Neutrophil-dependent augmentation of PAF-induced vasoconstriction and albumin flux in coronary arterioles

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Huang, Qiaobing, Mac Wu, Cynthia Meininger, Katherine Kelly, and Yuan Yuan. Neutrophil-dependent augmentation of PAF-induced vasoconstriction and albumin flux in coronary arterioles. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1138–H1147, 1998.—Platelet-activating factor (PAF) has been implicated in the pathogenesis of ischemic heart disease, reperfusion injury, and inflammatory reactions. Although neutrophils have been shown to primarily mediate PAF-induced microvascular dysfunction, the vasoactive effect of PAF and its neutrophil-dependent mechanism have not been directly and systematically studied in coronary resistance vessels. Therefore, the aim of this study was to examine the effects of PAF on coronary arteriolar function and neutrophil dynamics using an isolated and perfused microvessel preparation. Topical application of PAF to the vessels induced a dose-dependent decrease in the diameter but an increase in the apparent permeability coefficient of albumin. Disruption of the endothelium abolished the vasomotor response to PAF, and perfusion of neutrophils significantly augmented PAF-induced changes in vasomotor tone and permeability. Furthermore, the interaction between neutrophils and the endothelium was studied in the intact perfused coronary arterioles. Under control conditions, there were no adherent neutrophils observed in the vessels at varied intraluminal flow velocities. However, administration of PAF caused neutrophil adhesion to the endothelium of coronary arterioles at low flow velocities. Western blot analysis indicated that PAF upregulated the expression of intercellular adhesion molecule-1 in cultured coronary microvascular endothelial cells. Taken together, the results suggest that 1) PAF induces vasoconstriction and hyperpermeability in coronary arterioles via an endothelium-dependent and neutrophil-mediated mechanism, and 2) PAF is able to stimulate neutrophil adhesion in coronary arterioles under a condition of low flow rate.

microvascular permeability; coronary circulation; microcirculation; neutrophil adherence

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The inflammatory response represents a common pathophysiological consequence in the development of ischemic heart disease, reperfusion injury, atherosclerosis, and other types of vascular injury. The response is characterized by neutrophil adhesion and microvascular dysfunction, which impair coronary flow and local perfusion, leading to myocardial infarction (7, 26, 36). Although the deleterious role of neutrophil plugging and releasing of vasoactive agents in the microcirculation has been well documented, the direct relationship between the neutrophil dynamic and arteriolar function has not been established. Within this context, it is not clear whether neutrophils can adhere to the endothelium of coronary arterioles under a condition of flow. This issue is of importance in light of the fact that low flow or no reflow associated with neutrophil activation and arteriolar dysfunction often occur in ischemic heart diseases or during certain clinical procedures such as thrombolytic therapy (7, 28). Moreover, because most of the neutrophil-derived mediators are short lived and transported only downstream, the biological impact of activated neutrophils on the vascular wall appears to be localized to the area of adhesion. Therefore, the ability of neutrophils to adhere to the endothelium of a particular segment of microvessels is a critical factor in the pathophysiological modulation of microvascular function. Although most in vivo observations on the neutrophil-endothelium interaction have been focused on capillaries and venules, evidence is limited for the direct effect of neutrophils on arteriolar endothelium exposed to shear stress. The lack of understanding is partially because of technical difficulties in direct observation of neutrophil dynamics in the heart, a beating organ composed of thick tissues (36).

In addition to neutrophils, the vascular endothelial cell actively participates in the regulation of microcirculation. The endothelium-mediated vasoactivity is a complex process, in which the endothelial cell not only synthesizes and releases an array of vasoactive substances but also serves as a target of action of these substances (17, 34). An impaired local perfusion resulting from the loss of endothelium-dependent vasorelaxation has been observed in various vascular preparations including coronary microvessels during ischemic and inflammatory injuries (18, 21, 26, 36). However, the precise mechanism underlying the injury remains elusive.

The purposes of this study were 1) to examine neutrophil dynamics in coronary arterioles under varied flow conditions and 2) to directly investigate the endothelium-dependent and neutrophil-related mechanism of coronary microvascular responses to inflammatory stimulation. The interaction between neutrophils and the endothelium was studied using an isolated and perfused microvessel preparation, which enables clear visualization of moving cells and precise measurements of the vessel function under controlled physical forces (40). Platelet-activating factor (PAF) was selected to be used as an inflammatory stimulus because it is a typical lipid autacoid that is largely liberated in ischemic and inflammatory diseases (10). More importantly, it is released from the endothelial cell as well as...
isolation and labeling procedure did not significantly alter the neutrophil function.

Isolated and Cannulated Microvessel Preparation

Domestic pigs weighing 9–13 kg (male or female) were anesthetized with pentobarbital sodium (30 mg/kg iv) and heparinized (250 U/kg iv). After a tracheotomy and intubation, the animal was ventilated with room air. A catheter was inserted into a carotid artery, and 60 ml of blood were withdrawn for isolation of neutrophils. A left thoracotomy was then performed, and the heart was electrically fibrillated, excised, and placed in 4°C physiological saline. The left anterior descending artery was cannulated, and 5 ml indigo-cyanine-gelatin-physiological salt solution were infused under a low pressure to clearly define coronary microvessels. This solution was prepared by adding 0.2 ml indigo cyanine (Koh-I-Noor, Bloomsbury, NJ) and 0.35 g porcine skin gelatin (bloom 75–100, Sigma) to 30 ml of warm physiological saline solution and then filtered through P8 filter paper (Fisher Scientific, Pittsburgh, PA). Information regarding the validation and limitation of the ink-perfusion procedure has been provided in our previous publications (37, 39). Although previous experiments have suggested that the ink perfusion did not alter the vasoactivity of isolated coronary arterioles, its effect on arteriolar permeability was not directly evaluated in the current study. We do not rule out the possibility that the ink-perfusion procedure altered the basal barrier function, and thus our measurements of permeability response were performed in vessels that were already in a state of increased permeability.

Coronary arterioles ranging from 40 to 80 μm in diameter and 0.8 to 1.2 mm in length were dissected from surrounding myocardium in the dissecting chamber containing APSS at 4°C with the aid of an SV 11 stereo dissecting microscope (Carl Zeiss, Thornwood, NY). The vessel was transferred to a cannulating chamber that was mounted on a Zeiss Axiosvert 135 inverted microscope (Carl Zeiss). The isolated vessel was cannulated with a micropipette on each end and secured with 11–0 suture (Alcon, Fort Worth, TX). A third smaller pipette was inserted into the inflow pipette. The vessel was perfused with either APSS through the outer inflow pipette or APSS containing FITC-albumin or neutrophils through the inner inflow pipette. Each cannulating micropipette was connected to a reservoir so that the intraluminal pressure and flow velocity could be adjusted independently by simultaneously changing the height of inflow and outflow reservoirs in equal magnitude. The bath solution in the chamber was maintained at 37°C and pH 7.4 throughout the experiments. The image of vessels was projected onto a Hamamatsu charge-coupled device-intensified camera and was displayed on a high-resolution monochromatic video monitor and recorded onto a VHS video recorder. Vessel diameter was measured on-line with a video caliper, and the intraluminal flow velocity was measured with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University, College Station, TX).

Measurement of Albumin Permeability

The permeability of the cannulated vessels was measured with a fluorescence ratio technique (12). With the use of an...
optical window of a video photometer positioned over the vessel and adjacent space on the monitor, the fluorescent intensity from the window was measured and digitized on-line by a Power Macintosh computer. In each measurement, the vessel was first perfused with APSS through the outer inflow pipette to establish a baseline intensity. The vessel lumen was then rapidly filled with fluorochromes by switching the perfusion to the inner inflow pipette. This produced an initial step increase, followed by a gradual increase, in the intensity of fluorescence. There was a step decrease of intensity when the fluorescently labeled molecules were washed out of the vessel lumen by switching the perfusion back to the outer inflow pipette. The apparent solute permeability coefficient of albumin \( P_{A} \) was calculated using the equation \[ P_{A} = \frac{1}{D_{t}} \frac{\Delta I}{\Delta t} \frac{r}{2} \] where \( \Delta I \) is the initial step increase in fluorescent intensity, \( (\Delta I/\Delta t) \) is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel into the extravascular space, and \( r \) is the vessel radius.

Quantification of Neutrophil-Endothelium Interaction in Perfused Arterioles

We previously developed and validated an in situ model to observe neutrophil behavior and quantify the adhesive interaction between neutrophils and the endothelium of coronary microvessels under a condition of flow (40). Briefly, a suspension of PKH26-labeled neutrophils (-10⁷ cell/ml) was introduced into the lumen of coronary arterioles through a cannulating pipette. The neutrophils were allowed to freely flow under a controlled hydrodynamic shear force produced by a perfusion pressure gradient. The neutrophil dynamic was analyzed by replaying video recordings frame by frame on a high-resolution video monitor. The numbers of adherent and freely flowing cells were counted independently by two investigators, and the results were averaged. Adherence is defined as a cell that stops moving and attaches to the vessel wall longer than 30 s or permanently.

Cell Culture and Western Blot Analysis of Intercellular Adhesion Molecule-1 Expression

Sprague-Dawley rats (Charles River, Wilmington, MA) were anesthetized by the intraperitoneal injection of pentobarbital sodium, and the hearts were removed and placed in ice-cold MEM (Ipklik-modified buffer) containing 60 mM tauroine, 20 mM creatine, and 5 mM HEPES. The aorta was cannulated with 2-mm-ID stainless steel tubing and perfused from a static 40-mmHg hydrostatic pressure head. The oxygenated perfusate was supplemented with 0.1% dialyzed BSA and heparin sodium (1 U/ml) was passed through the heart once. After a 10-min washout period, collagenase (0.7 mg/ml) was introduced, and the perfusate was allowed to recirculate until the aortic perfusion pressure decreased below 40 mmHg. The ventricles were cut from the heart, minced, and placed in fresh collagenase-containing perfusate. The tissue was shaken at 250 rpm in a water bath under 100% O₂ for 10 min. CaCl₂ (50 μM) was added to the minced tissue, and digestion with collagenase continued for an additional 10 min. The cells were dispersed, filtered through a double layer of cheesecloth, and diluted 1:4 with buffer containing 0.1% dialyzed BSA. The cells were further purified by sedimentation and sequential filtration through a series of 90-, 45-, 25-, and 15-μm nylon screen. The endothelial identity of the cells was confirmed by the uptake of modified low-density lipoprotein. The cells were cultured under 10% CO₂ in 60-mm cultured dish containing 3 ml DMEM supplemented with 20% FBS, 2 mM L-glutamine, 0.4 mM L-arginine, 20 mM D-glucose, 20 U/ml heparin sodium, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were used at passages 5–7.

Endothelial cells were lysed in lysis buffer [containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mM Tris (pH 8), and 0.14 M NaCl] containing protease inhibitors (0.5 mg/ml Pefabloc, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Cell extracts were boiled with sample buffer (consisting of 63 mM Tris, pH 6.8, 12.5% glycerol, 2.5% SDS, and 0.3% bromphenol blue) and loaded on a 9.5–16% gradient gel. Proteins were transferred to nitrocellulose, and the blot was blocked with 5% nonfat dry milk. The blot was then incubated overnight at 4°C with monoclonal mouse anti-rat intercellular adhesion molecule-1 (ICAM-1) (Genzyme, Cambridge, MA) used at a concentration of 1 μg/ml. It was washed and incubated 1 h at room temperature with donkey anti-mouse IgG (H + L) (Jackson Immuno Research, West Grove, PA) diluted 1:20,000. Finally, the blot was washed and incubated with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Arlington Heights, IL). Proteins were detected using ECL hyperfilm (Amersham).

Experimental Protocols

Effect of PAF on coronary arteriolar tone. To examine whether PAF per se has a direct effect on the vasomotor function of coronary arterioles, the diameter of isolated coronary arterioles was compared before and after topical administration of the agonist. The cannulated arteriole was perfused with APSS at a pressure of 60 cmH₂O without flow. After the vessel developed spontaneous tone, the diameter was monitored before and after application of PAF (10⁻⁷ M) for 60 min to examine the time course of the PAF-induced tone response. Because a near-maximal effect was observed at 20–30 min after adding the drug, all of the following measurements were made at this time period of PAF treatment. Moreover, a dose-response curve was achieved by measuring the vessel diameter at a series of doses of PAF (10⁻⁹ to 10⁻⁵ M). To specify PAF-induced vasoactivity, the dose-response study was repeated in a group of vessels treated with 10⁻⁶ M of hexadecylamine-PAF, a putative PAF receptor antagonist that has been shown to selectively block the coronary vascular effects of PAF (22).

To further test whether PAF causes vasoconstriction through an endothelium-dependent mechanism, we examined the vasomotor response of isolated arterioles in the absence of endothelial function. The endothelium was disrupted by perfusing the vessel with calcium-free solution containing EGTA (4 × 10⁻³ M) for 40 min. The procedure has been shown to remove the endothelium lining the vessel wall and has been used widely for endothelium denudation in vitro as well as in isolated vessels (5, 35). In our previous studies (35), perfusion of arterioles with the calcium-free EGTA solution generally resulted in a temporary vasodilation. After being washed and equilibrated, the vessels developed tone and regained their original diameter. These vessels failed to respond to acetylcholine (10⁻⁷ M) but dilated to adenosine (10⁻⁴ M) to the same extent as intact vessels, indicating a selective disruption of the endothelium without affecting vascular smooth muscle function. In this study, after the EGTA solution was flushed out, the vessel was allowed to equilibrate for 30 min to regain tone, and then the diameter response to PAF (10⁻⁷ M) was measured. At the end of each experiment, the vasomotor responses of the vessels to the endothelium-dependent vasoactivator bradykinin (10⁻⁷ M) and the endothelium-independent vasoconstrictor adenosine...
(10^{-2} \text{ M}) were tested to confirm that the procedure abolished the endothelial activity but did not largely alter the smooth muscle function.

Vasomotor effect of PAF in the presence of neutrophils. After the spontaneous tone was established and the vessel was stabilized, perfusate containing 10^6 cells/ml of labeled neutrophils was introduced into the lumen of arterioles through a cannulating pipette. The diameter of vessels was measured in the presence of neutrophils before and after topical administration of PAF (10^{-8} \text{ M}) for 30 min. In a control study, the tone response to neutrophil perfusion without PAF was measured to ensure that the neutrophils were not significantly activated and that the basal tone of the arterioles was not altered in the presence of the nonactivated neutrophils. The dose of 10^{-8} \text{ M} was used for PAF because it induced a maximal stimulation of neutrophils as indicated by our in vitro analyses of neutrophil chemotaxis and oxygenation activity (data not shown) and by in situ measurements of neutrophil-endothelium adherence.

Effect of PAF on coronary arteriolar permeability. Although the capillary serves as the major site for blood-tissue exchange, coronary arterioles may participate in the exchange process in light of the fact that some metabolically active cardiac tissues lie in proximity to arterioles but are devoid of capillaries (12). More importantly, the increase in vascular permeability under pathophysiological conditions is an indicator of dysfunction or damage of the endothelial barrier. In the present study, we measured the permeability of arterioles to albumin using the isolated and perfused vessel preparation, which enabled precise measurements of protein transit under tightly controlled physical and chemical conditions. With the consideration of the structural characteristics of the arteriolar wall, an important feature of this approach is its sensitivity in detection of only modest changes in permeability that may occur in arterioles. To overcome the technical difficulties associated with diameter changes during permeability measurements, we maintained the suffusion bath solution at 15°C so that the vessels did not develop vasoactive tone in response to vasoactive agents. After the time course of PAF-induced changes in \( P_a \) was observed, different concentrations of PAF (10^{-8} to 10^{-5} \text{ M}) were added to the superfusate solution under controlled flow rates for 30 min, and the dose response of arteriolar permeability to PAF was achieved.

Effect of PAF on coronary arteriolar permeability in the presence of neutrophils. Although the above experiments provided information regarding the direct effect of PAF on arteriolar permeability, this study was undertaken to evaluate the involvement of neutrophils in PAF-induced increases in permeability. The permeability was measured in isolated coronary arterioles during application of PAF (10^{-8} \text{ M}) in the presence of neutrophils. The permeability responses to PAF in the presence of neutrophils were compared with those in the absence of neutrophils.

Neutrophil-endothelium interactions in coronary arterioles. The purposes of this experiment were 1) to observe whether neutrophils adhere to the endothelium of coronary arterioles especially under a condition of low flow and 2) to test the hypothesis that PAF affects arteriolar function partially by altering neutrophil-endothelium interactions. The fraction of adherent neutrophils was measured in isolated and perfused coronary arterioles at different intraluminal flow velocities. To produce a series of flow velocities while maintaining the intraluminal pressure relatively constant, the heights of the inflow and outflow reservoirs were adjusted in an equal magnitude but opposite direction. Our previous studies (39, 40) have shown that adjusting the perfusion pressure gradient between the inflow and the outflow pipettes (\( \Delta P \)) in a range of 2.5–20 cmH_2O produces a linear flow velocity ranging from 600 to 6,000 \mu\text{m/s}, which is in accordance with that measured in vivo in small coronary microvessels (1). The fraction of adherent neutrophils in the total flux of cells was plotted as a function of flow.

In vitro assay of adhesion molecules. To further emphasize the impact of PAF on endothelial cells in the modulation of neutrophil adhesive reactions, the expression of ICAM-1, an adhesion molecule that expresses on the surface of the endothelial cells, was measured. Western blot analysis was performed on cultured coronary microvascular endothelial cells treated with PAF (10^{-8} \text{ M}) for 30 min. Because interleukin-1\( \beta \) is a well-known activator of ICAM-1, it was used (10^{-9} \text{ M}) as a positive control. It is noted that neutrophil-endothelium interactions involve a group of adhesion molecules including the \( \beta_2 \)-integrin superfamily and selectins. However, we were unable to evaluate each of these molecules because of the limitation of cell sources and the lack of antibodies directed against the adhesion molecules.

Data Analysis

In the perfused vessel studies, \( P_a \) was measured two to three times for each vessel at each experimental intervention, and the values were averaged. For each experimental condition, the changes in the diameter and the permeability coefficient from different vessels were normalized to the control values obtained before treatments and were reported as percentages of the controls. All data were reported as means \( \pm \) SE. For all experiments, n is given as the number of vessels studied, with each vessel representing a separate animal. ANOVA was applied to evaluate the significance of intergroup differences. A value of P < 0.05 was considered significant for the comparisons.

RESULTS

PAF-Induced Vasoconstriction

Under control conditions in the absence of neutrophils, administration of PAF caused a modest decrease in the diameter of isolated and perfused porcine coronary arterioles (n = 6, diameter 67.3 \pm 8.4 \mu\text{m}). The vasoconstriction occurred quickly within 5 min, peaked at 20–30 min, persisted for 40–60 min, and was reversed by washing the suffusion bath free of PAF. The vasoactive effect of PAF displayed a dose-dependent characteristic (Fig. 1), whereas the diameter of arterioles was reduced to 97.2 \pm 1.1\% of control at 10^{-9} \text{ M} (n = 4), 93.7 \pm 0.6\% at 10^{-8} \text{ M} (n = 6), 85.2 \pm 4.2\% at 10^{-7} \text{ M} (n = 6), 77.0 \pm 4.4\% at 10^{-6} \text{ M} (n = 4), and 83.9 \pm 2.0\% at 10^{-5} \text{ M} PAF (n = 3), respectively. This effect was specific to PAF, because treatment of the vessels (n = 4) with the PAF receptor antagonist hexenalmine-PAF (10^{-6} \text{ M}) completely abolished the dose response (Fig. 1). To examine the role of endothelial cells in mediation of PAF-induced vasoactivity, the changes in arteriolar diameter were compared in isolated vessels before and after the endothelium was disrupted (Fig. 2). In the endothelium-intact vessels (n = 6), administration of PAF (10^{-7} \text{ M}) caused the vessels to constrict to 86.2 \pm 4.9\% of their control diameters. Disruption of the endothelium (n = 4) did not significantly alter the basal tone of the vessels but abolished the constrictor response to the same dose of PAF, suggesting that PAF acted via an endothelium-
dependent pathway. Furthermore, studies in isolated coronary arterioles showed that the presence of neutrophils augmented PAF-induced vasoconstriction (Fig. 3). For example, at $10^{-2}$ M PAF in the absence of neutrophils, no significant vasoconstriction was observed (the vessel diameter was $93.7 \pm 0.6\%$ of control value, $P > 0.05$). However, the same dose of PAF caused a significant vasconstriction in the vessels perfused with neutrophils: the vessel diameter was reduced to $78.7 \pm 5.9\%$ of control value ($n = 8$). Perfusion of neutrophils per se did not significantly alter the vascular tone (the vessel diameter was $101.1 \pm 4.2\%$ of control diameter, $n = 8$), indicating that before PAF treatment the neutrophils were in a relatively nonactivated state, and the enhanced vasoactivity was, therefore, due to PAF stimulation of neutrophils.

**PAF-Induced Increases in Albumin Permeability**

As shown in Fig. 4, treatment with PAF ($10^{-8}$ to $10^{-5}$ M) for 30 min increased the permeability of isolated and perfused coronary arterioles in a dose-dependent manner. Specifically, $P_a$ was $1.7 \pm 0.1 \times 10^{-6}$
cm/s under control conditions (n = 10, vessel diameter 56.8 ± 3.8 µm). PAF increased the P_a value to 3.3 ± 0.5 × 10^{-6} cm/s at 10^{-8} M (n = 4), 4.5 ± 0.8 × 10^{-6} cm/s at 10^{-7} M (n = 6), 5.6 ± 0.6 × 10^{-6} cm/s at 10^{-6} M (n = 4), and 5.0 ± 0.8 × 10^{-6} cm/s at 10^{-5} M (n = 4), respectively. Typically, the permeability responses to PAF were gradually seen in 15–20 min after addition of the drug and persisted for 60 min. The changes in permeability were reversible after washing the bath and equilibrating for 30 min. Similar to the tone response, an augmented permeability response to PAF was seen in the presence of neutrophils (Fig. 5). At 10^{-8} M, PAF increased P_a to 6.6 ± 0.6 × 10^{-6} cm/s (n = 4), which was significantly greater than that of PAF alone (3-fold increase with neutrophils vs. 2-fold increase without neutrophils). However, perfusion of neutrophils alone in the absence of the stimuli only slightly but insignificantly altered P_a (Fig. 5). There were no significant changes in the vessel diameter during the permeability measurements.

**PAF-Induced Neutrophil Adherence**

PKH26-labeled porcine neutrophils were clearly visualized using fluorescence microscopy. Under control conditions at flow velocities ranging from 600 to 6,000 µm/s, no rolling or adherent neutrophils were observed. Application of PAF (10^{-8} M, 30 min) to neutrophil-perfused arterioles caused neutrophil adherence to the endothelium, which was more significant under low-flow conditions (Fig. 6). In the presence of PAF, the fraction of adherent cells per 100 fluxed cells was 6.1 ± 2.3, 3.5 ± 0.8, 3.3 ± 1.5, 3.8 ± 2.6, and 0.6 ± 0.3 at a ΔP of 2.5, 5, 10, 15, and 20 cmH_2O, respectively (n = 5). Furthermore, Western blot analysis with monoclonal antibody directed against ICAM-1 indicated that PAF treatment (10^{-8} M, 30 min) induced a significant increase in the expression of ICAM-1 in coronary microvascular endothelial cells (Fig. 7). The in vitro effect of PAF on ICAM-1 expression was similar to that of interleukin-1.

**DISCUSSION**

The major findings of this study were as follows: 1) neutrophils were able to adhere to the endothelium of coronary arterioles under low shear conditions during stimulation by PAF; 2) PAF caused a vasoconstriction and an increase in albumin permeability in coronary arterioles by directly acting on the endothelium as well as through the release of additional mediators.
as through a neutrophil-mediated pathway; and 3) the PAF-stimulated adhesive interaction between neutrophils and coronary arteriolar endothelium may involve the upregulation of ICAM-1.

**Neutrophil-Endothelium Interaction in Coronary Arterioles**

The interaction between circulating neutrophils and vascular endothelium is determined by the adhesive force generated by adhesion molecules and the hemodynamic dispersal force generated by shear stress (7, 30, 31). The balance between the two forces is altered in pathological processes associated with a decreased local blood flow or an inflammatory reaction (11, 25, 28). Intraluminally accumulated neutrophils not only physically obstruct the microcirculation (7, 9, 30) but also release various vasoactive mediators that cause constriction of resistance vessels and protein extravasation across postcapillary venules (7, 16, 17). A body of in vivo evidence has demonstrated that blockage of neutrophil adherence effectively prevents endothelial dysfunction and venular hyperpermeability in ischemia reperfusion injury and inflammatory diseases (2, 3, 15, 16).

In this regard, a potential mechanism of adhesion-induced vascular injury relies on a prolonged exposure of the endothelium to locally concentrated vasoactive mediators released from adherent neutrophils, such as oxidative metabolites and lipid products. This concept well explains the damage of endothelial barrier function associated with neutrophil adherence often seen in postcapillary venules. Interestingly, a persistent increase in coronary resistance accompanied by an impaired vasodilator response has been observed in the early stage of ischemia reperfusion or inflammatory injury (3, 26, 36), which is believed to be largely dependent on activated neutrophils (7, 21, 27). However, there are no reports of neutrophil aggregation or adherence in coronary arterioles. In view of the fact that most of the neutrophil-derived mediators are quickly metabolized or are otherwise carried away by the blood flow, their effect must be localized to the areas of neutrophil attachment to the endothelium. This renders a puzzle of why neutrophils cause such potent and persistent vasoconstriction in the vessels where adherence is not observed. One possibility is that the vasoactive mediators produced by neutrophils accumulate in capillaries and venules can diffuse across the vessel wall and attack the adjacent arterioles. Alternatively, neutrophils do have the ability to adhere in arterioles, but such phenomenon is not easily observed with the current techniques, especially in parenchymal organs, such as the heart.

Our experiments in intact perfused coronary arterioles suggest that there is a close interaction between neutrophils and arteriolar endothelium under a low flow rate upon inflammatory stimulation. Although the precise molecular pathway leading to the interaction is not entirely clear from the present study, the ability of neutrophils to adhere to arteriolar endothelium argues against the hypothesis that arteriolar endothelial cells lack counter receptors for the neutrophil β2-integrins. In fact, many in vitro investigations on neutrophil adhesion were conducted in cultured endothelial cells harvested from large vessels, such as aorta or umbilical veins, which express adhesion molecules and display adhesive effects on neutrophils as well (31). Importantly, neutrophil rolling and adhesion have been observed in vivo in the cat mesentery arterioles in a low wall shear rate (25), confirming the existence of endothelial adhesion molecules in arteriolar microvessels. On the other hand, we agree that heterogeneous adhesive reactions occur in arterioles and venules, which may account for the preferential rolling and adhesion of leukocytes in venules (19, 24). This is supported by our finding that PAF induced neutrophil adhesion in coronary arterioles, whereas C5a did not show any effect (40). Considering the vascular effects of PAF and C5a, a discrepancy between the two stimuli is that PAF targets both neutrophils and endothelial cells (41) but C5a acts primarily through a neutrophil-dependent pathway (4, 23, 40). Taken together, our results support the concept that both the activation of cells and the decrease in shear force are required for neutrophil adhesion with microvascular endothelium in intact tissues (9, 11, 30, 31).

**Effect of PAF**

It has recently been proposed that during reperfusion injury the generation of acute inflammatory mediators acts as a trigger mechanism for endothelial dysfunction that is exacerbated by neutrophil adherence (18, 27). Of the mediators, PAF has been shown to be a primary one that is largely produced in inflammation or ischemic diseases (18) and has an even greater impact on myocardial injury than neutrophils per se (29). The biological effects of PAF are characterized by vasoconstriction, intravascular aggregation, oxidative reactions, and microvascular leakage (2, 8, 10, 15, 16, 34). Administration of PAF antagonists significantly reduces the changes in hemodynamics induced by PAF or inflammatory stimulation in various tissue preparations including the heart (17, 22). Different mechanisms, including a receptor-mediated signaling pathway, have been implicated in PAF-induced increases in coronary vascular resistance (32). The present study is in agreement with the idea that PAF directly constricts coronary arterioles via an endothelium-dependent and receptor-mediated pathway, because disruption of the endothelium or blockage of the PAF receptor abolished the vasmotor response to PAF. However, the autacoid per se did not seem to be a potent constrictor of coronary arterioles, and its vasmotor effect was significant only at high doses or in the presence of neutrophils. Similarly, a more profound increase in albumin flux was observed with PAF in the vessels perfused with neutrophils than in the absence of neutrophils. The results suggest that multiple interactions occur between endothelial cells and neutrophils during stimulation by PAF.

The precise mechanism of PAF-induced neutrophil-endothelium interactions has not been established. It appears that PAF promotes neutrophil adherence to
the microvascular wall by activating both neutrophils and endothelial cells. An example of the endothelial response to PAF is the overexpression of ICAM-1, the endothelial ligand of neutrophil β2-integrins. To our understanding, there has been no direct evidence to date showing the effect of PAF on ICAM-1 expression in coronary microvascular endothelial cells. On the other hand, in vivo studies have demonstrated that treatment with monoclonal antibodies directed against ICAM-1 attenuates PAF-induced endothelial oxidative stress, albumin leakage, and adhesion to neutrophils (16), supporting the involvement of ICAM-1 in PAF action. The importance of the ICAM-1-dependent adherence reaction has long been emphasized in the pathogenesis of postischemic no-reflow and inflammatory injury (14, 18, 33).

In addition to ICAM-1, a group of selectin and integrin molecules play an important role in the interaction between neutrophils and vascular endothelium. For example, anti-CD18 antibodies have demonstrated great protection against PAF-associated and neutrophil-mediated tissue injury (2, 15, 16). Interestingly, P-selectin has been found to coexpress with PAF receptor on the surface of stimulated endothelial cells and to potentiate the CD18-dependent adhesive response (20). In fact, both P-selectin and CD18 have been implicated in PAF-mediated neutrophil aggregation and adhesion (2, 6, 41). In the present study, we were unable to directly measure the expression of all these molecules in isolated vessels because of technical difficulties. Similarly, we did not test whether P-selectin was involved in neutrophil adherence observed in the coronary arterioles because of the lack of monoclonal antibodies directed against the porcine selectins. However, the main purpose of this study was to examine the neutrophil dynamic and its associated functional changes in coronary arterioles rather than to identify specific adhesion molecules that account for the adhesive reaction. In this regard, the Western blot analysis of ICAM-1 on cultured rat coronary microvascular endothelial cells was designed to provide supporting evidence for the regulatory effect of PAF on coronary microvascular endothelium.

Evaluation of Experimental Approaches

There are few experimental approaches that allow quantitative measurements of neutrophil-endothelium interactions in coronary microcirculation under flow conditions. The technical difficulty in visualization of circulating neutrophils is attributed to the thickness of myocardium, the high speed of freely flowing cells, and the beating motion of the heart (36). Recently, investigators have successfully observed the accumulation of activated leukocytes in postischemic coronary capillaries and venules at full-flow rates with the aid of intravital fluorescence microscopy (28), providing physiologically relevant information. Compared with this and other in situ models, the isolated coronary microvessel preparation features a direct and simplified evaluation of the interaction between neutrophils and endothelium. Perfusion of the vessels with fluorescently labeled neutrophils enables clear visualization of neutrophil behavior under carefully controlled physical forces with little influence from the parenchyma, interstitium, and blood components. Although we did not calculate the absolute values of shear rate in the vessels, setting different perfusion pressure gradients produced a series of intraluminal flow velocities (600 to 6,000 mm/s) that fall into the physiological range of coronary microvascular blood flow (1). The interesting point is that neutrophils had the ability to directly interact with the endothelium of coronary resistance vessels under a condition of inflammation combined with low flow. The observation provides a different view of the mechanism underlying neutrophil-dependent arteriolar dysfunc-

A major concern of the experimental approach is that the microvessels might be damaged during isolation and perfusion. The validation of the preparation has been discussed in detail in our previous papers (37, 39, 40). In the present studies, because the vessels displayed normal physiological functions including spontaneous tone and reversible vasoactivity, it is unlikely that the endothelium of the isolated vessels was severely damaged. However, with a comparison of the basal permeability, our values of permeability to albumin are relatively higher than those previously reported by Huxley and Williams (12) and are comparable to the values for α-lactalbumin obtained in isolated porcine coronary arterioles (13). Thus we could not exclude the possibility that the vessels experienced disturbance or stimulation during the experiments. In any event, the vessel was removed from the supporting tissue and lacked blood supply, and we regard it as a structurally intact preparation that bridges the gap between the cultured cell model and in vivo approaches.

The vasoactive and permeability responses of coronary resistance vessels to PAF have not been well documented. Compared with previous studies (8), the pattern and the maximal response of PAF-induced vasoconstriction and albumin flux in porcine coronary microvessels are slightly different from those observed in the skeletal muscle microvessels, indicating that there are heterogeneous responses to the autacoid in different vascular beds. It should be emphasized that we evaluated arteriolar permeability to albumin as an index of endothelial function with respect to PAF-associated vasoactivity. It is conventionally considered that arterioles do not participate in the exchange process. This concept is challenged by a recent study showing that coronary arterioles possess permeasele-

In summary, this study has demonstrated that PAF regulates vasoactive function and permeability of coronary arterioles by directly stimulating the endothelium. The vascular responses to PAF are augmented in
the presence of neutrophils, which may be attributed to the activation of neutrophils and the upregulation of adhesive interactions between neutrophils and the arteriolar endothelium.

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