Endothelium-Derived Nitric Oxide Attenuates Neutrophil Adhesion to Endothelium Under Arterial Flow Conditions

Patrick Provost, Jules Y.T. Lam, Lucie Lacoste, Yahye Merhi, David Waters

Abstract Nitric oxide (NO) synthesized from cultured endothelial cells inhibits platelet aggregation and adhesion to subendothelial extracellular matrix and may contribute to the thromboresistance of the endothelium. NO has also been shown to inhibit neutrophil aggregation and adherence to postcapillary venules. Whether NO derived from the intact endothelium of an arterial wall can influence platelet and neutrophil adhesion under whole-blood arterial flow conditions was evaluated in this study. Porcine aortic segments with intact endothelium were exposed to flowing porcine arterial blood for 5 minutes at a shear rate of 424 sec⁻¹. Pretreatment of the endothelium with the physiological precursor of NO, L-arginine (2 mmol/L), reduced ³¹¹In-labeled neutrophil adhesion by 32% from 10.2±1.6 to 6.9±1.3x10⁶/cm² (P<.05), relative to control. This effect was reversed by the inhibitor of NO synthesis, N⁵-nitro-L-arginine methyl ester (L-NAME, 5 mmol/L) (8.2±3.0 versus 8.6±3.2x10⁶/cm² for control; P=NS). Pretreatment of the endothelium with d-arginine (2 mmol/L) did not influence neutrophil adhesion (8.7±2.0 versus 8.6±2.0 x10⁶/cm² for control; P=NS). The intact endothelium, which is normally thromboresistant, shows a low basal level of ³¹¹Cr activity, corresponding to a platelet adhesion less than 0.5x10⁶/cm², and this thromboresistance was not significantly influenced by l-arginine. These results indicate that NO derived from an intact arterial endothelium under whole-blood arterial flow conditions may be an important modulator of neutrophil interaction with the intact endothelium. (Arterioscler Thromb. 1994;14:331-335.)

Key Words • endothelium • L-arginine • nitric oxide • neutrophil • platelets

Endothelial cells are able to synthesize nitric oxide (NO) from the terminal guanido nitrogen atom of the amino acid l-arginine by the NO synthase enzyme.¹ NO, which accounts for the biological properties of endothelium-derived relaxing factor (EDRF),² acts as a transduction mechanism underlying several physiological responses. Abolishment of EDRF or endothelium-derived NO exerts vasodilatory effects on underlying vascular smooth muscle³ by stimulating soluble guanylate cyclase and increasing intracellular cyclic GMP (cGMP) levels.⁴ Additionally, EDRF or endothelium-derived NO is released luminally⁵-⁶ where it can influence the interaction of circulating blood cells with the vessel wall. NO has been shown to inhibit the aggregation of platelets⁷⁻⁸ and to cause disaggregation of aggregated platelets⁹ in washed platelet suspensions under static conditions in an aggregometer. Recently, De Graaf et al¹⁰ have shown that NO derived from cultured endothelial cells is a potent inhibitor of platelet adhesion to subendothelial matrix under flow conditions. This finding may have important clinical implications if the normal vascular endothelium can also be shown to influence the adhesion of circulating blood cells to the endothelial cells. Thus, the effect of NO derived from an intact arterial vessel endothelium on whole-blood platelet adhesion to the endothelial surface under arterial flow conditions was studied.

More recently, the role of NO on neutrophil interaction with the vessel wall has been reported. Adherence of neutrophils to the vascular endothelial cells is the first step in the diapedesis of leukocytes from the vasculature and the maintenance of an acute and chronic inflammatory response that may aggravate reperfusion injury. Kubis et al¹¹ have shown that infusion of specific inhibitors of NO synthesis, such as N⁵-monomethyl-l-arginine (L-NMMMA) or N⁵-bromo-L-arginine methyl ester (L-NAME), promotes neutrophil adhesion in postcapillary venules through a CD11/CD18-dependent mechanism. In addition, neutrophils may influence platelet–vessel wall interactions and thrombosis¹²; however, the interactions among NO, neutrophils, and platelets are unclear, and the effect of NO on both platelet and neutrophil adherence in intact vessels exposed to arterial flow conditions is unknown.

Therefore, we investigated the influence of NO derived from an intact porcine arterial endothelium on platelet and neutrophil adhesion under controlled arterial flow conditions using whole blood¹³,¹⁴ at a shear rate of 424 sec⁻¹. The exposed endothelium was pretreated with vehicle, d-arginine, or its active enantiomer and the physiological precursor of NO synthesis, l-arginine, in the absence or presence of L-NAME, a specific inhibitor of NO synthesis in endothelial cells.¹⁵ It was demonstrated that enhancing endothelium-derived NO production modulates neutrophil interaction with the endothelium under arterial flow conditions, without altering the thromboresistant properties of the endothelium.

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Methods

Animal Preparation
Twenty-nine nonheparinized normal Yorkshire pigs of either sex (mean weight, 15.8±2.07 kg) were sedated by intramuscular injection of 225 mg ketamine (Rogarsetic, Rogar/STB Inc) and 125 mg azaperone (Stresnil, Janssen Pharmaceuticals). The pigs were intubated and mechanically ventilated with ambient air, and anesthesia was maintained with 0.5% halothane (Fluothane, Ayerst). Animals were treated in accordance with the guidelines of the Canadian Council on Animal Care regulations. To quantitate the adhesion of platelets and neutrophils to the vascular endothelium, these cells were radiolaabeled with 51Cr and 111In, respectively.

Isolation and 51Cr Labeling of Platelets
On the day of the experiment, 50 mL autologous blood anticoagulated with acit-citrate dextrose (ACD, Abbott) was collected from the pigs to obtain a platelet concentrate by differential centrifugation as previously described.13,14,16,17 The platelet suspension was incubated with 350 μCi of [51Cr] sodium chromate (Merck Frosst Canada Inc) for 40 minutes. The suspension was then centrifuged to remove unbound 51Cr. The radiolabeled platelets were resuspended in plasma and then reintroduced into the animal through the ear vein.

Isolation and 111In Labeling of Neutrophils
Normal porcine aortas were dissected free of surrounding connective tissues, kept in an HBSS-HEPES buffer at pH 7.4, containing 440 mg/L/L penicillin (Ayerst) and 40 mg/L gentamycin (Garamycin, Schering) at 4°C, and used within 4 days. No differences were observed between vessels that were used immediately versus those used at 4 days. Immediately before the superfusion experiment, the aorta was cut into rings and then longitudinally opened and cut into segments measuring 10x35 mm for use in the superfusion chambers. Care was taken not to touch the luminal surface. The aortic segments with intact endothelium were then incubated with D- or L-arginine hydrochloride (2 mmol/L) (Sigma Chemicals); or physiological saline vehicle (Abbott) for 60 minutes, after which unbound 111In was removed by centrifugation. The labeled neutrophil preparation was resuspended in 5 mL platelet-poor plasma and reintroduced into the animal, and the superfusion experiment was performed 1 hour later. This procedure yields a neutrophil population that is 95% pure and more than 90% viable, as assessed by the trypan blue exclusion test. The amount of platelets (x10^9/cm^2) adhering to the porcine aortic endothelium was calculated as previously described,13,16,17 knowing the blood platelet count and the 51Cr activity in blood and the endothelial tissue. The number of neutrophils (x10^3/cm^2) adhering to the endothelium was calculated in a similar fashion, knowing the blood neutrophil count and the 111In activity in blood and the endothelial tissue. Neutrophil adhesion was then expressed as a percentage of control.

Endothelial Tissue for Superfusion Experiment
Normal porcine aortas were dissected free of surrounding connective tissues, kept in an HBSS-HEPES buffer, at pH 7.4, containing 440 mg/L/L penicillin (Ayerst) and 40 mg/L gentamycin (Garamycin, Schering) at 4°C, and used within 4 days. No differences were observed between vessels that were used immediately versus those used at 4 days. Immediately before the superfusion experiment, the aorta was cut into rings and then longitudinally opened and cut into segments measuring 10x35 mm for use in the superfusion chambers. Care was taken not to touch the luminal surface. The aortic segments with intact endothelium were then incubated with D- or L-arginine hydrochloride (2 mmol/L) (Sigma Chemicals); the specific inhibitor of NO synthesis, L-NAME (5 mmol/L) (Sigma Chemicals); or physiological saline vehicle (Abbott) for 1 hour before the experiment. To ascertain that NO accounted for the observed effects induced by L-arginine, the L-arginine–treated endothelium segments were coincubated with L-NAME (5 mmol/L) before the experiment.

Superfusion Experiments
Experiments were performed using a Plexiglas superfusion chamber that mimics the tubelike or cylindrical shape of blood vessels.14 The upper wall or roof of this cylindrical chamber was removed, resulting in a window (2.0 x 25 mm) permitting direct exposure of flowing blood to the intact endothelial surface of a porcine aortic segment. The right femoral artery and vein were cannulated and connected to the Plexiglas chambers, which were immersed in a thermostatically controlled water bath at 37°C.13 Arterial blood was drawn through the superfusion flow chambers at a constant flow of 20 mL/min by a peristaltic pump (model 7014, Masterflex, Cole-Parmer Instruments Co) and recirculated back into the animal through the femoral vein. A 5-minute superfusion was performed at a shear rate of 424 sec^-1. Aortic segments with intact endothelium pretreated with L-arginine, D-arginine, the combination of L-arginine and L-NAME, or L-NAME alone were placed in the superfusion chamber on one side of a Y connector on the arterial inflow line, whereas the chamber containing control endothelium pretreated with vehicle was placed on the other side of the Y connector to allow simultaneous parallel pairwise superfusion of the treated and control endothelium.

Quantitation of Platelet and Neutrophil Adhesion
At the end of the experiment, the amount of platelets or neutrophils that adhered to the exposed endothelium within the superfusion chamber was quantitated by determining the specific 51Cr or 111In radioactivity associated with the endothelium, respectively. The radioactivity of reference blood samples was quantitated as well, using a gamma counter (Minaxi 5000, Packard Instruments) equipped with a computer and multiscint analysis program to correct for background and for overlapping activity of 51Cr and 111In. The amount of platelets (x10^3/cm^2) adhering to the porcine aortic endothelium was calculated as previously described,13,16,17 knowing the blood platelet count and the 51Cr activity in blood and the endothelial tissue. The amount of neutrophils (x10^3/cm^2) adhering to the endothelium was calculated in a similar fashion, knowing the blood neutrophil count and the 111In activity in blood and the endothelial tissue. Neutrophil adhesion was then expressed as a percentage of control.

results
Aortic segments with intact endothelium were exposed to flowing arterial blood at a shear rate of 424 sec^-1, which corresponds to values for those of large and healthy arteries where the shear rates range from 106 to 500 sec^-1.18 Under these arterial flow conditions, 51Cr activity associated with the endothelium was very low, and platelet adhesion averaged 0.38±0.08 x10^3/cm^2, which represents the background activity level and the sensitivity limit for the technique. This was similar to our previously reported value of <0.5 x10^3/cm^2 obtained in vivo on intact endothelium and suggested that the arterial endothelial preparation had maintained its thromboresistance.16,17,19 By scanning electron microscopy, the endothelium had a normal appearance, and no platelets were seen adhering to the endothelial surface. The endothelial integrity was not altered by treatment
with L-NAME. In previous studies, we have shown that injury to the arterial wall, exposing the thrombogenic media to the same arterial flow conditions ex vivo, yields a platelet deposition averaging 60 to 70 × 10⁶/cm², which is similar to the platelet deposition on the damaged arterial wall whenever the media is exposed to the circulation in vivo (60 to 100 × 10⁶/cm²). With milder arterial injury causing endothelial denudation without exposing the arterial media, a monolayer of adherent platelets (6 to 8 × 10⁶/cm²) is observed whenever the subendothelium only is exposed. Pretreatment of the endothelium with D-arginine, L-arginine, or the combination of L-arginine and L-NAME did not alter these thromboresistant properties of the intact endothelium, and ⁵¹Cr activity remained low (Fig 1). In ex vivo whole-blood aggregation studies, L-NAME did not induce aggregation of porcine platelets, relative to control.

In contrast, exposure of vehicle-pretreated intact endothelium to flowing arterial blood leads to the adherence of neutrophils that average 6.7 ± 1.0 × 10⁶/cm². This likely represents the amount of neutrophils interacting with or marginating on the endothelium under this arterial flow condition. A similar level of spontaneous baseline neutrophil adhesion to cultured endothelial cells is also observed in vitro. Pretreatment of the intact endothelium with the physiological precursor of endothelial NO, L-arginine, decreased neutrophil deposition by 32% from 10.2 ± 1.6, n = 10, to 6.9 ± 1.3 × 10⁶/cm², relative to a parallel control (P <.05). This inhibitory effect of L-arginine was abolished in the presence of the specific inhibitor of endothelial NO synthesis, L-NAME, resulting in a neutrophil adherence averaging 8.2 ± 3.0, n = 9, relative to a parallel control value of 8.6 ± 3.2 × 10⁶/cm² (P = NS) (Fig 2). In additional experiments, treatment of the intact endothelium with L-NAME alone led to a 17% increase in neutrophil adhesion (n = 7; P < .05) without altering the thromboresistance of the endothelium. To demonstrate the specificity of L-arginine action, incubation studies were also performed with D-arginine, a nonphysiological precursor of NO and an inactive stereoisomer of L-arginine. Pretreatment of the intact endothelium with D-arginine did not influence neutrophil deposition relative to control (8.7 ± 2.0, n = 10, versus 8.6 ± 2.0 × 10⁶/cm², P = NS).

Discussion

These results indicate that endothelium-derived NO attenuates neutrophil interaction with the endothelium under whole-blood arterial flow conditions. Thus, by synthesizing NO from the amino acid precursor L-arginine, vascular endothelial cells play an important role not only in the relaxation of the underlying vascular smooth muscle but also in the modulation of circulating blood cell interaction with the vessel wall. These effects of the endothelium have been attributed to EDRF, a diffusible substance released by the endothelial cells and with properties similar to those of NO. In vitro studies using washed platelets, cultured endothelial cells, or static conditions have shown that NO inhibits platelet aggregation and causes disaggregation of aggregated platelets. Because flow or shear forces modulate the adhesion of platelets and the release of EDRF and because red blood cells influence platelet reactivity, the response of platelets or neutrophils in the absence of blood in an aggregometer could be different from whole-blood cell adhesion under flow conditions. In addition, evidence for the role of NO in regulating circulating blood cell adhesion to the endothelium has come mainly from endothelial cell culture studies. Whether the intact endothelium from an arterial wall can influence the adhesion of both platelets and neutrophils in whole-blood circulating under arterial flow conditions was unclear. This study shows that enhancing NO production from an intact arterial endothelium inhibits the circulating neutrophil-endothelial interaction occurring under arterial flow conditions. Under these basal control conditions, no platelets were found to adhere to the intact endothelium, suggesting that the endothelium has maintained its thromboresistant properties. This model thus appears to accurately simulate important aspects of the functioning of the normal vascular endothelium under well-controlled flow.
conditions. It was not surprising, therefore, that enhancement of endothelium-derived NO production with L-arginine did not alter this resistance of the endothelium to platelet adhesion and deposition. Similarly, pretreatment with D-arginine and cotreatment with L-arginine and L-NAME were without further effects. It is interesting to note that De Graaf et al.31 have also noted the absence of platelet adherence to endothelial cells in culture even when endothelial NO production was maximally inhibited, raising the possibility that NO may not be the predominant thromboresistant factor produced by the endothelial cells. However, these studies were performed at a shear rate of 100 sec \(^{-1}\), where higher shear rates may detach the endothelial cells cultured on cover slips.3 Our study, however, does confirm the thromboresistance of the intact endothelium even at the higher shear rate of 424 sec \(^{-1}\).

In this study, neutrophil interaction with endothelium was inhibited by pretreatment of the endothelium with L-arginine but not with its stereoisomer D-arginine, suggesting that the inhibition was stereospecific to L-arginine. In vascular endothelial cells, L-arginine is the physiological precursor of NO synthesis,1 which can be blocked by L-arginine analogues like L-NMMA or L-NAME.15 In the present study, inhibition of NO synthesis was accomplished by pretreating the endothelium with the L-arginine analogue L-NAME, which unlike L-NMMA is not metabolized into L-arginine by endothelial cells.25 At the concentration used, the specific inhibitor of NO synthesis L-NAME reversed the L-arginine–induced attenuation of neutrophil adhesion to the intact endothelium. Recently, Ma et al.28 have shown that inhibition of endothelium-derived NO from an intact arterial wall was achieved with millimolar concentration of L-NAME, which was reversed by the presence of millimolar concentration of the precursor L-arginine. In our studies, less than 5% of the injected neutrophils were activated, as shown by the nitro blue tetrazolium test.

The mechanism whereby NO modulates leukocyte adherence is probably through the leukocyte adhesion molecules. Thus, Kubes et al.11 have recently shown that a monoclonal antibody (MoAb IB) directed against the common \(\beta\)-subunit (CD18) of the leukocyte-adhesion glycoprotein complex prevented the leukocyte adherence induced by L-NAME, suggesting that NO mediates its antiadhesive effect through the leukocyte adhesion molecule CD11/CD18.12

In some pathophysiological states such as myocardial ischemia and reperfusion, activated neutrophils can induce significant endothelial dysfunction by releasing oxygen free radicals, especially the superoxide anion, which is known to rapidly inactivate NO.27 In atherosclerotic vessels, reduced endothelium-derived NO release is observed28 and may promote vasoconstriction and facilitate platelet aggregation and release of platelet-activating mediators (eg, thromboxane A\(_2\), serotonin, ADP, and platelet-activating factor), which may contribute to the progression of the disease. Recently, Lefer and Ma29 have shown that decreased NO release in hypercholesterolemia is associated with enhanced neutrophil adherence. In the present study, we observed that in normal arteries, enhanced NO production and release attenuate neutrophil adhesion under arterial flow conditions. These results have important potential pathophysiological significance, since adherent neutrophils may influence platelet thrombosis32 and may produce and release superoxide radicals,30 arachidonic acid metabolites (leukotrienes A\(_4\) and B\(_4\)),30 and proteolytic enzymes (elastase and collagenase),30,31 all of which may amplify endothelial dysfunction and promote arterial wall damage. Enhanced NO production may be protective by interfering directly with neutrophil function. A study by McCall et al.13 has shown that NO can inhibit rabbit neutrophil aggregation induced by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, an effect that is potentiated by superoxide dismutase and by a phosphodiesterase inhibitor. In addition, 3-morpholino-sydnonimine (SIN-1), which spontaneously releases NO, has also been shown to inhibit neutrophil function, an effect mediated by cGMP.33

In summary, this study shows that pretreatment of the intact porcine arterial endothelium with L-arginine, the physiological precursor of NO, attenuated whole-blood porcine neutrophil adhesion to the endothelium, an effect reversed by the specific inhibitor of NO synthesis, L-NAME. These results show that endothelium-derived NO attenuates neutrophil interaction with endothelium under arterial flow conditions and raise the possibility that enhanced NO production may modulate endothelial function, atherosclerosis, and inflammation, processes in which leukocyte and platelet adhesion may be important. These studies may be important in better understanding the role of the normally functioning arterial endothelium and of endothelial NO in regulating platelet and neutrophil–vessel wall interaction under arterial flow conditions.

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