Influence of Platelet-Activating Factor on Cerebral Microcirculation in Rats
Part 1. Systemic Application

Eberhard Uhl, MD; Sven Pickelmann, MD; Alexander Baethmann, MD; Ludwig Schürer, MD

Background and Purpose—Platelet-activating factor (PAF) has been demonstrated to have a mediator function in shock, with some of its deleterious effects being attributed to its influence on microcirculation. Systemic PAF concentrations as found in shock could also compromise the cerebral microcirculation. Our purpose in the present study was to examine the influence of systemically applied PAF on microvascular perfusion and leukocyte-endothelium interactions in cerebral microvessels.

Methods—A closed cranial window technique was used for intravital fluorescence microscopy of the brain surface. PAF was infused in concentrations of $10^{-12}$, $10^{-9}$, and $10^{-6}$ mol/L into the carotid artery (5 mL/h for 20 min) of Sprague-Dawley rats (n=30). The selective PAF receptor antagonist WEB 2170BS (2 mg/kg body weight) was used to inhibit specific PAF effects.

Results—The number of leukocytes (cells/100 μm² · min) rolling along or adhering at the venular endothelium increased following infusion of PAF $10^{-6}$ mol/L from 7.7±2.5 to 24.4±8.9 ($P<0.05$) and from 1.9±0.5 to 6.9±2.2 ($P<0.05$), respectively, within 2 hours. Mean arterial pressure decreased from 92±22 mm Hg to 49±17 mm Hg ($P<0.05$). The lower concentrations of PAF were less effective to decrease mean arterial pressure but also induced leukocyte-endothelium interactions. The intravenous administration of WEB 2170BS 15 min before the infusion of PAF $10^{-6}$ mol/L prevented both systemic hypotension and activation of leukocyte-endothelium interactions.

Conclusions—Increased systemic blood levels of PAF as found during shock can not only cause systemic arterial hypotension but also induce leukocyte-endothelium interactions in cerebral venules. The activation of leukocytes was found to be independent of PAF-induced arterial hypotension. The specificity of these results is confirmed by the findings that WEB 2170BS could inhibit the PAF-induced systemic hypotension as well as the activation of leukocytes.

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

Platelet-activating factor (PAF) is an endogenous phospholipid with potent mediator functions in a variety of allergic and inflammatory diseases as well as in trauma and shock.1 PAF seems also to be involved in the development of secondary brain damage after traumatic or ischemic brain injury.2 The exact mechanisms of its deleterious effects, however, are still not completely understood. Besides its well-known systemic hypotensive properties, PAF has been shown to elicit marked changes in the microcirculation of peripheral organs, eg, dilation and constriction of arterioles,3 extravasation and edema,4 and induction of leukocyte-endothelium interactions.5 Whether these effects following PAF administration can also be observed in cerebral microcirculation has not yet been shown. Because PAF is released in excessive quantities during shock, we hypothesized that cerebral microcirculation is directly affected by the mediator, leading to an impairment of the perfusion independent of its hypotensive action in the systemic circulation. Therefore, the objective of the current experiments was to quantitatively assess the influence of PAF injected into the carotid artery on the cerebral microvessels, focusing on the induction of leukocyte-endothelium interactions and the integrity of the blood-brain barrier (BBB), by means of intravital fluorescence microscopy.

Materials and Methods

Animals
Thirty adult male barrier-bred Sprague Dawley rats (body weight, 300±26 g) were used in this study. The animals had free access to tap water and pellet food. The experiments were conducted according to institutional guidelines and were approved by the state government of Bavaria.

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Surgical Preparation

Anesthesia was induced by pentobarbital 3.6% (10 mL/kg body weight intraperitoneally). The animals were placed on a feedback-controlled heating pad (Effenberger). The rectal temperature was continuously controlled and maintained at 37.4 ± 0.2°C. Polyethylene catheters (PE-50, Portex) were inserted into the left femoral artery and vein for continuous measurement of the arterial blood pressure, blood sampling, and infusion of anesthetics and fluorescence dyes. Another catheter was inserted into the left carotid artery with placement of the tip at the bifurcation of the common carotid artery for retrograde infusion of PAF or vehicle, respectively, into the internal carotid artery. After tracheostomy the animals were intubated with a modified 16-gauge cannula (B. Braun). After immobilization with pancuronium bromide (initial bolus of 1.2 mg/kg body weight followed by continuous infusion of 1.2 mg/h), the rats were mechanically ventilated (Harvard ventilator model 683; PacO₂ 36 to 40 mm Hg; PacO₂ 100 to 120 mm Hg). Anesthesia was continued by intravenous α-chloralose (Merck; bolus of 5 mg/kg body weight). Arterial blood samples (0.1 mL) were obtained in 20-minute intervals for measurement of arterial blood gases, pH, base excess (BE), and hemoglobin (Hb) concentration (ABL 300, Radiometer A/S). For assessment of systemic leukocyte counts and hematocrit (Hct) (Coultor Counter T450), blood samples (300 μl) were taken immediately after implantation of the catheters, before application (0 minutes), at the end of the application of PAF (20 minutes), and at the termination of the experiment (120 minutes). In case of a negative BE < 10 mmol/L, the half dose of NaHCO₃ (2 to 5 mm Hg) was infused. This was the case in 2 animals treated with PAF 10⁻⁶ mol/L, receiving a total volume of 0.6 mL of NaHCO₃. The arterial blood pressure (MAP), intracranial pressure (ICP), and body temperature were used as control. Because of the tachyphylactic properties of PAF, only 1 concentration was used in each individual animal. For testing the specificity of the effects induced by PAF, the competitive receptor antagonist WEB 2170BS (Boehringer Ingelheim) was administered in additional experiments. The antagonist (2 mg/kg body weight) was dissolved in isotonic saline and injected intravenously 15 minutes before the infusion of PAF 10⁻⁶ mol/L. The latter concentration was found to elicit the strongest response of the microcirculatory parameters.

In 3 control and 3 treated animals, respectively, systemic PAF levels were analyzed with use of a commercially available [¹²⁵I]PAF radioimmunoassay (DuPont de Nemours GmbH, NEN Division). For that purpose, blood samples were taken before application (0 minutes), at the end of the application of PAF (20 minutes), and at the termination of the experiment (120 minutes). Animals used for the radioimmunoassay were not subjected to intravital microscopy.

Intravital Fluorescence Microscopy

The animals were placed on a computer-controlled microscope stage for repeated analysis of identical segments of cerebral blood vessels over extended time periods. The intravital fluorescence microscope (Leitz) was equipped with a 75-W xenon lamp and a Plomer filter block for epi-illumination. Leukocytes were stained in vivo before each measurement by intravenous injection of 0.1 mL of 0.1% Rhodamine 6G (Sigma Chemical). Leukocyte-endothelium interactions were observed by use of a N₂ filter block and a salt water immersion objective (×25). The intravital microscopic images were recorded by an SIT video camera (C2400, Hamamatsu Photonics). Offline analysis of the videotapes was performed by frame-to-frame analysis with a television monitor (Trinitron PVM-2130QM, Sony) at a total magnification of ×998. To test the integrity of the blood-brain barrier at the end of the experiment (online), 0.5 mL of a 5% Na⁺-fluorescein solution (Sigma) was injected intravenously. For observation of the extravasation of the fluorescent marker, an I₂ filter (Leitz) and an objective with a magnification ×10 were used. After the injection of the fluorescent marker, the preparation was observed in short intervals for another 20 minutes.

Analysis of Microcirculatory Parameters

The analysis of the pial microcirculation included measurements of the arteriolar and venular diameters (in micrometers), the number of rolling and adherent leukocytes in venules (cells per 100 micrometers per minute), the velocity of freely moving leukocytes in venules (millimeters per second), and the integrity of the BBB (yes/no). Diameters were measured with a computer-assisted microcirculation analysis system (CAMAS). The white blood cells were classified according to their interaction with the venular endothelium as adherent, rolling, or freely moving leukocytes. Rolling leukocytes were defined as cells having multiple intermittent contacts with the venular endothelium and thereby moving definitely more slowly than the freely moving leukocytes in the center line of the microvessel. Adherent leukocytes were defined as cells attached to the venular wall for >20 seconds. Vessel segments with a length of 100 μm were studied during an observation period of 1 minute. Furthermore, the velocity of freely flowing leukocytes (millimeters per second) in the central flow axis was quantified. In a given vessel segment, the velocities of at least 30 leukocytes were measured at each time point, and the harmonic mean h [mm/s] was calculated. The result was used as an estimate for the blood flow velocity in venules. Using the diameters of the venules (D) and the velocity of the freely moving leukocytes (V), the shear rate (seconds⁻¹) in each single venule was calculated as γ = (D × Vₜₚₚₚ) × 8.0 mm Hg.

Platelet-Activating Factor and WEB 2170BS

Platelet-activating factor (C-16 PAF, 1-O-hexadecyl-2-(R)-acyctethyl-sn-glycero-3-phosphocholine; molecular weight: 525.7 Da; Bachem AG) was dissolved in isotonic saline containing endotoxin-free bovine serum albumin (0.5% solution; Sigma). Albumin was added to prevent PAF from adsorption at unphysiological surfaces. Concentrations of 10⁻¹⁰, 10⁻¹⁰, and 10⁻¹⁰ mol/L PAF were used. Using an infusion rate of 5 mL/h for 20 minutes, a total dose of 0.88 pg (10⁻¹² mol/L; 44 fg/min), 0.88 ng (10⁻⁹ mol/L; 44 pg/min), or 0.88 μg (10⁻⁶ mol/L; 44 ng/min) was administered, respectively. Infusion of isotonic saline with bovine serum albumin (0.5% solution) was used as control.
Experimental Design

Animals (n=6) were randomly assigned to the control and the different treatment groups with infusion of PAF at concentrations of 10^-12 to 10^-6 mol/L, including the group to study the PAF antagonist. After implantation of the cranial window and start of the superfusion with mock CSF, the animals were allowed to stabilize during a control period of 60 minutes. During this time 4 baseline measurements at 20 minute intervals were performed. Before the first measurement, 2 to 3 regions of interest with at least 1 pial arteriole and 1 pial venule, respectively, were selected. Thus, in each animal at least 2 to 3 arterioles and 2 to 3 venules were observed and analyzed. Subsequently, PAF was infused intra-arterially for 20 minutes. Further intravital microscopic measurements were performed 5 minutes after the start of PAF infusion and at the end of the infusion after 20 minutes. The infusion of PAF of the vehicle was followed by a 2-hour observation period with intravital microscopic measurements at 20 minute intervals. At the end of the experiment, the integrity of the BBB was investigated by intravenous injection of Na^-fluorescein as barrier marker.

Data Analysis

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

Results

Systemic Parameters

Intracranial and airway pressures remained constant in all groups throughout the experiment. The MAP transiently decreased during the infusion of PAF. Following a dose-response relationship, hypotension was most pronounced after application of the highest concentration of PAF (decreasing from 92±22 mm Hg at 0 minutes to 49±17 mm Hg at 20 minutes after PAF infusion; P<0.01; Figure 6). PAF also induced a dose-dependent systemic acidosis with a decrease of blood pH and the BE, whereas the PCO_2 remained unchanged (Table). Furthermore, a slight transient increase of the hemoglobin concentration (in milligrams per deciliter) unchanged (Table). Furthermore, a slight transient increase of the hemoglobin concentration (in milligrams per deciliter) from 15.3±1.6 to 16.4±1.2 (NS) and of the hematocrit (%) from 41.3±5.6 to 47.9±10.3 (Table; NS) was found 20 minutes after the start of the infusion of PAF at a concentration of 10^-6 mol/L. The systemic leukocyte count increased significantly after the infusion of PAF but returned to baseline values at the end of the experiment (Table). PAF concentrations in blood remained constant throughout the experiment in the control group (baseline value, 7.0±1.8 ng/mL). Twenty minutes after start of the infusion of PAF 10^-6 mol/L, the concentration in blood had increased from 4.0±3.0 ng/mL to 56.8±32.3 ng/mL compared with 6.8±1.7 ng/mL in the control group. The PAF concentration was found to be decreased again at the end of the experiment (120 minutes; 17.5±15.9 ng/mL versus 10.9±2.6 ng/mL in the control group).

Microcirculatory Parameters

Infusion of PAF led to an almost-immediate increase in the number of rolling and adherent leukocytes in cerebral venules (Figures 1, 2, and 3). Leukocyte-endothelium interactions were never observed in arterioles, nor was plugging of capillaries by leukocytes. These leukocyte-endothelium interactions elicited by PAF were not limited to the period of infusion of the phospholipid but instead continued to increase until the end of the experiment. The maximum of leukocyte-endothelium interactions was observed after infusion of PAF 10^-6 mol/L, with the number of rolling and adherent leukocytes increasing from 7.2±2.2 to 24.4±8.8 and from 1.9±0.5 to 6.9±2.2 cells/100 mm·min, respectively (P<0.01), 100 minutes after the start of the infusion. The same concentration also elicited a significant (P<0.05) dilation of arterioles during the infusion of PAF (Figure 4), whereas the arteriolar diameters remained unchanged in the control group and in the

Hematocrit, Systemic Leukocyte Count, Blood pH, Pco_2, and Base Excess Before and 20, 60, and 120 Minutes After the Start of the Infusion of PAF at Various Concentrations or Vehicle for 20 Minutes into the Carotid Artery

<table>
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<th>Time After Infusion</th>
<th>Hct, %</th>
<th>WBC, g/L</th>
<th>pH</th>
<th>Pco_2</th>
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For all groups, n=6. Animals pretreated with WEB 2170BS (2 mg/kg body weight IV) were infused with PAF at a concentration of 10^-6 mol/L. Values are mean±SD. Mann-Whitney U test. *P<0.05, **P<0.01 vs control. NM indicates not measured at 60 minutes.
animals exposed to the lower PAF concentrations. The dilation of arterioles disappeared as soon as the administration of PAF was discontinued. The venular diameters remained unchanged in all groups throughout the experiment (data not shown).

Except during the infusion of PAF 10^{-6} mol/L, leukocyte velocity (data not shown) and shear rate in the pial venules (Figure 5) increased slightly in the control as well as in the treated animals until to the end of the experiment, without attaining statistical significance, however. During the application of PAF 10^{-6} mol/L, the centerline velocity of leukocytes decreased from 1.1±0.5 to 0.63±0.2 mm/s (NS) but returned to baseline levels upon termination of the infusion. The shear rate was reduced by 37% during the infusion (Figure 5). A statistically significant correlation, however, between the decrease in shear rate and the increase in the number of rolling and adherent leukocytes was not found in any of the experimental groups. Control of BBB integrity with Na^+-fluorescein at the end of the experiment revealed no extravasation of the fluorescence marker in either in the control or in the PAF group.

**Pretreatment With WEB 2170BS**

Administration of the PAF receptor antagonist before infusion of PAF 10^{-6} mol/L inhibited the PAF-induced hypotension (Figure 6) as well as the dilation of arterioles (Figure 4). Moreover, the induction of leukocyte-endothelium interactions by PAF was markedly inhibited. Whereas infusion of PAF 10^{-6} mol/L alone led to a significant increase in the number of rolling and adherent leukocytes, the response was attenuated by WEB 2170BS and did not differ from the findings of the control group (Figures 1 and 2). Systemic acidosis could only partially be prevented, while the antagonist completely antagonized the increase in Hct, Hb, and in the systemic leukocyte count (Table).

**Discussion**

**Systemic Effects**

Increased blood levels of PAF have been found during shock under experimental and clinical conditions. Systemic application of PAF induces severe hypotension comparable to septic or anaphylactic conditions already in subnanomolar concentrations. In accordance with previous studies, infusion...
The hypotensive response to PAF is attributed to a direct relaxation of arterioles, resulting in the reduction of the peripheral vascular resistance. In addition, PAF has negative inotropic effects on the myocardium. PAF-induced extravasation leading to hemoconcentration and reduction of the circulating blood volume has also been discussed. We also found a transient hemoconcentration after the infusion of PAF \(10^{-6}\) mol/L. Furthermore, our results confirm observations concerning the development of a systemic acidosis and the increase of both the hemoglobin concentration and the systemic leukocyte count after PAF administration in rats.

**Vessel Diameters**

The effect of PAF on the vasomotor response of cerebral arterioles is a matter of controversy. Edwards et al studied parenchymal arterioles of rat brain in vitro but failed to demonstrate any changes induced by PAF \((10^{-12} \text{ to } 10^{-6} \text{ mol/L})\), in contrast to respective observations by Uski and Reinstrup with isolated feline or human pial arterioles. Depending on the baseline vasomotor tone, PAF in high concentration caused vasoconstriction and in low concentrations caused vasodilation. The superfusion to the brain of newborn pigs by PAF induced constriction of pial arterioles. In our study no direct effect of PAF on arteriolar diameters could be observed. The dilation of pial arterioles after infusion of PAF \(10^{-6}\) mol/L seems to be a compensatory mechanism to the compromised cerebrovascular autoregulation following the hypotensive effect of PAF rather than a direct effect of the agent on the arterioles. Therefore, our results do not support the hypothesis of Kochanek et al, who attributed the PAF-induced decrease in cerebral blood flow in their study to vasoconstriction. With regard to the varying results, it can be assumed that local administration of PAF may result in both constriction as well as dilation of cerebral vessels depending on the segment, the baseline vascular tone, and the dose administered.

**Leukocyte-Endothelium Interactions**

The current experiments demonstrate for the first time that systemic administration of PAF induces leukocyte-endothelium interactions in cerebral venules. Although all concentra-
tions were effective, the activation of leukocytes was most pronounced after the infusion of PAF 10⁻⁶ mol/L. No clear dose-response relationship was found, since leukocyte-endothelial interactions were more pronounced after infusion with PAF 10⁻¹² mol/L than PAF 10⁻⁷ mol/L. This finding could be related to the somewhat lower shear rate after exposure with PAF 10⁻¹² mol/L. However, the existence of such a dose-related activation of leukocyte-endothelial interactions by PAF remains unclear. Dillon et al. superfused the hamster cheek pouch with PAF in concentrations from 10⁻¹¹ up to 10⁻⁵ mol/L. They could observe a slight increase in the number of rolling leukocytes in venules that was not dose dependent. In contrast, the number of adherent leukocytes in venules increased inversely with the PAF concentration. PAF 10⁻⁹ and 10⁻¹¹ mol/L elicited the most pronounced response compared with concentra[tions of PAF 10⁻⁵ and 10⁻⁷ mol/L. There was no difference between PAF 10⁻¹¹ and PAF 10⁻⁹ mol/L. On the other hand, Tonnesen et al. found an enhanced neutrophil adherence to cultured human microvascular endothelial cells in a dose-dependent manner.

Margination of leukocytes, rolling, adherence, and finally emigration into the surrounding tissue are considered to be the initial steps in the process of inflammation. Margination, which seems to depend on the fluid-mechanical forces, is a major factor for the induction of leukocyte-endothelial interactions. In vitro and in vivo studies have shown that the frequency of rolling and adherent leukocytes is influenced by the shear rate. The reduction of the shear rate in postcapillary venules increases leukocyte rolling even when adhesion receptors are blocked and facilitates the margination of leukocytes.

Above a shear rate of 400 s⁻¹, leukocyte-endothelium interactions are at a minimum because cell elements, e.g., leukocytes, are forced toward the central flow axis. We did not, however, find any correlation between the shear rate and the frequency of rolling or adherent leukocytes. The maximum of leukocyte-endothelium interactions did not occur before 1 to 2 hours after the shear rate was found to be transiently decreased by the infusion of PAF 10⁻⁹ mol/L. At the end of the experiment, leukocyte-endothelium interactions continued despite high shear rates. Therefore, an increase of leukocyte-endothelium interactions secondary to fluid-mechanical factors can be excluded, which makes an enhanced expression of leukocyte adhesion receptors much more conceivable. This conclusion is further supported by the fact that the induction of leukocyte-endothelium interactions was inhibited by PAF treatment.
also occurred after the application of the lower concentrations of PAF, which did not lead to dramatic changes in the systemic parameters, eg, MAP, pH, and BE.

Blood-Brain Barrier
Kumar et al.27 demonstrated an increased permeability of the BBB to lactate after PAF exposure of isolated perfused rat brains. In the present study, however, we did not observe an increase in the permeability of the BBB at the end of the experiment. Nevertheless, a transient opening of the BBB during the infusion of PAF or at a later time point cannot be excluded. Because activation of leukocytes may lead to an opening of the BBB,28 it is conceivable that the current increase of leukocyte adherence to the venular endothelium eventually enhances vascular permeability at a time beyond the observation period of 2 hours in our experiments.

Inhibition of the PAF-Induced Effects by WEB 2170BS
WEB 2170BS is a competitive receptor antagonist that has been found to inhibit PAF effects in a variety of studies.29 In our experiments, pretreatment with this substance almost completely antagonized PAF-induced hypotension and the increase in the number of circulating leukocytes, whereas the development of the metabolic acidosis was only partially inhibited. The antagonist also inhibited PAF-induced rolling and adhesion of leukocytes to the venular endothelium, which indicates that these effects can be specifically initiated by PAF.

In summary, the present findings provide evidence that increased concentrations of PAF in the systemic circulation, besides lowering blood pressure, may lead to disturbances of the cerebral microcirculation, including activation of leukocyte-endothelium interactions. Thus, in shocklike conditions the microvascular perfusion of the brain may not only be affected by severe impairment of the macrohemodynamics but also by a PAF-specific activation of leukocyte-endothelium interactions in cerebral blood vessels, leading to margination and emigration of leukocytes as the initial steps of an inflammatory reaction. The specificity of the present findings is demonstrated by the experiments with the administration of the PAF receptor antagonist WEB 2170BS, which was blocking the PAF-related effects. It may thus be concluded that deterioration of brain function in hemorrhagic or septic shock may be at least in part attributable to the systemic release of PAF resulting in alterations of the cerebral microcirculation and induction of inflammatory reactions in cerebral microvessels.

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