Rabbit Polymorphonuclear Neutrophils Elicit Endothelium-Dependent Contraction in Vascular Smooth Muscle

Eliot H. Ohlstein and Andrew J. Nichols

The present studies were designed to investigate the interaction between activated polymorphonuclear neutrophils (PMNs) and endothelial regulation of vascular smooth muscle function. Rabbit peritoneal PMNs (4x10^3–4x10^5 cells/ml) added to muscle bath chambers containing phenylephrine-precontracted rabbit isolated aortic rings produced no effect on vascular tone. However, when PMNs were activated with the chemotactic peptide, f-met-leu-phe (0.1 μM), PMNs produced concentration-dependent vascular contraction, which was dependent on the presence of the endothelium. Aortic rings denuded of endothelium were unaffected by activated PMNs. Superoxide dismutase (100 units/ml) treatment of tissues blocked completely PMN-induced vascular contraction, whereas mannitol (20 mM) had no significant effect on PMN-induced vascular contraction. Pyrogallol (a generator of superoxide anion) produced a response that was similar to that observed with activated PMNs. Superoxide anion production was measured separately, and the time of peak rate of superoxide anion production corresponded to the time of the maximal vascular contractile responses. Activated PMNs added to vascular tissues undergoing endothelium-dependent relaxation mediated by either acetylcholine or A23187 produced a reversal of vascular relaxation. Furthermore, activated PMNs did not have any effect on endothelium-independent vascular relaxation produced by either isoproterenol or nitroglycerin. The present investigation reveals that activated PMNs can release superoxide anion and produce endothelium-dependent contraction. The endothelium-dependent contraction may be the result of superoxide anion inactivation of endothelium-derived relaxing factor.

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Interaction of polymorphonuclear neutrophils (PMNs) with the vascular endothelium is one of the initial events during the induction of the acute inflammatory reaction in response to ischemic damage (e.g., during coronary artery occlusion). After adherence to the vascular endothelium, PMNs migrate to sites of tissue damage and release oxygen free radicals, arachidonic acid metabolites, and lysosomal proteases, which exacerbate tissue injury.1,2 PMN adherence to the vascular endothelium also enhances vascular permeability and the recruitment of additional PMNs to the site of injury.3 Furthermore, following reperfusion of ischemic tissue, PMN migration and adherence to the vascular endothelium is greatly enhanced.2

The emigration and accumulation of PMNs at sites of ischemic damage may modify local blood flow by a number of mechanisms.4 The physical obstruction of capillary flow by PMNs and changes in PMN deformability, which produces rheological changes, can result in the increase of vascular resistance.5 In addition, the release of PMN-derived vasoactive factors, including oxygen free radicals, may affect vascular smooth muscle directly or may influence the activity of endothelial-derived autacoids, such as prostacyclin or endothelium-derived relaxing factor (EDRF). Thus, PMNs have the potential to produce profound effects on vascular smooth muscle and endothelial function during ischemia and reperfusion.

The vascular pharmacology and physiology of EDRF is being researched extensively. It has been reported that superoxide dismutase (SOD) potentiates the vascular relaxant effects and half-life of EDRF6,7 and that the free radical superoxide anion destroys EDRF activity.8,9 Since activated PMNs produce superoxide anion, the present studies were designed to investigate the interaction between rabbit PMNs and the vascular endothelium. Vascular reactivity of rabbit isolated aortic rings was assessed...
after exposure to rabbit PMNs and the influence of the vascular endothelium was determined.

Materials and Methods

Rabbit Isolated Aortic Rings

New Zealand White male rabbits (2–3 kg) were killed by cervical fracture and exsanguination. Thoracic aortae were excised immediately, cleaned of connective tissue, cut into rings 4 mm in length, and mounted for isometric tension recordings in 25-ml organ bath chambers containing Krebs-bicarbonate buffer of the following millimolar composition: NaCl 112, KCl 4.7, KH2PO4 1.2, MgSO4 1.2, CaCl2 2.5, NaHCO3 25, dextrose 11.0, and disodium EDTA 0.3. Tissue baths were maintained at 37°C and aerated continuously with 95% O2-5% CO2. The resting tension of the aortae was maintained at 2 g, and the tissues were allowed to equilibrate for 2 hours. Isometric tensions were recorded on dynographs (model R-611, Beckman Instruments, Fullerton, California) with force-displacement transducers (model FT03c, Grass Instruments, Quincy, Massachusetts). Endothelial integrity was assessed as described by the method of Furchgott and Zawadzki.10 The presence of a functional vascular endothelium was confirmed by the ability of acetylcholine (ACh; 0.1 µM) to produce vascular relaxation. Vascular responses to PMN and other vasoactive agents were obtained by submaximally contracting aortic rings with phenylephrine (PE; approximately 1-2 minutes later, PMNs or other vasoactive agents were added to the bath chambers. Approximately 1–2 minutes later, PMNs or other vasoactive agents were added to the bath chambers and vascular responses were recorded.

Rabbit Peritoneal PMN

Male New Zealand White rabbits (2–3 kg) were given 100 ml of 0.1% oyster glycogen intraperitoneally. Sixteen hours later, the rabbits were euthanized by barbiturate overdose, and the peritoneal cavity was washed with 100 ml phosphate buffered saline (PBS), pH 7.4, containing 5 units/ml heparin. The cell suspension was filtered through four layers of cheesecloth and centrifuged at 250g for 5 minutes at room temperature. Contaminating erythrocytes in the leukocyte pellet were destroyed by ice-cold hypotonic (0.15% NaCl) lysis for 20 seconds. The cells were washed and resuspended in PBS to a concentration of 1x10^7/ml and kept on ice. Using this procedure, the leukocyte preparation was greater than 80% PMN as determined by Wright-Giesma staining and was greater than 95% viable as determined by trypan blue exclusion. PMN preparations did not contain measurable levels of hemoglobin (<10 nM) as determined by scanning spectrophotometric analysis.

Determination of Superoxide Production

The rate of superoxide anion production by peritoneal PMNs was measured spectrophotometrically by the reduction of ferricytochrome C.11 PMNs were resuspended in Hanks balanced salt solution. Five hundred microliter samples of the rabbit PMN suspension containing 5x10^6 cells were preincubated with cytochalasin B (5 µg/ml) and ferricytochrome C (100 µM) in a total volume of 900 µl for 15 minutes at 37°C in 1.5-ml spectrophotometric cells in a temperature-controlled Beckman DU-5 spectrophotometer with automatic sample changer. The PMNs were stimulated with 0.1 µM f-met-leu-phe in a final reaction volume of 1.0 ml. For each assay, duplicate samples were run containing an excess (100 µl) of SOD as blanks. The absorbance at 550 nm was measured every 30 seconds, and superoxide anion production (nanomoles per 5x10^6 PMNs) was calculated by dividing the SOD-sensitive absorbance of the samples by the extinction coefficient for the reduction of ferricytochrome C (21.1 mM⁻¹ cm⁻¹).

Data Analyses

PMN-induced vascular contractions were expressed in grams contraction above the PE-induced tone, and vascular relaxation results were calculated as a percentage of the decrease of PE-induced tone. Data are expressed as the mean±SEM. Statistical significance was evaluated by applying an unpaired Student's t test to groups of data, and data were considered statistically significant when p<0.05.

Drugs

The following drugs were obtained from Sigma Chemical, St. Louis, Missouri: phenylephrine hydrochloride, acetylcholine chloride, calcium ionophore A23187 (free acid), sodium nitroprusside, isoosperitro- nol hydrochloride, f-met-leu-phe, pyrogallol, indomethacin, oyster glycogen, cytochalasin B, ferricytochrome C (horse heart type VI), methylene blue, and mannitol. Nitroglycerin was purchased from Eli Lilly and Company, Indianapolis, Indiana. Superoxide dismutase (5,000 units/mg, from bovine erythrocytes) was purchased from Boehringer Mannheim, Indianapolis, Indiana. All concentrations are expressed as the base and are final bath concentrations.

Results

Effect of PMNs on Vascular Function

PMNs (10^4–5x10^6 cells/ml) added to muscle bath chambers containing rabbit isolated aortic rings under basal tension had no effect on vascular tone (data not shown). Aortic rings contracted with PE were also not significantly affected by PMNs (Figure 1A). However, when aortic rings were pretreated with the chemotactic peptide f-met-leu-phe (0.1 µM), PMNs produced a concentration-dependent contraction of the vascular smooth muscle, which was dependent on the presence of the vascular endothelium (Figure
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1B). f-met-leu-phe by itself did not significantly affect vascular tone. PMN-induced contraction was 15.4±1.9% of PE-induced tone (n=18). Aortic rings denuded of endothelium were unaffected by activated PMN (Figure 1C). The concentration-dependent contractile response to activated PMN is illustrated in Figure 2. Maximal contraction was produced by approximately 1×10⁵ cells/ml, and maximal contraction was approximately 0.53±0.12 g. The contractile responses to activated PMN were reproducible in the same endothelium intact vascular ring segment; a first exposure to activated PMNs produced 0.52±0.08 g contraction, and a second exposure 30 minutes later to activated PMN produced 0.59±0.11 g contraction (n=4).

Effect of Activated PMNs on EDRF

Activated PMNs added to aortic rings undergoing endothelium-dependent relaxation mediated by either ACh or A23187 produced a reversal in the relaxation (Figures 3A and 3B). ACh (0.1 μM) and A23187 (0.1 μM) produced a 58±3% and a 75±4% relaxation, respectively. PMNs (4×10⁶ cells/ml) produced a 57±12% (n=6) and an 18±5% (n=5) reversal of ACh (0.1 μM) and A23187 (0.1 μM) endothelium-dependent relaxation, respectively. The presence of f-met-leu-phe did not influence ACh- or A23187-mediated vascular relaxation (data not shown). The guanylate cyclase inhibitor, methylene blue (10 μM), reversed ACh-mediated endothelium-dependent relaxation 147±31% (n=3) (Figure 3C). Activated PMNs did not have any effect on vascular relaxation produced by either nitroglycerin (Figure 3D) or isoproterenol (Figure 3E). The relaxant effect of nitroglycerin was not significantly affected by endothelium-dependent contraction to activated PMNs. Under control conditions, nitroglycerin (1 μM) produced 84±5% (n=5) vascular relaxation, whereas after addition of activated PMN, vascular relaxation was 77±11% (n=3). Aortic rings pretreated with indomethacin (10 μM; 30 minutes) did not affect endothelium-dependent contraction mediated by activated PMNs, indicating that prostaglandins were not involved in the observed endothelium-dependent contractile responses (data not shown).

Generation of Superoxide Anion by Activated PMNs

The ability of PMNs to generate superoxide anion in vitro in response to activation by f-met-leu-phe was studied. The addition of f-met-leu-phe to rabbit PMNs induced a time-dependent production of superoxide anion (Figure 4A). The rate of superoxide anion production was calculated and is illus-
FIGURE 2. Concentration-response curve for endothelium-dependent contraction mediated by activated polymorphonuclear neutrophils (PMNs). Concentration-response curves to PMNs were initiated approximately 1–2 minutes after the addition of f-met-leu-phe (0.1 μM) to bath chambers. n=3–6.

Trated in Figure 4B. The peak rate of production occurred at approximately 1 minute after addition of the chemotactic peptide. The time at which the peak rate of superoxide anion production occurred corresponded to the time at which maximal contractile responses were observed after addition of PMNs.

Role of Superoxide Anion—Activated PMN-Mediated Endothelium-Dependent Contraction

Inhibitors and generators of superoxide anion were used as tools to investigate further the mechanism whereby activated PMNs were producing endothelium-dependent contraction. Activated PMNs did not produce endothelium-dependent contraction in vascular tissues treated with SOD (100 units/ml) (Figure 5A). However, vascular relaxation mediated by ACh was not inhibited by SOD (Figure 5B). Endothelium-dependent relaxation mediated by ACh was 52±1% (n=5), and in the presence of SOD (100 units/ml), ACh-mediated relaxation was 59±3% (n=5).

Pyrogallol (a generator of superoxide anion) produced a contractile response (760±92 mg; n=6) that was similar to that observed with activated PMNs (Figure 6A). ACh-mediated endothelium-dependent relaxation after treatment with pyrogallol was inhibited to 37±7% (n=5) (Figure 6A). When PMNs were added to tissues treated with pyrogallol, no further contraction was produced (Figure 6B). Furthermore, ACh-mediated endothelium-dependent relaxation was markedly diminished after PMN-mediated endothelium-dependent contraction (Figure 6C).

Role of Hydroxyl Radical in Activated PMN-Mediated Endothelium-Dependent Contraction

Mannitol, a scavenger of hydroxyl radical, was tested for activity in influencing activated-PMN-mediated endothelium-dependent contraction. PMN-induced contraction of rabbit aorta was 0.43±0.05 g tension (n=5) after treatment with mannitol (20 mM) (Figure 7A), which was not significantly different from the contraction obtained in the absence of mannitol. In addition, mannitol did not prevent the reversal of ACh-mediated endothelium-dependent contraction (53±7%; n=3) (Figure 7B). These data indicate that hydroxyl radical did not contribute to the PMN-mediated contractile response.

Discussion

The present studies revealed that activated rabbit peritoneal PMNs can influence vascular smooth muscle function through interaction with the vascular endothelium. PMNs produced no effect on smooth muscle tone unless preactivated with the chemotactic peptide f-met-leu-phe. PMN-induced endothelium-dependent contraction appeared to be mediated through the generation of superoxide anion, which resulted in inactivation of EDRF. This is the first demonstration that activated PMNs produce endothelium-dependent contraction that is mediated through inhibition of EDRF.

Activation of neutrophils results in a respiratory burst, which produces up to a 20-fold increase in oxygen consumption, and greater than 90% of the oxygen consumed by neutrophils can be accounted for by superoxide anion secretion. Associated with the increase in oxygen consumption is the secretion of both superoxide anion and hydrogen peroxide. f-met-leu-phe can induce the secretion of nanomole quantities of superoxide anion and hydrogen peroxide; however, it has been reported that in order for f-met-leu-phe to induce significant secretion of these free radicals into the extracellular environment, PMNs must be pretreated with cytochalasin B (an inhibitor of microfilaments). The present studies demonstrate that the chemotactic peptide can activate PMNs without prior treatment with cytochalasin B. It has previously been demonstrated that human PMNs are not activated by f-met-leu-phe when in suspension in the absence of cytochalasin, but they are activated by this and other PMN activators when they adhere to biological surfaces, including endothelial cells. Thus, it is likely that adherence of PMNs to the vascular endothelium primed the PMNs for activation by the chemotactic...
peptide. This will result in a very localized production of superoxide anion at the endothelial cell surface and will preclude the chemical demonstration of superoxide anion production in the organ bath.

The data demonstrating that pyrogallol mimics the endothelium-dependent contractile effects of PMNs supports the hypothesis that superoxide anion generated from activated PMNs is inactivating EDRF. Ignarro and colleagues have reported that pyrogallol will produce endothelium-dependent contraction of vascular smooth muscle by generating superoxide anion, which will cause the inactivation of EDRF. As illustrated in Figure 6, pyrogallol produced contraction of endothelium-intact rabbit aortic rings, and subsequent addition of PMNs failed to produce further vascular contraction. The reason for this was that superoxide anion generated from pyrogallol destroyed the EDRF already present, and any subsequent generation of superoxide anion by PMNs was ineffective because of continuing inactivation of EDRF by superoxide anion generated by pyrogallol.

The inability of PMNs to reverse the relaxation produced by isoproterenol or nitroglycerin is due to the fact that these agents produced vascular smooth muscle relaxation that is totally independent of the vascular endothelium. It has been demonstrated that chemical generation of superoxide anion inactivates EDRF but does not affect nitroglycerin-mediated vascular relaxation. Therefore, it was not expected that activated PMNs would have any effect on vascular relaxation produced by these agents.

There is considerable support for the concept that neutrophil-derived oxygen radicals play a role in myocardial ischemia and reperfusion injury. Agents that interact with reactive oxygen species (e.g., SOD and mercaptopropionyl glycinne) or agents that prevent the production of oxygen free radicals (e.g., prostacyclin, BW 755C, and allopurinol) have been shown to attenuate tissue destruction resulting from myocardial ischemia and reperfusion. Furthermore, there have been several studies that have revealed a correlation between the severity of ischemic damage resulting from reperfusion and the extent of neutrophil infiltration. The observations of the present study provide a potential mechanism whereby neutrophils exacerbate tissue destruction. The release of superoxide anion from PMNs...
FIGURE 4. Concentration-response curves for the production (Panel A) and the rate of production (Panel B) of superoxide anion by polymorphonuclear neutrophils (PMNs). PMNs were activated by f-met-leu-phe (0.1 μM). Standard errors of the mean were sufficiently small that they were contained within symbols.

FIGURE 5. Representative recorder tracings illustrating the effect of superoxide dismutase (SOD, 100 μl) on endothelium-dependent contraction produced by activated rabbit polymorphonuclear neutrophils (PMNs) (4×10⁴ cells/ml) (Panel A) and on endothelium-dependent relaxation produced by acetylcholine (ACh) (Panel B). PE, phenylephrine; F, f-met-leu-phe; ACh, acetylcholine.

FIGURE 6. Representative recorder tracings illustrating the effect of pyrogallol (PYRO, 100 μM) on endothelium-dependent contraction produced by activated rabbit polymorphonuclear neutrophils (4×10⁴ cells/ml). PE, phenylephrine; F, f-met-leu-phe; ACh, acetylcholine.

adhering to the vascular endothelium will counteract the protective effects of endothelial released EDRF. Besides being vasodilatory, EDRF is also antiaggregatory on platelets. Inactivation of EDRF by superoxide anion released from activated PMNs may result in local vasoconstriction and platelet
activation. Platelet activation can lead to even further vasoconstriction and cardiac ischemia since thromboxane A2 is synthesized and released by activated platelets.

Engler et al20-21 have speculated that the no-reflow phenomenon following myocardial ischemia is attributable to physical obstruction of capillary blood flow by accumulating neutrophils and that these cells remain "plugged" in myocardial capillaries upon reperfusion. After reperfusion, myocardial blood flow is reduced, and neutrophils accumulate further in the ischemic area. Under experimental conditions when neutrophils are removed, the no-reflow phenomenon is eliminated and myocardial blood flow is increased.5 An extension of this hypothesis is that, in addition to the physical obstruction of capillary blood flow by neutrophils, accumulating neutrophils inactivate EDRF through the production and release of superoxide anion. The inactivation of this endogenous vasodilator will result in an increase in total coronary vascular resistance and a decrease in blood flow. Thus, the inactivation of EDRF by activated neutrophils may contribute to the no-reflow phenomenon.

The question arises if the endothelium-dependent contractile responses to PMNs are attributable to the release of an endothelium-derived contracting factor from the endothelium, rather than from superoxide anion released from PMNs. Vanhoutte and Katusic22 have recently speculated that an endothelium-derived contracting factor may be superoxide anion. These investigators have reported that in canine basilar artery, A23187 produced endothelium-dependent contractions that were blocked by SOD plus catalase. The source of superoxide anion was speculated to be through the activation of endothelial cyclooxygenase. However, in the present studies, the presence of indomethacin in the organ bath did not affect PMN-mediated endothelium-dependent contraction. In addition, PMNs activated by f-met-leu-phe were capable of generating superoxide anion in the absence of endothelium or vascular tissue (Figure 4). The possibility also exists that PMNs mediate the release of endothelin, a newly discovered contractile peptide that is produced by endothelial cells.23 We believe this unlikely because of the transient nature of PMN-induced vascular contraction. Endothelin produces slow and sustained endothelium-independent contraction of rabbit aorta that is not readily washed out (data not shown), whereas PMN-induced endothelium-dependent contraction returns to precontraction levels within several minutes. Furthermore, the ability of SOD to attenuate PMN-induced vascular responses suggests that the mediator involved is superoxide anion. However, other possible endothelium-derived contracting factors cannot be entirely ruled out.

There have been several reports of vasoactive factors, released from PMNs, that affect vascular smooth muscle activity. Sessa and Mullane24 have described a protein-like factor released from purified peripheral rabbit PMNs that produced concentration-dependent contraction of vascular smooth muscle independent of the vascular endothelium, which was not affected by SOD, free-radical scavengers, or cyclooxygenase inhibitors. This PMN-derived vasoactive factor is clearly different from the endothelium-dependent contractile factor (superoxide anion) observed in the present studies. Rimele and colleagues25 have reported recently a neutrophil-derived relaxing factor from Lewis rat periosteal PMNs. This factor resembled EDRF in that it produced in vitro endothelium-
dependent relaxation of precontracted rat aortic rings. The relaxant activity of this factor was augmented by SOD and cyclic GMP selective phosphodiesterase inhibitors and was antagonized by guanyl cyclase inhibitors. In addition, rat PMNs were capable of expressing this neutrophil-derived relaxing factor independent of PMN activation (e.g., f-met-leu-phe). In the present investigations, we were unable to demonstrate vascular smooth muscle relaxation with rabbit PMNs. This neutrophil-derived relaxing factor may be specific for rats.

In conclusion, the present investigation reveals that rabbit PMN activated by the chemotactic peptide f-met-leu-phe can release superoxide anion and interact with the vascular endothelium producing endothelium-dependent contraction. The endothelium-dependent contraction appears to be mediated through superoxide anion inactivation of EDRF.

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