukocyte accumulations of these dogs were considered with those of the rest of the control animals, 2 significant correlations emerged. The accumulation of leukocytes was correlated with the severity of the ischemic insult measured by the number of readings <10% of baseline during ischemia (r = 0.817, Figure 1) and also was correlated with the number of areas of very low blood flow in each dog (r = 0.912, Figure 2).

**Discussion**

Despite the elimination of areas of very low blood flow with therapeutic manipulation of the cyclooxygenase pathway of arachidonic metabolism, we demonstrated no change in leukocyte accumulation 4 hours after ischemia induced by air embolism. These results appear to reject our initial hypothesis and suggest either very little effect on lipoxygenase product formation by indomethacin in this model or only a minor role for lipoxygenase products (leukotrienes and hydroxy acids) as granulocyte chemotaxins in the early postischemic reperfusion period.

It is not possible to use the results of this experiment to completely reject a role for lipoxygenase products in the postischemic accumulation of leukocytes. Heparin may have an effect on granulocyte accumulation although this is variable,12-13 and Higgs et al14 demonstrated in vivo inhibition of granulocyte adherence using PGI₂ in doses similar to those employed in our study. The net result of the competing effects of PGI₂, heparin, and indomethacin may therefore result in no change in leukocyte accumulation. In addition, the effectiveness with which indomethacin increases lipoxygenase product formation varies depending on dose and species used,6,11,15-17 and therefore we cannot be certain that the dose of indomethacin we used would have been effective. Finally, air embolism causes extensive endothelial injury,18,19 which may promote granulocyte adhesion without the need for chemotaxins.10,20 Other models of ischemia may not cause such extensive endothelial damage and may therefore be more sensitive to changes in chemotactic mediators.

Although we did not influence granulocyte accumulation with the therapy used, we were able to show a correlation of leukocyte accumulation with both the severity of ischemia and with the number of areas of very low blood flows. The presence of granulocytes in ischemic areas within 4 hours of injury supports but does not prove a role for granulocyte participation in postischemic reperfusion injury. Work by Romson et
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al10 in leukopenic dogs, and Mullane et al12 using a lipoxigenase inhibitor, suggest that granulocytes worsen damage in models of myocardial infarction. Granulocytes produce arachidonic acid metabolites such as prostaglandins, leukotrienes, and hydroxy acids, capable of producing local vasoconstriction and increasing microvascular permeability.8,9,23 The potent granulocyte chemotactic properties of the leukotrienes and hydroxy acids allow granulocytes to function in a self-amplifying fashion. Granulocytes can also produce cellular injury through superoxide anion and other free radical products, hydrolytic enzyme release from intracellular granules, and by the synthesis of platelet activating factor.9,23–27 These theoretical considerations, as well as our data, suggest that a search for therapies that modify granulocyte accumulation may yield important benefits in postischemic neuronal recovery.

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References


Key Words • granulocytes • stroke • prostaglandins • leukotrienes • air embolism
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