**Title:** Platelet Activating Factor Receptor Blockade Enhances Recovery After Multifocal Brain Ischemia

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**Abstract:**
Platelet activating factor; stroke; ischemia; air embolism
PLATELET ACTIVATING FACTOR RECEPTOR BLOCKADE ENHANCES RECOVERY AFTER MULTIFOCAL BRAIN ISCHEMIA

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Summary

We treated four anesthetized dogs (Canis familiaris) with the platelet activating factor (PAF) receptor antagonist kadsurenone prior to 60 min of multifocal ischemia induced by air embolism, and measured neuronal recovery, blood flow and autologous 111In-labeled platelet accumulation for 4 h after ischemia. Four anesthetized animals with identical ischemia served as controls. Kadsurenone (3 mg/kg) administered 5 min prior to ischemia and continuously (1 mg/kg/hr) throughout ischemia and recovery significantly enhanced recovery of cortical somatosensory evoked response (CSER) amplitude (7 of baseline) when compared to controls (27-36% vs 9-14%, p < 0.05). We estimated platelet accumulation as radioactivity (cpm/g tissue) in the injured hemisphere minus that in the non-injured hemisphere. Kadsurenone-treated animals did not exhibit significantly altered 111In-labeled platelet accumulation when compared to controls (6158 ± 2380 vs 9979 ± 3852, mean ± SEM). Beneficial effects of PAF receptor blockade other than those on platelet accumulation may be involved.

Experimental evidence supports the accumulation of platelets in regions of low blood flow following multifocal cerebral ischemia and the production of platelet aggregates following stroke (1,2). Inhibition of platelet activation is a logical approach to prevention and therapy of cerebral vascular disease. The unstable metabolites of arachidonic acid initiate one pathway of platelet activation, and inhibition of their production improves outcome after multifocal ischemia and may prevent stroke (1,2). Platelet activating factor (PAF) is a very powerful stimulus to platelet aggregation in rabbits, humans and dogs, with effective doses in the nanomolar range (5,6). PAF has a specific membrane protein receptor on platelets, leukocytes, and smooth muscle (6) and produces leukocyte activation and smooth muscle contraction, in addition to aggregating platelets (6). We report the effectiveness of a specific PAF receptor blocking agent, kadsurenone, in improving the outcome of multifocal
brain ischemia induced by air embolism (7,8,9).

Methods

Eight conditioned male mongrel dogs were anesthetized with alpha chloralose and mechanically ventilated. Appropriate catheters were inserted to allow for monitoring of blood pressure, sampling of arterial blood for periodic hematocrit and blood gas measurements, and infusion of anesthesia and drugs. Temperature was maintained between 37.5 and 38.5 °C with warming pads. A PE-50 catheter was placed in the right internal carotid for the injection of air. After initial preparation, 102 mls of blood were withdrawn into 18 mls citrate and the platelets were separated and labelled with ^111 In as previously described (1). The red cells were reinfused, and electrodes mounted in the skull for recording the cortical response evoked by left median nerve stimulation (CSER).

The PI-NI amplitude of five responses was averaged as a baseline value, then the kadsurenone or the kadsurenone vehicle was infused. The evoked responses were then re-recorded to be certain there was no direct effect of the drug. The ischemic period then began with the injection of 50 microliters (µl) of air into the right internal carotid. Every 30 seconds for the next hour, the CSER was measured, and doses of 20-40 µl were given according to a predetermined, rigid rule system designed to maintain the PI-NI amplitude between 10 and 20% of the baseline value (3).

Immediately after ischemia, the autologous ^111 In-labeled platelets were infused and CSER measurements were made every 10 min during a 4 h recovery period. At the end of ischemia, ^14 C iodoantipyrine was infused for the autoradiographic determination of blood flow after euthanasia with saturated KCl, the brain was removed, frozen in freon at -70 °C and each hemisphere divided coronally into 3 segments. Samples of watershed area cortex from each segment and each hemisphere were excised, weighed, and counted on a gamma counter without further preparation. The right-left hemisphere difference (cpm/g) of ^111 In activity was then calculated for each segment and averaged to give a mean for each animal (1). The frozen brain was then cut into 20 micron sections for autoradiography.

Four animals were treated with an intravenous bolus of the PAF receptor antagonist kadsurenone (3 mg/kg) followed by a continuous intravenous infusion (1 mg/kg/hr) for the entire 5 h duration of each experiment. Each 10 mg aliquot of kadsurenone was dissolved in 200 µl of dimethyl sulfoxide (DMSO) and then suspended in 20 ml of phosphate buffered saline (PBS) by rapid vortexing at 50-60 °C immediately prior to infusion (7). Four control animals were administered an equal volume of DMSO/PBS vehicle in an identical manner. The control and treated animals were done alternately.

Samples of citrated blood were removed prior to drug administration and at 5, 15, 60 and 240 min after therapy from all treated animals and from 2 controls. Platelet rich plasma (PRP) obtained from these samples was employed in aggregation studies with threshold concentrations of PAF (0.1-0.15 µM L phosphatidyicholine, B-acetyl, O-alkyl; Sigma, St. Louis) to assay for evidence of in vivo drug effect (7). Percent inhibition was calculated using the maximal transmittance at 1 min after stimulation with PAF. Two-way analysis of variance was applied to the CSER data to test the effect of time and treatment. Individual comparisons were made using Wilcoxon rank sum. All results are reported as mean ± standard error.

Results
The mean evoked response control amplitude prior to administration of kadsurenone was $233.6 \pm 31.9 \mu V$ (4 dogs). Five min after injection of kadsurenone, the mean amplitude was $231.6 \pm 30 \mu V$; there is no significant difference between these values.

We estimated the severity of ischemia in both groups by comparing the following indices: 1) the amount of air administered ($0.31 \pm 0.10 \text{ ml control, vs } 0.30 \pm 0.04 \text{ ml treated}$); 2) the percent of readings during ischemia that were $< 20\%$ of baseline CSER amplitude ($84 \pm 6 \text{ vs } 82 \pm 7$); 3) the percent of readings during ischemia that were $< 10\%$ of baseline CSER amplitude ($31 \pm 11 \text{ vs } 43 \pm 11$); 4) the CSER amplitude ($\%$ of baseline) at the end of ischemia ($9 \pm 1 \text{ vs } 11 \pm 1$). No difference between groups was detected. Platelet aggregation induced by $0.1 \mu M$ PAF in animals receiving kadsurenone was inhibited $95 \pm 5\%$ at 15 min and $46 \pm 18\%$ at five hours following initial infusion of the drug. The response of platelets in PRP obtained from control animals ($n = 2$) to PAF at all 4 time points was unchanged from pre-ischemic aggregation.

**FIG. 1**

Effect of Kadsurenone treatment on CSER recovery ($\%$ of baseline PI-N1 amplitude) versus time after onset of ischemia. The abscissa shows time after onset of ischemia; the ordinate is the percent of baseline evoked response. The difference between treated and control groups was significant $p < 0.05$. 
The time course of CSER amplitude recovery is shown below. A two-way ANOVA for repeated measures applied to this data shows a significant difference in favor of treatment ($p < 0.05$) with no significant effect of time, although the treatment effects appeared most marked during the first hour of recovery. The Wilcoxon rank test shows a significant difference in recovery 20 min after ischemia which persists at 1 hr. Percent recovery at 20 min, 1 hr, and 4 hr was $35 \pm 3\%$, $36 \pm 3\%$, and $27 \pm 3\%$ in treated animals. The control recoveries at the same time points were $11 \pm 3\%$, $12 \pm 2\%$, and $14 \pm 7\%$. Despite enhanced CSER recovery, kadsurenone did not significantly alter autologous 111In-labeled platelet accumulation after 4 h of recovery. The injured hemisphere minus control hemisphere CPM/g value (average of anterior posterior and middle segments), mean and SEM for four animals, was $6138 \pm 2386$ (treated) and $9779 \pm 3632$ (controls).

The method used to cut the brain yielded sections that allowed identification of 9 gray matter areas and 6 white matter areas for each animal. The blood flow in each area was calculated from densitometer readings and averaged for the gray and white matter areas for each group. There was no difference in the blood flow in any single area between the groups, nor was there any difference in average blood flow. The average gray matter flow (ischemic hemisphere, ml/min/100g) in the control group was $68 \pm 15$ and $120 \pm 58$ in the treatment group. White matter flow (ischemic hemisphere) was $18 \pm 7$ in the control and $33 \pm 14$ in the treatment group. Since air embolism produces areas of very low flows surrounded by areas of higher flows, which tend to average to normal, we also calculated the number of animals in both groups that had flows below $6\text{ ml/min/100g}$ in the white matter, and $15\text{ ml/min/100g}$ in the gray matter. These flows are associated with severe neuronal dysfunction (3). The treatment group had no flows in this range, but two animals in the control group had very low flows, one having 10 areas of severe damage, another having one area.

**Discussion**

Our results demonstrate that kadsurenone administered prophylactically improved outcome after multifocal cerebral ischemia induced by air embolism. The improved outcome is manifested by improved evoked response amplitude, but this is not accompanied by any change in platelet accumulation. The estimate of platelet accumulation has a large variance, however, and thus this result is not statistically powerful. Kadsurenone infusion had no effect on evoked response amplitude prior to ischemia, thus its effect would seem to be on pathophysiological mechanisms of ischemia.

We have previously shown that platelets accumulate in areas of low blood flow following ischemia (1). Platelet aggregates may well contribute to worsening blood flow in the ischemic region, thereby impeding recovery. The apparent dissociation improved outcome and continued platelet accumulation with a therapy designed to inhibit platelet function is therefore surprising, but there are several reasonable explanations for this dissociation. Although we were able to show that the doses used in the study inhibited platelet aggregation induced by 0.1 micromolar PAF in peripheral arterial blood, kadsurenone is a reversible competitive inhibitor of the PAF receptor (7,8,9), and we have no data on the concentration of the drug in the affected brain area. Hence, it is possible that local concentrations of PAF in the injured zone may have exceeded the threshold...
for aggregation. In addition, it is probable that PAF is not the only mediator of platelet aggregation involved in the process of platelet accumulation during ischemia. Metabolites of arachidonic acid have powerful effects on platelets and manipulation of this system improves recovery following ischemia (3,10,11). Exposure to subendothelial proteins is a powerful stimulus to platelet aggregation (12). It is likely that inhibition of multiple systems of platelet activation would be required to completely eliminate accumulation. It is even possible that accumulation of platelets represents adhesion to damaged endothelium that does not require platelet activation (13), and therefore would not be affected by inhibitors of platelet aggregation. Finally, it is possible that platelet accumulation may have differed at earlier time points when the evoked response recovery differed.

The effects of PAF are not limited to platelet activation, and inhibition of these other effects may be responsible for the improved CSER recovery. PAF stimulates leukocyte aggregation and production of superoxide anion, leukotriene B4, and PAF (14). We have shown that leukocytes accumulate in the ischemic area within four hours following air embolism (15). Inhibition of PAF induced activation may therefore reduce the damage caused by these leukocytes. In the absence of platelets or leukocytes, PAF causes coronary vasoconstriction and decreased contractility (16,17) and mediates the hypotension produced by endotoxin (7). Injected into the skin, it produces edema (18). Kadsurenone antagonizes the hypotensive response and binds to receptors on leukocytes and smooth muscle (5,19). Thus, it is possible that the beneficial effect of kadsurenone may be due to reduction of edema or to improvements in local collateral circulation, although the extent to which PAF is involved in these phenomena in the brain is unknown. There is no direct evidence for the existence of PAF receptors in the brain, although the peripheral anti-PAF receptor effects of triazolobenzodiazepines, which are psychoactive, have led to speculation about receptor similarity (20).

Inhibitors of PAF represent a novel approach to treatment of ischemia. Results of this study suggest that further work in this area might be promising, but do not prove that PAF is involved in brain injury following ischemia.

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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, DHHS Publ. No. (NIH) 85-23.

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