

Infarct Reduction by the Platelet Activating Factor Antagonist Apafant in Rats

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Background and Purpose: Recent findings suggest a key role for platelet activating factor in neuroinjury. For this reason we evaluated the effects of the platelet activating factor antagonist apafant (4-(2-chlorophenyl)-9-methyl-2[3(4-morpholinyl)-3-propanon-1-yl][6*H*-thieno[3.2-*f*][1.2.4]triazolo]4,3-1][1.4]diazepine on infarct volume and local cerebral blood flow following irreversible occlusion of the left middle cerebral artery in rats to assess the direct and vascular components of apafant's action.

Methods: We measured infarct volume 48 hours after middle cerebral artery occlusion. The effect of multiple doses of apafant (30 mg/kg p.o.) was tested in both pretreatment ($n=8$) and posttreatment ($n=8$) groups. In the pretreatment group apafant was given 30 minutes before and 2, 6, and 18 hours after occlusion. Rats of the posttreatment group received apafant 1, 6, and 18 hours after middle cerebral artery occlusion. We also examined the effect of a single dose of apafant given 30 minutes prior to occlusion ($n=9$) on local cerebral blood flow determined 2 hours after middle cerebral artery occlusion.

Results: Both regimens of apafant effectively decreased infarct volume. The reduction in cortical infarct volume was 59% ($p<0.01$; *H* test, *U* test) when the rats were treated before and after vessel occlusion whereas the decrease was 47% ($p<0.05$; *H* test, *U* test) when treatment began 1 hour after occlusion. Apafant did not change local cerebral blood flow after occlusion compared with controls.

Conclusions: We suggest that the cytoprotection afforded by apafant occurs mainly via a direct effect on brain tissue and has no major vascular component. (*Stroke* 1992;23:98-103)

In recent years efforts to develop drugs for preventing or treating brain ischemia have, in the main, focused on two classes of drugs: calcium antagonists and *N*-methyl-D-aspartate antagonists. However, it seems unlikely that brain damage can be prevented or ameliorated most effectively by employing a single therapeutic agent. It could be more promising to interrupt different pathobiochemical pathways simultaneously.

For this reason, newly synthesized triazolobenzodiazepines that block the biological effects of platelet activating factor (PAF) could be valuable therapeutic tools. These triazolobenzodiazepines are potent antagonists of PAF while they lack affinity for central benzodiazepine receptors.^{1,2}

An acetylglyceryletherphosphorylcholine, PAF is synthesized in brain tissue³ and is believed to be a potent mediator of inflammation. As such, PAF has emerged as a key mediator of neuroinjury.⁴⁻⁷ A direct neurotoxic effect of PAF in cell culture has been demonstrated recently.⁸

These findings argue in favor of a direct involvement of PAF in the biochemical sequelae of cerebral ischemia; accordingly, in models of global ischemia evidence for the beneficial effects of the PAF antagonists ginkgolide B and kadsurenone has been presented.⁹⁻¹¹ However, delayed neuronal death of hippocampal pyramidal cells following global ischemia is not unanimously believed to be a feature pertinent to human cerebral ischemia.¹² For this reason we decided to evaluate the cytoprotective properties of PAF antagonists in a clinically more relevant model of focal cerebral ischemia in rats.¹³ We hypothesized that application of PAF antagonists might lead to an amelioration of focal ischemic damage in the brain. This putative amelioration by PAF antagonists could be mediated via an effect on brain parenchyma as well as on the cerebral circulation since PAF itself leads to constriction of cerebral arterioles and a substantial decrease in cerebral blood flow.^{8,14}

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We report infarct volume and local cerebral blood flow (LCBF) in rats following administration of the PAF antagonist apafant before and after middle cerebral artery occlusion (MCA-O).

Materials and Methods

Focal cerebral ischemia was induced by irreversible occlusion of the left middle cerebral artery by micro-bipolar coagulation in Fischer 344 rats according to Tamura et al.¹³ The spontaneously breathing rats were anesthetized using a small animal mask with 1% halothane in a 70:30 (vol:vol) mixture of nitrous oxide and oxygen. The tail artery was cannulated for arterial blood sampling and blood pressure recording. Temperature was closely held at 37°C by using a closed-circuit rectal thermoprobe connected to a heating lamp. Physiological variables were measured 5 minutes before and immediately after MCA-O. The rats underwent left subtemporal craniectomy, the dura mater was incised, and under a high-power operating microscope the stem of the artery and its lenticulo-striate branches were permanently occluded by micro-bipolar electrocoagulation. The wounds were sutured, anesthesia was discontinued, and external heating was maintained until the rats had regained complete consciousness and motility.

For determination of infarct volume, after 48 hours the rats were anesthetized with 1.5% halothane in a 70:30 (vol:vol) mixture of nitrous oxide and oxygen and transcardially perfusion-fixed with 4% paraformaldehyde freshly dissolved in phosphate buffer at pH 7.4; the brains were processed for embedding in paraffin. Coronal sections were taken every 0.5 mm. Slices were stained with cresyl violet, and the infarcted area was determined planimetrically by an investigator blinded to the treatment of the rat. Infarct volume was calculated from the infarcted area on each slice and the distance between succeeding slices.

For measurement of LCBF, rats underwent surgery for MCA-O as described above without cannulating the tail artery. Both femoral veins and arteries were cannulated, and the rat was immobilized with plaster casts covering the lower abdomen and hind limbs. The animals were allowed to recover from anesthesia. Body temperature was held at 37°C with a heating lamp. Physiological variables as mentioned above were checked routinely. Two hours after MCA-O, 150 $\mu\text{Ci}/\text{kg}$ of [¹⁴C]iodoantipyrine at steadily increasing rates was infused over 1 minute using a peristaltic pump. Arterial blood samples were obtained from the free-flowing arterial catheter and collected in pre-weighed vials. The samples were weighed and solubilized, and the amount of radioactivity was determined by liquid scintillation counting. The rats were decapitated at 1 minute, and the brains were immediately dissected out and frozen in isopentane chilled to -50°C. Ten-micrometer slices were cut in a cryostat, dried on a hot plate at 50°C, and together with carbon-14 methacrylate standards exposed to Kodak SB 5 x-ray film (Rochester, N.Y.) for 10 days. By using

the operational equation of Sakurada et al,¹⁵ LCBF was computed from the radioactivity in a given brain structure and the arterial plasma concentration curve of the tracer by taking into account corrections for the time lag and washout in the catheter.¹⁶

In the infarct volume study, 30 mg/kg p.o. apafant (4-(2-chlorophenyl)-9-methyl-2[3(4-morpholinyl)-3-propanon-1-yl][6*H*-thieno[3.2-*f*][1.2.4]triazolo[4,3-1]]1.4]diazepine; former code name WEB 2086) was administered either 30 minutes before and 2 hours after MCA-O ($n=8$) or 1 hour after MCA-O ($n=8$). In both cases, additional doses were given 6 and 18 hours after MCA-O. In the LCBF study, apafant was given 30 minutes prior to MCA-O ($n=9$).

Data are presented as mean \pm SEM. Physiological variables were compared using analysis of variance. Since variances of the infarct volumes were not homogeneous, the statistical significance of infarct volume reductions was assessed with the Kruskal-Wallis *H* test and the Mann-Whitney *U* test.

Results

Apafant did not change the physiological variables significantly (data not shown).

Apafant significantly reduced cortical and total infarct volumes by 59% ($p<0.01$) and 50% ($p<0.05$), respectively, when treatment was started 30 minutes before MCA-O and additional doses were given until 18 hours after MCA-O (Figure 1, Table 1). Postocclusion treatment significantly reduced cortical infarct volume by 47% ($p<0.05$) and left total infarct volume unchanged. Striatal infarct volume remained unchanged in both groups (Table 1).

In a different series of experiments the influence of apafant on LCBF 2 hours after MCA-O was evaluated. While MCA-O drastically reduced LCBF in the frontal, parietal, temporal, and occipital cortices of the ipsilateral hemisphere compared with the nonlesioned contralateral side, there was no significant difference between controls and rats treated with apafant. In all other structures measured no inter-hemispheric differences were observed (Table 2).

Discussion

Our data show that the PAF antagonist apafant can effectively reduce the volume of a cerebral infarct, not only when given before, but also when given after MCA-O. The data suggest that the beneficial effect of apafant is not related to changes in LCBF immediately after occlusion.

A naturally occurring etherphospholipid (1-*O*-alkyl-2(*R*)-acetylglycerol-3-phosphorylcholine), PAF has been shown to bind to specific receptors in a process that is affected by the presence of Mg^{2+} and/or Na^+ .¹⁷ Though the signaling processes and effector systems involved on the cellular level are not fully understood at present, PAF is known to induce a variety of biochemical effects such as opening of Ca^{2+} -dependent K^+ channels,¹⁸ stimulation of guanosine triphosphatase activity in platelets,¹⁷ increasing cytosolic Ca^{2+} concentrations, and, consequently, elevating phospho-



FIGURE 1. Nissl-stained coronal section of rat brain at level of caudate 48 hours after occlusion of left middle cerebral artery. Top: Untreated control animal. Note zone of pallor indicating infarcted area in neocortex and caudate. Bottom: Rat treated with 30 mg/kg p.o. apafant 30 minutes prior to vessel occlusion and 2, 6, and 18 hours after occlusion. Note that zone of pallor indicating infarcted area in neocortex is considerably smaller than in untreated control rat.

tidic and free fatty acid production.^{19,20} Moreover, PAF is a potent inflammatory mediator as shown by its action on polymorphonuclear leukocytes.²¹ Extremely low (picomolar) concentrations of PAF are sufficient

to sensitize polymorphonuclear leukocytes by inducing the production of oxygen radicals and leukotrienes.²² These processes may precede and contribute to endothelial damage and ensuing microcirculatory failure.

TABLE 1. Effect of Apafant on Infarct Volumes 48 Hours After Occlusion of Left Middle Cerebral Artery in Rats

Treatment group	Total dose apafant (mg/kg)	n	Infarct volume (μ l)		
			Cortex	Striatum	Total
Control	0	12	88.7 \pm 10.0	17.9 \pm 2.3	106.7 \pm 11.8
Pretreatment	30 mg/kg \times 4	8	36.2 \pm 11.1*	19.5 \pm 1.9	53.5 \pm 13.4†
Posttreatment	30 mg/kg \times 3	8	52.2 \pm 12.2†	18.2 \pm 1.4	70.1 \pm 12.8

Values are mean \pm SEM.

* $\dagger p < 0.01$ and $p < 0.05$, respectively, different from control by Kruskal-Wallis H test, Mann-Whitney U test.

TABLE 2. Local Cerebral Blood Flow in Conscious Rats With Middle Cerebral Artery Occlusion After Pretreatment With 30 mg/kg p.o. Apafant

Structure	Left hemisphere		Right hemisphere	
	Control (n=9)	Apafant (n=9)	Control (n=9)	Apafant (n=9)
Frontal cortex	24.7±4.3	28.8±3.8	82.2±8.2	90.4±14.4
Parietal cortex	22.6±3.7	26.7±4.1	92.5±8.5	92.5±10.3
Temporal cortex	39.1±3.9	36.7±8.2	113.0±12.3	121.2±12.6
Occipital cortex	32.9±8.2	30.8±4.4	84.2±6.2	78.1±5.7
Thalamus	63.7±4.4	59.6±4.1	78.7±6.2	73.9±4.6
Hypothalamus	65.8±6.2	57.5±3.5	74.0±6.2	59.6±4.8
Hippocampus	39.0±3.5	39.5±3.7	47.3±2.9	47.7±3.2
Substantia nigra	69.9±8.2	57.5±4.3	71.9±8.2	63.7±6.1

Data are mean±SEM. No significant differences between treated and control rats by Mann-Whitney *U* test.

Therefore, PAF is involved in a number of pathophysiological processes contributing to tissue damage. In the central nervous system the permeability of the blood-brain barrier is increased and cortical blood flow is reduced, presumably either due to direct vasoconstriction of cortical vessels or mediated by leukotrienes, since PAF is a potent stimulator of leukocytes.^{6,14} Though blood flow is reduced, the metabolic demand of the tissue as measured by the oxygen consumption is elevated after PAF administration,⁸ a fact that could lead to further aggravation of ischemic damage.

To date, the most convincing data in favor of a key role of PAF during cerebral ischemia is derived from experiments with specific PAF antagonists in various models of global brain ischemia. Spinnewyn et al¹⁰ demonstrated a significant improvement of neurological scores in gerbils during the recirculation period following a 10-minute bilateral carotid artery occlusion. Applying different PAF antagonists, the authors found a correlation between the PAF antagonist's potency and the degree of cytoprotection it afforded. A key result of the study was the finding that application of ginkgolide B effectively prevented a drop in neurological scores even when applied as much as 1 hour after the insult.

In a model of multifocal ischemia induced by air embolism in dogs, the combined preischemic and postischemic application of the PAF antagonist kadsurenone significantly enhanced the recovery of cortical somatosensory evoked responses.²³ Recently, in a model of spinal cord ischemia in rabbits the PAF antagonist BN 50739 prevented delayed postischemic hypoperfusion and edema formation,⁷ and in rats ginkgolide B reduced hippocampal cell damage in the two-vessel occlusion model.¹¹ The injection of BN 50739 30 minutes before induction of a focal brain lesion with a neodymium:yttrium-aluminum-garnet laser drastically reduced edema formation 2 hours after injury and led to a decrease in neuronal death in the cortex and hippocampus. However, the data cover only the first 24 hours and it is not evident whether this cytoprotective effect could be quantified after longer postlesion survival times.²⁴

At present no data are available on the reduction of infarct volume by PAF antagonists following MCA-O. Our data show that blockade of PAF receptors leads to a substantial decrease of cortical infarct volume 48 hours after MCA-O, when the infarct is well established. It is not surprising that striatal infarct volumes were not reduced by treatment with apafant, probably because the vessel supply of the caudate seems devoid of interarterial anastomoses and therefore receives almost no blood flow once the lenticulostriate arteries are occluded, while the cortical branches of the middle cerebral artery have extensive anastomoses with distal branches of the anterior and posterior cerebral arteries.²⁵

The most important finding that bears direct clinical relevance is the fact that apafant reduces infarct volume even when the compound is administered 1 hour after MCA-O. Determination of the IC₅₀ values in an assay of PAF-induced human platelet aggregation in platelet-rich plasma indicates that apafant is about four times more potent than ginkgolide B.¹ Apafant competitively inhibits [³H]PAF binding to human platelets with an equilibrium dissociation constant K_D of 15 nM.²⁶ So far, apafant has not been tested in models of central nervous system injury even though extensive work has been carried out in a variety of animal models featuring PAF-induced disease states such as asthma, anaphylaxis, and endotoxin-induced shock, thereby proving the efficacy of this drug conclusively²⁷ (for a review see Weber and Heuer¹). Doses have to be compared carefully because considerable differences exist due to species and route of administration. In rats, the ratio between intravenously and orally effective doses is rather high. In a model of PAF-induced hypotension in rats the ED₅₀ was roughly 10 mg/kg for the oral route, but only 0.061 mg/kg for the intravenous route.²⁸ Our dose of 30 mg/kg p.o. is well beyond the threshold of 800 mg/kg p.o. at which apafant induces sedative effects in rats.²⁷ The half-life of apafant in rats is 3.1 hours after oral administration.²⁹ Our dosage intervals of 2.5–5 hours are within a range that should provide sufficiently high plasma levels.

Considering the diversity of the pathophysiological and biochemical effects of PAF, it seems likely that the cytoprotection afforded by PAF antagonists is the result of the interruption of multiple pathways triggered by PAF rather than a single mechanism. While in models of global ischemia ginkgolide B induced increases in cerebral blood flow during recirculation,³⁰ our data do not indicate a difference in LCBF 2 hours after MCA-O in apafant-treated and untreated rats. Hence, an effect of apafant on cerebral blood flow during the early postocclusion period seems relatively unlikely. It is believed that infarction is complete 4 hours after MCA-O and that little benefit is obtained from recirculation during the next 20 hours.³¹ Thus, there is an only remote possibility that we could have missed a substantial increase in cerebral blood flow during the crucial early postocclusion time when the infarct as characterized by LCBF measurements is still changing.

In contrast, our data support the notion that the reduction of infarct volume in this stroke model caused by apafant is linked to a direct effect of the drug on cerebral parenchyma. This view is corroborated by the fact that PAF has been shown to increase calcium influx into neuronal cells in tissue culture and may exert a direct toxic effect on neurons.⁹ Calcium influx activates phospholipase A₂, and the ensuing lipolysis may lead to membrane damage and cell death. The importance of this pathway is underscored by the findings of Birkle et al,²⁰ who demonstrated recently that ginkgolide B reduced the activation of phospholipase A₂ after electroconvulsive shock or decapitation ischemia in mice.

Our data suggest that PAF antagonists are important new therapeutic tools for the treatment of cerebral ischemia. The triazolobenzodiazepine apafant reduces infarct volume when applied after occlusion and thus may be a valuable drug for clinical use.

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