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SECURITY CLASSIFICATION OF THIS PAGE

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AD-A204 679

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NMRI 88-27	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Naval Medical Research; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION: Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code): Bethesda, Maryland 20814-5055	7b. ADDRESS (City, State, and ZIP Code): Department of the Navy, Washington, D.C. 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Naval Medical Research and Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code): Bethesda, Maryland 20814-5055	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
61153N	MR04101	01.1005	DN277001

11. TITLE (Include Security Classification)
Effects of Prostacyclin, Indomethacin, and Heparin on Cerebral Blood Flow and Platelet Adhesion after Multifocal Ischemia of Canine Brain

12. PERSONAL AUTHOR(S)
Kochanek,P.M.; Dutka,A.J.; Kumaroo,K.K.; Hallenbeck,J.M.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1988	7

16. SUPPLEMENTARY NOTATION
reprinted from: Stroke v.19, n.6, June 1988, pp.693-699

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Cerebral blood flow; Cerebral ischemia; Heparin; Dogs; Indomethacin; Platelets; Prostaglandins

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Phyllis Blum, Information Services Division	202-295-2188	ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

Effects of Prostacyclin, Indomethacin, and Heparin on Cerebral Blood Flow and Platelet Adhesion After Multifocal Ischemia of Canine Brain

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K.K. Kumaroo, PhD, and John M. Hallenbeck, MD

Seven anesthetized dogs treated with prostaglandin I_2, indomethacin, and heparin were compared with 12 controls to test the hypothesis that the salutary effect of treatment on recovery of neuronal function and cerebral blood flow (CBF) after ischemia is coupled to the inhibition of platelet accumulation. In this model of right hemisphere multifocal ischemia, cortical somatosensory evoked response (CSER) amplitude, ^{14}C autoradiographic blood flow, and ^{111}In-labeled platelet accumulation were measured. The ratio of injured to noninjured hemispheric ^{111}In activity (cpm/g) provided an index of platelet accumulation. Treatment improved CBF of the injured hemisphere compared with control after 4 hours of reperfusion (74 ± 17 versus 53 ± 13 ml/100 g/min, $p < 0.05$), and it enhanced recovery of CSER amplitude (percent of baseline) after 1 hour of reperfusion compared with control (27.1 ± 4.7% [treatment] versus 15.5 ± 2.8% [control], $p < 0.05$). However, the effect on CSER was not sustained after 4 hours of recovery. Despite these effects on CSER and CBF, treatment failed to inhibit ^{111}In-labeled platelet accumulation in the injured hemisphere (1.7 ± 0.3% [treatment] versus 1.5 ± 0.1% [control], $p > 0.05$). Platelets may adhere to damaged endothelium despite aggressive platelet antiaggregant therapy. (*Stroke* 1988;19:693–699)

The administration of prostaglandin (PG) I_2 and indomethacin with heparin enhances early recovery of cortical somatosensory evoked response (CSER) and prevents the development of zones of impaired reperfusion in models of multifocal and global brain ischemia.[1,2] Previous experiments[1-7] support the concept that an altered balance of thromboxane A_2 (TXA_2) and PGI_2 contributes to postischemic hypoperfusion. The therapeutic combination is designed to modify this balance.

Altered prostaglandin synthesis after ischemia represents one aspect of a broader hypothesis that tissue damage in the brain during ischemia causes a multifactorial sequence of events resulting in a focal increase in microcirculatory resistance during reperfusion.[1,2,8] This sequence is termed the blood–damaged tissue interaction. The platelet–endothelial interaction represents one part of this process potentially important to postischemic reperfusion.[8] TXA_2 and PGI_2 production at the platelet–endothelial interface occurs through selective metabolism of the cyclic endoperoxide PGH_2.[9-12] Platelet thromboxane synthetase converts PGH_2 to TXA_2 during platelet aggregation.[10,11] TXA_2 is a potent stimulant of platelet aggregation and vasoconstriction[6,10,11] and is produced during reperfusion after cerebral ischemia.[5,13,14] Endothelial PGI_2 synthetase converts PGH_2 to the vasodilatory PGI_2,[12] which strongly inhibits platelet aggregation.[15,16]

Platelets accumulate in the injured hemisphere of the brain after embolic and vaso-occlusive ischemia.[9,17,18] Accumulation is prominent after 4 hours of reperfusion in areas with low blood flow.[9]

In light of platelet TXA_2 synthesis and the likelihood that platelet accumulation is related to platelet aggregation, we hypothesized that the salutary effect of PGI_2, indomethacin, and heparin on cerebral blood flow (CBF) and CSER during reperfusion is coupled to the inhibition of platelet accumulation. To test this hypothesis, we examined the effect of PGI_2, indomethacin, and heparin treatment on ^{111}In-labeled platelet accumulation, CBF, and CSER after severe multifocal brain ischemia in dogs.

Materials and Methods

Twenty-two male mongrel dogs (9–15 kg) were anesthetized with α-chloralose according to previous methods.[1] Dogs were mechanically ventilated and monitored for mean aortic blood pressure (MAP), hematocrit, arterial blood gases, and end-tidal CO_2 and O_2 tensions, and they were prepared for recording of CSER[1,9,19] and for blood sampling during the CBF study.[2,8] Rectal temperature was maintained at

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Supported by the Naval Medical Research and Development Command under Work Unit MR040101-1126. The opinions and assertions contained herein are the private views of the investigators and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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Received April 20, 1987; accepted January 6, 1988.

37.1 ± 0.1° C (mean ± SEM). A thermodilution catheter was placed via the femoral vein into the pulmonary artery to determine pulmonary capillary wedge pressure (PCWP) and cardiac output (CO) according to previous methods.[1] A catheter was inserted into the right cephalic vein to infuse PGI_2. The right internal carotid artery was catheterized with PE-50 tubing.

Before ischemia, 102 ml blood was collected in 18 ml anticoagulant citrate dextrose solution (ACD-Formula A, Fenwall Laboratories, Deerfield, Illinois). ^{111}In-labeled platelets were prepared from this blood sample.[9,18] Platelet reactivity was periodically checked by aggregation studies with ADP.[9,18] To restore blood volume, erythrocytes obtained from the initial 102-ml blood sample were reinfused 1 hour before ischemia. Labeled platelets were infused during the final 5 minutes of ischemia.

The dogs were placed in a stereotaxic apparatus and prepared for CSER recording.[1,9,19] After exposure of the skull, screw electrodes were positioned over the right sensorimotor cortex and the nasal bones. Stimulating electrodes were positioned in the left upper foreleg such that the median nerve was between them. Potentials were generated and recorded with a Nicolet CA-1000 evoked response system (Madison, Wisconsin).[1,19,20]

Focal ischemia was induced in the right hemisphere by infusing 50 μl air into the right internal carotid artery. CSERs were measured every 90 seconds during the 1-hour ischemic period. Intermittent boluses of 20–50 μl air were injected into the right internal carotid artery to maintain suppression of the P1–N1 amplitude of the CSER at 10–20% of its baseline value. Immediately after ischemia, nine dogs were treated with PGI_2, indomethacin, and heparin, while 13 dogs received no therapy. CSER was measured every 10 minutes during the 4-hour recovery period, and the P1–N1 amplitude (percent of baseline) was recorded. Additional control groups treated with PGI_2, indomethacin, or heparin alone or in any combination of two agents were previously shown not to significantly affect CSER or CBF when given after ischemia in this model.[1]

PGI_2 (Upjohn, Kalamazoo, Michigan; 25 μg/ml in 0.1 M Tris-HCl/0.15 M NaCl at pH 8.5) was continuously infused during the first hour of recovery at 100 ng/kg/min.[1,2] Thereafter, the PGI_2 infusion rate was increased by 10 ng/kg/min every 10 minutes as long as MAP remained > 100 mm Hg. Indomethacin (Indocin; gift of Merck, Sharp & Dohme, West Point, Pennsylvania) was administered as an initial 4 mg/kg bolus immediately after the start of the PGI_2 infusion,[1,2] and after 2 hours of treatment a 2 mg/kg bolus was administered. Heparin (American Biologics, Philadelphia, Pennsylvania) was given as a 300 unit/kg bolus, and after 1 hour this bolus was followed by a continuous infusion of 25 unit/kg/hr (Figure 1).

After the 4-hour recovery period, a 1-minute [^{14}C]iodoantipyrine autoradiographic CBF study was performed.[2,8,9] Later, the brain was divided coronally into three segments, each containing symmetric portions of the right and left hemispheres, which were

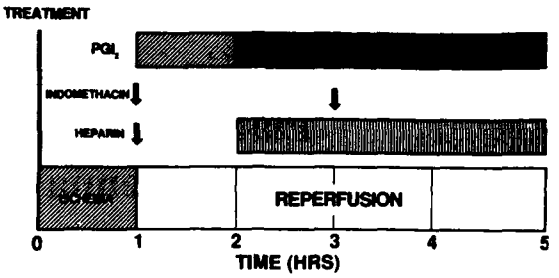

FIGURE 1. *Drug regimen used in treatment group. Prostaglandin (PG) I_2 (100 ng/kg/min), indomethacin (4 mg/kg), and heparin (300 unit/kg) were administered immediately after ischemia. Because cortical somatosensory evoked response recovery was unable to be maintained beyond the 1st hour of reperfusion in previous studies,[20] supplemental dose of indomethacin, continuous heparin infusion, and progressive increase in PGI_2 infusion rate were begun after the 1st hour of reperfusion.*

labeled "anterior" (containing the head of the caudate), "middle" (containing the thalamus), and "posterior" (containing the hippocampus). CBF was calculated from 20-μm sections, while 40-μm sections allowed visual detection of platelet accumulation in the tissue and relative CBF rates to be determined (dual-label autoradiography).[9,18,21] Elution of the [^{14}C]iodoantipyrine from the 40-μm sections with methanol enhanced visual detection of ^{111}In-labeled platelet accumulation.[9,18] After sectioning, cortical samples were excised from homologous watershed areas of the right and left hemispheres of each segment (anterior, middle, and posterior). The right hemisphere constituted the injured side, and the left hemisphere constituted the noninjured side. Samples were weighed and counted on a gamma counter (LKB Wallac CompuGamma, Turku, Finland). Radioactivity, expressed as counts per minute per gram of tissue, provided an index of platelet accumulation in the three defined brain segments, and a right: left ratio was calculated for each brain segment. A mean hemispheric right: left ratio was determined for each dog from the mean of the three segmental right: left ratios.

Three dogs were excluded from data analysis. One control dog was unable to be consistently suppressed below 20% of baseline CSER during ischemia. One treated dog experienced a large intraparenchymal hemorrhage early in treatment. The PGI_2 solution infiltrated subcutaneously in one dog.

Individual comparisons between control and treated groups were made with the Wilcoxon rank sum test and Student's t test. Controlled variables and CSER data from both groups were compared with the two-way analysis of variance for repeated measures. Results were considered significant at $p < 0.05$.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication No. (NIH) 86-23.

Table 1. Controlled Physiologic Variables in Dogs

Variable	Before ischemia	After 1 hour reperfusion	Before cerebral blood flow study
pH			
Control	7.36 ± 0.03	7.35 ± 0.05	7.35 ± 0.03
Treatment	7.38 ± 0.05	7.34 ± 0.06	7.35 ± 0.04
Hematocrit			
Control	43 ± 5	43 ± 4	41 ± 7
Treatment	40 ± 6	40 ± 8	40 ± 5
$Paco_2$ (mm Hg)			
Control	36 ± 3	35 ± 3	33 ± 3
Treatment	36 ± 3	36 ± 4	34 ± 2
Pao_2 (mm Hg)			
Control	92 ± 5	95 ± 7	97 ± 8
Treatment	93 ± 7	91 ± 8	95 ± 4
Mean aortic blood pressure (mm Hg)			
Control	130 ± 24	116 ± 18	128 ± 15
Treatment	123 ± 22	108 ± 20	112 ± 31
Cardiac output (l/min)			
Control	1.36 ± 0.23	1.50 ± 0.37	1.50 ± 0.38
Treatment	1.47 ± 0.21	1.88 ± 0.36	1.31 ± 0.17
Temperature (°C)			
Control	37.8 ± 1.2	36.6 ± 1.3	37.5 ± 1.0
Treatment	37.1 ± 1.1	36.2 ± 1.4	37.0 ± 0.6
Pulmonary capillary wedge pressure (mm Hg)			
Control	8 ± 4	8 ± 3	7 ± 2
Treatment	8 ± 3	8 ± 4	7 ± 3

Values are mean ± SD for 12 control, 7 treatment dogs.

Results

Hematocrit, pH, $Paco_2$, Pao_2, MAP, PCWP, and temperature (Table 1) did not differ significantly between the two experimental groups at any of the three sampling times. Although there was no significant difference between the treated and control groups in CO at any time (Table 1), there was a significant interaction between treatment and time for CO ($p < 0.0025$). CO increased 29% after 1 hour of treatment ($p < 0.02$). In contrast, there was no significant change in CO during the same period in untreated dogs.

The amount of air administered, the percentage of readings <20% and <10% of baseline CSER amplitude during ischemia, and the CSER amplitude at the end of ischemia were used as indexes of severity of ischemia (Table 2).[20] There were no significant differences between groups with any index.

There was a significant interaction between treatment and time for CSER amplitude recovery ($p < 0.025$) (Figure 2). To compare these results with our previous studies,[1,9,20] the two groups were compared at 1 and 4 hours after ischemia. The percent recovery of baseline CSER amplitude at 1 hour after ischemia

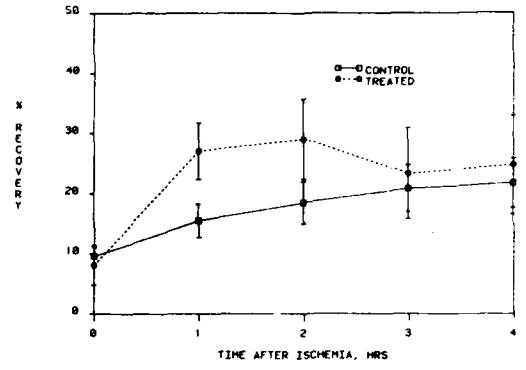

FIGURE 2. *Effect of prostaglandin (PG) I_2, indomethacin, and heparin treatment on recovery of cortical somatosensory evoked response (CSER) (% baseline P_1–N_1 amplitude) versus time after completion of ischemia. There was significant interaction between treatment and time for recovery* ($p < 0.025$). *Treatment significantly enhanced CSER amplitude during 1st hour of reperfusion compared with control* ($p < 0.05$). *Effect could not be sustained to 4 hours.*

was 27.1 ± 4.7% versus 15.5 ± 2.8% (mean ± SEM) in the treated and control groups, respectively. This represents significantly enhanced CSER amplitude recovery after 1 hour of treatment ($p < 0.05$). Even if the two treated dogs that met protocol for ischemia but were excluded are considered, recovery at 1 hour was still significantly enhanced when compared with control (25.4 ± 3.7% [treatment] versus 15.5 ± 2.8% [control]). However, this effect on CSER was not sustained, and no difference between the treated and control groups was observed after 4 hours of recovery (24.8 ± 8.2% versus 21.8 ± 4.1%, respectively, mean ± SEM, NS).

The mean ± SEM hemispheric right : left ratios of ^{111}In activity after 4 hours of reperfusion were 1.5 ± 0.1 and 1.7 ± 0.3 in the control and treated groups, respectively (Figure 3). These did not differ significantly.

Nine cortical and subcortical gray matter areas and five white matter areas were selected for blood flow readings. The average blood flows for dogs in the control and treated groups are shown in Table 3, subdivided by gray and white matter structures and by injured and noninjured hemispheres. When the two groups are compared by hemisphere and tissue type using Student's *t* test, the injured hemisphere gray

Table 2. Indexes of Severity of Ischemia in Dogs

	Last reading during ischemia	% CSER <10% baseline	% CSER <20% baseline	Air injected (μl)
Control ($n = 12$)	9.6 ± 1.5	85 ± 2	35 ± 6	259 ± 42
Treatment ($n = 7$)	8.1 ± 3.3	86 ± 3	44 ± 7	220 ± 20

Values are mean ± SEM.
CSER, cortical somatosensory evoked response.

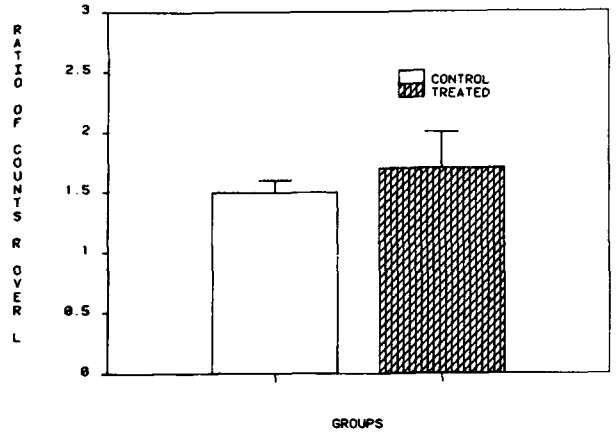

FIGURE 3. *Effect of prostaglandin I_2, indomethacin, and heparin treatment on ^{111}In-labeled platelet accumulation (mean hemispheric right: left ratio in ^{111}In activity) 4 hours after ischemia. Difference between groups was not significant.*

matter had significantly greater blood flow in the treated group than in the control group. The overall significance level is $p<0.05$ after applying the Bonferroni correction for multiple comparisons. In addition, if we consider the number of dogs that had neuron-disabling blood flows as previously defined (<6 ml/100 g/min in white matter and <15 ml/100 g/min in gray matter),[1] we find five control and only one treated dogs with these low flows.

Dual-isotope autoradiography with ^{111}In-labeled platelets and [^{14}C]iodoantipyrine permitted assessment of any relation between CBF and platelet accumulation. After elution of the [^{14}C]iodoantipyrine with methanol,[9,18,21] a hemispheric right–left difference in punctate platelet images was noted in six of 12 control and three of seven treated dogs. Simultaneous examination of the dual-label and methanol-extracted autoradiograms revealed two apparent patterns of platelet accumulation. In four of six untreated dogs with a visible right–left difference, a blush of ^{111}In activity appeared in severely oligemic areas (Figure 4).[9] In contrast, treated dogs exhibited punctate ^{111}In activity in a linear pattern that appeared to correspond to platelet accumulation in large blood vessels (Figure 5).

Discussion

Three general conclusions from this work will be discussed. First, treatment with PGI_2, indomethacin, and heparin produces early enhancement of CSER amplitude that cannot be sustained to 4 hours after ischemia. Second, treatment improves postischemic CBF in the injured hemisphere even as late as 4 hours after ischemia. Third, treatment fails to inhibit platelet accumulation in the injured hemisphere, although dense zones of platelet accumulation in areas of low blood flow are eliminated.

The enhanced recovery of CSER during the 1st hour of reperfusion in treated dogs confirms earlier studies with this regimen, although the lower percent CSER recovery reflects more severe ischemia in our study.[1,2] Similarly, the inability to sustain this effect beyond early reperfusion substantiates our more recent work.[20] Because it was unclear whether the inability to maintain enhanced CSER recovery was related to a waning drug effect after the 1st hour,[20] supplementation of the treatment regimen with an additional bolus of indomethacin, continuous heparin drip, and escalation of the PGI_2 infusion was instituted after the 1st hour of treatment. Supplementation did not sustain CSER recovery. During the infusion of the vasodilatory PGI_2, CO and PCWP were monitored as was reinfusion of erythrocytes from the initial 102-ml sample. With this protocol, intravascular volume was maintained as demonstrated by stable PCWP in both groups. However, there was a significant interaction between treatment and time for CO that paralleled recovery of CSER. The reason that significantly enhanced CSER amplitude recovery could not be maintained after 1 hour of reperfusion is unclear. However, the inability to maintain significantly enhanced CSER amplitude occurred despite sustained elimination of neuron-disabling blood flows. One possibility is that detrimental aspects of reperfusion not blocked by this treatment operate in the zones of ischemic damage that continue to be perfused.

Although the presence of neuron-disabling blood flows correlates with poor CSER recovery in this model,[1,9,20] it is not a necessary condition for poor recovery because only 50% of the untreated dogs had blood flows in this range. In addition, significantly enhanced CSER amplitude could not be maintained to 4 hours after ischemia despite sustained elimination of neuron-disabling blood flows throughout the 4-hour recovery period in all but one treated dog. This suggests that postischemic hypoperfusion is not the principal cause of neuronal injury in the postischemic period. Instead, hypoperfusion appears to be only one manifestation of a more fundamental process that is deleterious to the restoration of neuronal function in a postischemic zone. Instead of leading to tissue damage primarily through interference with oxygen and substrate delivery and through impaired clearance of metabolic wastes due to microcirculatory shutdown, the critical effect of the blood–damaged tissue interaction might be the production of mediators of direct tissue injury. Prime candidates for these mediators include free radicals, calcium, leukotrienes,

TABLE 3. **Average Blood Flow in Nine Cortical and Subcortical Gray Matter Areas and Five White Matter Areas**

Area	Treated ($n=7$)	Control ($n=11$)
Injured hemisphere gray	73.9±17.4*	53.0±12.9
Noninjured hemisphere gray	69.4±19.4	51.2±10.6
Injured hemisphere white	15.9±2.0	13.8±2.1
Noninjured hemisphere white	15.9±2.0	14.7±1.5

Values are mean ± SEM.
*Significantly higher than control by t test with Bonferroni correction for four comparisons ($p<0.05$).

FIGURE 4. *Dual-label autoradiograms* (top) *demonstrating cerebral blood flow and ^{111}In-labeled platelet deposition in brain sections from two representative control (ischemia without treatment) dogs* (left *and* right). *Blush of platelet accumulation is clearly observed in each methanol-extracted autoradiogram* (bottom) *corresponding to area of low blood flow in native autoradiogram.*

TXA_2, prostaglandins, platelet activating factor (PAF), and leukocyte and platelet accumulation with elaboration of their diverse mediators and activation of the complement, coagulation, and fibrinolytic systems.[5,9,13,14,17,19–32]

Platelet aggregation at sites of endothelial damage leads to increased vascular resistance and thrombosis through unbalanced TXA_2 synthesis.[1,10,33] However, platelets may produce tissue injury during reperfusion by other mechanisms. Platelets can trigger the intrinsic coagulation pathway through the activation of Hageman factor,[1,6,33] and platelet factor 3 can accelerate coagulation.[34] Platelets can increase vascular permeability by releasing granular constituents and by producing PAF.[35–37] Hydroxy acids and PAF are produced by platelets during aggregation and are potent granulocyte chemotaxins, as is platelet-derived complement activating factor.[38–40] Superoxide anion is also produced by platelets.[41]

Despite aggressive therapy directed at inhibiting platelet aggregation, platelet accumulation in the injured hemisphere after 4 hours of reperfusion was not inhibited. The failure to inhibit platelet accumulation is surprising in that studies support almost complete inhibition of platelet aggregation to all stimuli with the doses of PGI_2 used in our study. PGI_2 (30–100 ng/kg/min) inhibited platelet aggregation in dogs[42,43] and blocked ^{111}In-labeled platelet accumulation in canine pulmonary venous thrombosis.[44] In addition to the effects of PGI_2, indomethacin (4 mg/kg) decreased brain TXB_2 levels after ischemia,[5] and heparin (100–200 unit/kg) inhibited ^{111}In-labeled platelet accumulation in canine pulmonary embolism.[45]

Platelet adherence to damaged endothelium rather than platelet aggregation may be the major determinant of hemispheric platelet accumulation in this model. Subendothelial collagen, fibronectin, and Factor VIII/von Willebrand factor exposed on the damaged endothelium are determinants of local platelet adhesion in vitro.[46–50] PGI_2 inhibits platelet aggregation at concentrations 200 times lower than those required to inhibit platelet–endothelial adhesion,[51] suggesting in our study that PGI_2 may allow platelets to stick to damaged vascular tissue while limiting thrombus formation. Aspirin has been shown to inhibit thrombus formation in a carotid endarterectomy model, but a carpet of platelets remained on the vascular endothelium.[52] Although we were unable to detect a numerical difference in platelet accumulation, the autoradiograms differed in the two groups. Control dogs had large areas of low blood flow with a blush of platelets in the damaged area. Treated dogs had scattered punctate accumulations of activity in the damaged hemisphere. This difference is coincident with the elimination of areas of low blood flow with treatment, and it suggests that the production of areas with low blood flow may be related to platelet aggregation. Accumulation in treated dogs may represent adhesion to widely scattered areas of endothelial damage, which may be particularly apparent in this model of multifocal ischemia induced by air emboli.[53,54] That inhibition of another pathway for platelet aggregation (PAF) failed to block platelet accumulation further supports the role of platelet adhesion in this model.[55] In addition, Factor VIII/von Willebrand factor-depleted dogs demonstrated improved postischemic CBF and CSER recovery.[19,31,32]

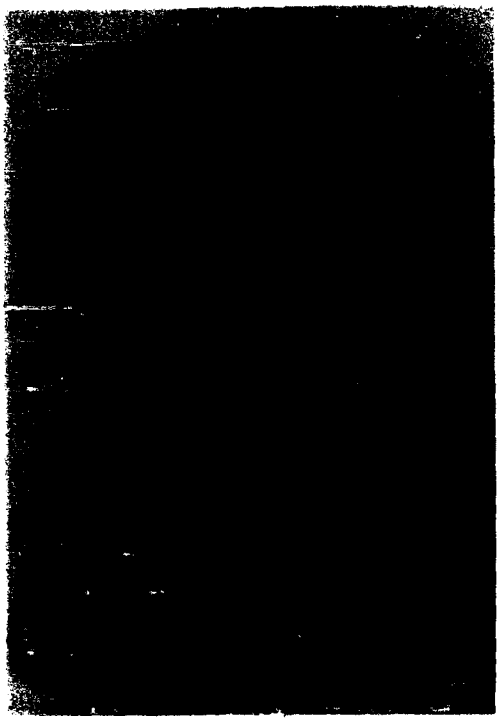

FIGURE 5. *Dual-label autoradiogram* (top) *demonstrating cerebral blood flow and ^{111}In-labeled platelet deposition in brain section from dog treated with prostaglandin I_2, indomethacin, and heparin. Punctate ^{111}In activity appears predominately in injured hemisphere on methanol-extracted autoradiogram* (bottom) *but in a vascular pattern. Areas with neuron-disabling blood flow are not observed.*

Although previous studies in this model[9,18] showed that platelet accumulation in areas of low blood flow in untreated animals was not due to hemorrhage or an increase in local brain blood volume, it is possible that selective vasodilation of the damaged hemisphere during treatment accounts for the enhanced right: left ratio in the treated group. This would be most likely if platelet adhesion was not inhibited. Vasodilation alone would unlikely account for the hemispheric difference in platelet accumulation since CBF in the damaged hemisphere was the same as that in the control hemisphere in treated dogs.

Aggressive platelet antiaggregant therapy with PGI_2, indomethacin, and heparin improves early CSER recovery and postischemic CBF, but it does not inhibit platelet accumulation or sustain the level of CSER recovery. Further research in the treatment of stroke might profitably involve inhibitors of platelet adhesion, including manipulation of Factor VIII/von Willebrand factor, fibronectin, or specific platelet adhesion receptors.[49,50,55]

Acknowledgments

The superb technical assistance of G.E. Sloan, J. DeJesus, C. Jones, M. Routh, J. Boogaard, and A. Winton is gratefully acknowledged. We also thank J. Santucci for secretarial assistance and R. Hays for data analysis.

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KEY WORDS • cerebral blood flow • cerebral ischemia • heparin • indomethacin • platelets • prostaglandins • dogs