Influence of Platelet-Activating Factor on Cerebral Microcirculation in Rats
Part 2. Local Application

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Background and Purpose—Platelet-activating factor (PAF) is involved in the development of secondary brain damage after ischemic and traumatic brain injury. On the basis of data from studies in peripheral organs, we hypothesized that PAF-mediated effects after cerebral injury could be secondary to alterations in cerebral microcirculation.

Methods—Changes in cerebral microcirculation focusing on leukocyte-endothelium interactions were quantified with the use of a closed cranial window model in Sprague-Dawley rats (n = 33) by means of intravital fluorescence microscopy. The brain surface was superfused with PAF in concentrations from $10^{-4}$ (n = 3) to $10^{-12}$ mol/L (n = 6) for 20 minutes (5 mL/h).

Results—PAF $10^{-4}$ mol/L (n = 4) increased the number of rolling and adherent leukocytes in venules from 9.7 ± 0.4 to 19.7 ± 2.3 cells/100 mm · min ($P = \text{NS versus control}$) and from 2.2 ± 0.5 to 4.3 ± 0.7 cells/100 mm · min ($P < 0.05$ versus control), respectively. Lower concentrations did not elicit leukocyte-endothelium interactions. Vessel diameters remained unchanged except for a transient increase of arteriolar diameters during superfusion with PAF $10^{-4}$ and $10^{-6}$ mol/L (n = 6). Although only a limited area of the brain surface was exposed to PAF, the mediator induced a significant dose-dependent transitory arterial hypotension and caused irreversible circulatory shock at the high concentration (PAF $10^{-3}$ mol/L). Arterial hypotension after administration of PAF $10^{-3}$ mol/L could be attenuated by the intravenous pretreatment with the PAF antagonist WEB 2170BS.

Conclusions—PAF, when locally released after brain injury, can penetrate the blood-brain barrier and induce systemic effects, including arterial hypotension. Its role as a mediator in the development of secondary brain damage seems, at least in the initial phase, not to be associated with disturbances of cerebral microcirculation or activation of leukocytes. (Stroke. 1999;30:880-886.)

Key Words: cerebral circulation ■ leukocytes ■ microcirculation ■ platelet activating factor ■ rats

Platelet-activating factor (PAF) is an endogenous phospholipid considered to be an important mediator in inflammatory and allergic diseases as well as in shock. Several reports have also provided evidence for a role of PAF in ischemic and traumatic brain injury. PAF can be synthesized by brain cells and is found in small quantities in normal brain tissue. Increased levels of PAF were found in experimental models of ischemia as well as in patients with ischemic stroke. Furthermore, pretreatment and posttreatment with PAF antagonists in global and focal ischemia can improve the survival of neurons and the neurological outcome. The exact underlying pathophysiological mechanisms of the various effects mediated by PAF are still under discussion. Intravital microscopic studies in peripheral tissues have shown that local application of PAF increases vascular permeability, causes arteriolar vasoconstriction, and induces leukocyte-endothelium interactions in venules. In a previous intravital microscopic study using a closed cranial window preparation in the rat, we have observed that the intra-arterial infusion of PAF into the internal carotid artery in concentrations found during shock induces leukocyte-endothelium interactions in cerebral venules independent of changes in microvascular flow or of systemic parameters such as arterial blood pressure. The specificity of these effects was proven by the pretreatment of the animals with the PAF receptor antagonist WEB 2170BS, which could inhibit the PAF-induced decrease in systemic blood pressure as well as the induction of leukocyte-endothelium interactions. Whether a local release of PAF would also elicit leukocyte-
endothelium interactions in the cerebral microcirculation has not been studied thus far. Therefore, the objective of the present in vivo study was to assess the effects of locally administered PAF on cerebral microcirculation and the induction of leukocyte-endothelium interactions in pial venules.

Materials and Methods

Animals

Thirty-three male Sprague-Dawley rats (body weight, 295 ± 23 g) were used. The laboratory-acclimated animals had free access to tap water and standard pellet food. The experiments were conducted according to institutional guidelines and were approved by the state government of Bavaria.

Surgical Preparation

The surgical preparation has been described in detail previously. Briefly, after induction of anesthesia with pentobarbital 3.6% (10 mL/kg body wt IP), the animals were placed on a feedback-controlled heating pad (Effenberger) with the rectal temperature maintained at 37.5 ± 0.6°C. Femoral arterial and venous catheters were surgically inserted for continuous measurement of arterial blood pressure, blood sampling, and infusion of anesthetics and fluorescent dyes. After tracheotomy and intubation, the animals were immobilized with pancuronium bromide (initial bolus of 1.2 mg/kg body wt IV followed by continuous infusion of 1.2 mg/h) and mechanically ventilated (Harvard ventilator model 683; PaCO₂ 35 ± 5 mm Hg; PaO₂ 100 to 120 mm Hg). Anesthesia was continued by intravenous α-chloralose (Merck; bolus of 5 mg/kg body wt). Arterial blood samples (0.1 mL) were obtained in 20-minute intervals for measurement of arterial blood gases, pH, base excess, and hematocrit (ABL 300, Radiometer A/S). In cases of a negative base excess >10 mmol/L, animals received NaHCO₃. Systemic leukocyte count and hematocrit were assessed immediately after implantation of the catheters; before (0 minutes), during (10 minutes), and at the end of the PAF application (20 minutes); and at the end of the experiment (120 minutes). The mean arterial blood pressure, intracranial pressure, and airway pressure were continuously monitored (Honeywell, model 3260 recorder). After fixation of the skull in a stereotaxic frame (model 900, David Knopf Inc), a closed cranial window for intravital microscopy equipped with an inflow and outflow catheter for superfusion of the brain and monitoring of intracranial pressure was implanted over the left parietal brain hemisphere, as described.

Platelet Activating Factor and WEB 2170BS

PAF (C-16 PAF; 1-O-hexadecyl-2-((R)-sn-glyceryl-3-phosphocholine; molecular weight, 525.7 Da; Bachem AG) dissolved in artificial cerebrospinal fluid containing endotoxin-free bovine serum albumin (0.5% solution; Sigma) was administered in concentrations of 10⁻¹², 10⁻⁹, 10⁻⁶, 10⁻³ mol/L. With the use of a superfusion rate of 5 mL/h for 20 minutes, total doses from 0.88 pg (10⁻¹² mol/L; 44 fg/min) to 0.88 mg (10⁻³ mol/L; 44 μg/min) were superfused. Control animals were superfused with mock cerebrospinal fluid without PAF. Because of the known tachyphylaxis of PAF, only one concentration was administered in each individual animal.

For specific inhibition of the effects induced by PAF, the competitive PAF receptor antagonist WEB 2170BS (provided by Boehringer Ingelheim) was used. The antagonist (2 mg/kg body wt) was dissolved in isotonic saline and injected intravenously 15 minutes before superfusion of the brain with PAF 10⁻³ mol/L. The latter concentration was chosen because it was found to elicit the most deleterious effects. In 3 animals, PAF concentrations in blood were analyzed after superfusion of the brain with PAF 10⁻¹⁰ mol/L with the use of a commercially available standardized [125]labeled PAF radioimmunoassay (DuPont de Nemours GmbH, NEN Division). Blood samples of 1 mL were taken before superfusion with PAF (0 minutes), at the end of superfusion with PAF (20 minutes), and at the termination of the experiment (120 minutes). These animals were not subjected to intravital microscopy.

Intravital Fluorescence Microscopy

The workstation for intravital fluorescence microscopy has been described in detail previously. Before each measurement, leukocytes were labeled in vivo by intravenous injection of 0.1 mL of a 0.1% rhodamine 6G solution (Sigma Chemical). The intravital microscopic images were recorded by a video camera and stored on videotapes for offline evaluation, as reported. The integrity of the blood-brain barrier (BBB) was studied online at the end of the experiment by the intravenous injection of 0.5 mL of a 5% Na⁺-fluorescein solution (Sigma Chemical).

Analysis of Microcirculatory Parameters

The measurements included arteriolar and venular diameters (micrometers), the number of rolling or adherent leukocytes in venular vessel segments of 100-μm length during an observation period of 1 minute (cells/100 μm · min), and the opening of the BBB (yes/no). Diameters were measured with the use of a computer-assisted analysis system (CAMAS). In each venular vessel segment, the velocities (millimeters per second) of at least 30 leukocytes freely moving in the central flow axis were measured at each time point, and the harmonic mean was calculated. The result served as an estimate of the blood flow velocity in venules. Using the diameters of the venules (D) and the velocity of freely moving leukocytes (V), we calculated the shear rate γ (per second) according to the formula γ = (V_length/D) × 8.

Experimental Design

After implantation of the closed cranial window and start of superfusion with mock cerebrospinal fluid, the animals were allowed to stabilize during a control period of 60 minutes. During this period, 4 control measurements were performed at 20-minute intervals. Before the first measurement, 2 to 3 regions of interest with at least 1 pial arteriole and 1 pial venule were selected. Thus, in each animal at least 2 to 3 arterioles and 2 to 3 venules were observed and analyzed. After the control period, the brain was superfused with PAF for 20 minutes. Further intravital microscopic observations were performed 5 minutes after the start and at the end of the superfusion (20 minutes) of PAF or the vehicle, then every 20 minutes for 2 hours. At the end of the experiment, the integrity of the BBB was investigated by the intravenous injection of Na⁺-fluorescein.

Six animals were randomly assigned to the different groups with the superfusion of PAF at concentrations of 10⁻¹² to 10⁻⁴ mol/L. All animals exposed to PAF 10⁻³ mol/L experienced irreversible circulatory shock. Therefore, the number of animals in this group was limited to 3. Since PAF at a concentration of 10⁻³ mol/L had the most deleterious systemic effects, another 3 animals received WEB 2170BS (2 mg/kg body wt IV) 15 minutes before superfusion with PAF.

Data Analysis

Statistical analysis was performed with SigmaStat 1.0 software (Jandel Inc). Because of the limited number of animals, nonparametric distribution of the data was assumed. Therefore, the Kruskal-Wallis test, followed by the Mann-Whitney U test, together with the Bonferroni-Holm procedure for repeated measurements was used to analyze differences between control and treated groups. Statistical significance was assumed at P < 0.05. All values are reported as mean ± SD.

Results

Systemic Parameters

PAF induced a dose-dependent decrease of mean arterial pressure leading to an irreversible hypotensive shock after superfusion with PAF 10⁻³ mol/L in all animals (Figure 1).
These animals were not included in the evaluation of the microcirculatory parameters. The hypotension induced by PAF 10^{−3} mol/L could be partially inhibited by pretreatment of the animals with the specific PAF receptor antagonist WEB 2170BS (Figure 1). PAF 10^{−2} and 10^{−4} mol/L induced a transient decrease of arterial blood pressure, which recovered after the superfusion was discontinued (Figure 1). Two animals superfused with PAF 10^{−4} mol/L also experienced irreversible circulatory shock and were excluded from microcirculatory analysis. The lower concentrations (PAF 10^{−9} and 10^{−12} mol/L) had no effect on blood pressure (data not shown). Furthermore, superfusion with PAF at a concentration of 10^{−4} mol/L led to acidosis; pH dropped from 7.37±0.04 to 7.23±0.08 within 60 minutes, and base excess decreased by 9.2 mmol/L. The lower concentrations had no influence on base excess or pH. Intracranial and airway pressure, hemoglobin, hematocrit, and the systemic leukocyte count remained constant in all groups and did not differ from the control group (data not shown). After superfusion of the brain surface with PAF 10^{−4} mol/L, PAF concentration in blood increased from 8.5±2.5 to 16.6±7.7 ng/mL 20 minutes after start of the superfusion. The PAF concentration in blood returned to baseline at the end of the experiment (9.2±3.4 ng/mL).

**Microcirculatory Parameters**

Whereas the venular diameters did not change throughout the experiment (data not shown), a dilation of arterioles was observed during the superfusion with PAF 10^{−4} and 10^{−6} mol/L (Figure 2). This was found to be coincident with reduction of the mean arterial pressure. Lower concentrations of PAF had no effect on the arteriolar diameters (Figure 2). The number of rolling or adherent leukocytes did not change in either the control group or in animals superfused with PAF in concentrations from 10^{−6} to 10^{−12} mol/L (Figures 3 and 4). A significant increase (P<0.05 versus control) in the number of adherent leukocytes from 2.2±0.5 to 4.3±0.7 cells/100 mm · min was found 60 minutes after superfusion with PAF 10^{−4} mol/L (Figure 4). The number of rolling leukocytes increased from 9.7±0.4 to 19.7±2.3 cells/100 mm · min without reaching significance compared with control (Figure 3). Leukocyte velocity (data not shown) and subsequently the shear rate in venules (Figure 5) were significantly reduced (P<0.05) within 40 minutes after the application of PAF 10^{−4} mol/L.
mol/L and remained low throughout the experiment. In all other groups, neither parameter changed significantly. No extravasation of Na\textsuperscript{+}-fluorescein at the end of the experiment was observed, indicating complete integrity of the BBB (Figure 6a). Opening of the BBB was only observed in the animals superfused with PAF 10\textsuperscript{−3} mol/L (Figure 6b) and could not be prevented by pretreatment with the PAF antagonist.

**Discussion**

**Systemic Parameters**

Arterial hypotension was the most striking response to superfusion of the brain surface with PAF. Thus far, however, this hypotensive effect has only been described after systemic application of the phospholipid.\textsuperscript{13,15} It is nevertheless surprising that the exposure of only a limited area of the brain surface to PAF resulted in a significant dose-dependent and transient systemic hypotension. At high concentrations (10\textsuperscript{−3} mol/L), circulatory shock was irreversible. Lower concentrations (PAF 10\textsuperscript{−9} and 10\textsuperscript{−12} mol/L) had no effect on arterial blood pressure. A similar observation was reported in 1988 by Armstead et al,\textsuperscript{16} who superfused the brain surface of newborn pigs with up to 0.1 μg of PAF, a dose that corresponds to the concentration of 10\textsuperscript{−9} mol/L in our study. The blood pressure of the pigs remained unaffected. The systemic hypotension after local cerebral application does not seem to be mediated by local receptors of cerebral blood vessels but may be attributed to a secondary increase in systemic levels of vasoactive mediators\textsuperscript{17,18} and a direct relaxant effect of PAF on arteriolar smooth muscle cells in the peripheral circulation, with a consecutive reduction of systemic vascular resistance.\textsuperscript{19} This conclusion is supported by findings following the pretreatment with the PAF antagonist WEB 2170BS, which could at least partially inhibit the hypotension after superfusion with PAF 10\textsuperscript{−3} mol/L.

With the exception of decreases in pH and base excess, no changes in systemic parameters, including intracranial pressure, airway pressure, hemoglobin concentration, hematocrit, or systemic leukocyte count, were found, in contrast to observations in the same model after intra-arterial injection of PAF.\textsuperscript{12} We believe that the superfusion of the brain did not result in systemic concentrations of PAF high enough to cause the changes observed after intra-arterial application.
The PAF concentration in blood 20 minutes after the start of superfusion reached only 29% (16.6±13.3 ng/mL) of the concentration found after intra-arterial infusion (56.8±22.9 ng/mL).

**Vessel Diameters**

In contrast to studies in peripheral organs, local superfusion of the brain surface with PAF did not result in a significant change of vessel diameters. The increase in arteriolar diameters during the superfusion with PAF 10^{-4} mol/L and PAF 10^{-6} mol/L may be attributed to an autoregulatory reaction to the reduction of systemic blood pressure rather than to a direct local dilatory effect of PAF. A similar observation was made after the intra-arterial infusion of PAF into the carotid artery. Furthermore, PAF concentrations from 10^{-12} to 10^{-6} mol/L did not change diameters of cerebral arterioles of rats in vitro. These results are in contrast to studies with isolated feline and human pial arteries: high concentrations of PAF induced vasoconstriction, whereas the low concentrations (10^{-7}, 10^{-6} mol/L) induced vasodilatation of prostaglandin F_{2\alpha}-pretreated arterioles. In newborn pigs, superfusion of the brain surface with PAF was followed by vasoconstriction of pial arterioles. The reason for these discrepant findings remains unclear. It is, however, well known that the microvascular effects of PAF may vary considerably between species and also between different tissues in one species.

**Blood-Brain Barrier**

Although Kumar et al have reported that PAF increases the permeability of the BBB, effects of PAF on BBB integrity remain controversial. In peripheral organs, superfusion of PAF was found to increase vascular permeability. In the present study, extravasation of Na\(^+\)-fluorescein was only observed after superfusion with PAF in an extremely high concentration (10^{-3} mol/L). Although it inhibited systemic hypotension, supposedly by blocking PAF receptors of peripheral vessels, pretreatment with the PAF receptor antagonist did not prevent opening of the BBB. These findings indicate that the opening of the BBB in our study seems to be due to a direct detergent-like effect of the high concentration on the cell membranes and not to a PAF-mediated morphological change of endothelial cells, since PAF in a dose >4 \(\mu\)mol can cause disintegration of the lipid bilayers of cell membranes. In contrast to other published data, the increased concentration of PAF in blood after PAF superfu-
sion indicates that PAF may penetrate the BBB, although it cannot be completely excluded that some of the mediator reaches the systemic circulation via the arachnoid granulations. Additional indirect evidence is derived from the hypotensive response, which may be blocked by the intravenous injection of the PAF antagonist during local superfusion of the brain surface with PAF.

**Leukocyte-Endothelium Interactions**

In recent years, increasing evidence has been provided that leukocytes may play a crucial role in the development of secondary brain damage after ischemia or traumatic brain injury. Leukocyte depletion or blocking of leukocyte adhesion receptors was found to reduce secondary brain damage and to improve the functional outcome. Studies with PAF antagonists have indicated a role of PAF in the activation and accumulation of leukocytes after cerebral injury. In peripheral organs, PAF induces margination and adhesion of leukocytes in postcapillary venules, which has been considered to be the initial step in the inflammatory reaction. In contrast, a marked effect of PAF on leukocyte-endothelium interactions in the brain could not be detected in the present studies. Except after superfusion with PAF 10^{-4} mol/L, an increase in the number of rolling or adherent leukocytes was not observed. Concentrations leading to leukocyte-endothelium interactions in cerebral venules after infusion of the mediator into the internal carotid artery were not effective when superfused onto the brain. The reason for these discrepant results is unclear. For activation of leukocyte-endothelium interactions, leukocytes as well as endothelial cells must be exposed to PAF. The fact that during superfusion of the brain the endothelial cells are initially exposed to the mediator with their abluminal side might have prevented PAF-initiated presentation of adhesion molecules on these cells, in contrast to the primary intraluminal contact between PAF and the endothelial cells on intra-arterial infusion. Alternatively, it may be hypothesized that during superfusion of the brain, intravascular levels of PAF were not sufficient to elicit leukocyte-endothelium interactions because of the rapid inactivation of the substance by the brain acetyl-hydrolase together with inhibition of diffusion by the BBB. The present findings, however, do not exclude that continuous release of PAF after brain injury can induce leukocyte-endothelium interactions. Indeed, blood levels of PAF were found to be increased in patients with ischemic stroke. Furthermore, since our experiments have been performed with an intact BBB, a primary disruption of the BBB after ischemia or trauma may likely facilitate leukocyte-endothelium interactions from a local release of PAF in the brain.

In summary, in contrast to the systemic administration of PAF and in contrast to respective effects in peripheral organs, local superfusion of the brain with PAF neither affects the cerebral microcirculation nor induces leukocyte-endothelium interactions, except in extremely high concentrations that cause irreversible circulatory shock. Therefore, the present findings do not support the hypothesis that the release of PAF in the early phase after cerebral injury contributes to the activation of leukocytes or the impairment of cerebral microcirculation. Nevertheless, it cannot be ruled out that rolling, adherence, and emigration of leukocytes together with changes in the microvascular perfusion occur in case of BBB disruption and continuous release of PAF. Furthermore, the dose-dependent hypotensive response after the superfusion of only a small area of the brain surface indicates that PAF penetrates the BBB. Local release of PAF at high concentrations in ischemic or traumatic brain injury could have deleterious effects on systemic parameters such as arterial blood pressure.

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**References**

Platelet-activating factor (PAF) is a phospholipid mediator of inflammation that is synthesized by many cell types. PAF is known to alter cerebral vascular tone\(^1,2\) and has been implicated in disease states, including ischemia\(^3\) and subarachnoid hemorrhage.\(^4\)

These two accompanying articles summarize studies that examined effects of intravascular (intracarotid) and topical application of PAF in cerebral venules. There were several findings from these experiments. First, intravascular PAF increased leukocyte-endothelial interactions (rolling and adhesion of leukocytes to endothelium) in cerebral venules. The effects of PAF were inhibited by WEB 2170BS, suggesting that the response was receptor mediated. Second, the data suggest that PAF more effectively increase leukocyte-endothelial interactions when administered by the intravascular route compared with ablumenally applied PAF. Third, in contrast to some studies,\(^1,2\) PAF had no apparent direct effect on arteriolar tone or microvascular permeability in these experiments.

An important unanswered question is what mechanism(s) mediate the increase in leukocyte-endothelial interactions in response to PAF. The increase in leukocyte rolling and adhesion to endothelium in response to PAF began relatively quickly (within minutes), but the maximum effect was observed nearly 2 hours after the start of infusion. Mechanisms that could potentially be involved include formation of reactive oxygen species. More slowly developing responses to PAF may include increased DNA binding activity of nuclear factor-kappa,\(^5\) a major transcription factor for a variety of inflammatory mediators, including endothelial-leukocyte adhesion molecules.

**Frank M. Faraci, PhD, Guest Editor**

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