Intracellular Oxidative Stress Induced by Nitric Oxide Synthesis Inhibition Increases Endothelial Cell Adhesion to Neutrophils

Xiao-fei Niu, C. Wayne Smith, Paul Kubes

Abstract The objective of the present study was to determine whether prolonged inhibition of nitric oxide synthesis in endothelial cells increased the surface adhesion of these cells for neutrophils. Human umbilical vein endothelial cells (HUVECs) were grown to confluence in 48-well microtiter plates. Exposure of HUVECs to the nitric oxide synthesis inhibitor N⁶-nitro-l-arginine methyl ester (L-NAME) did not cause neutrophil adhesion at 1 hour but increased adhesion at 4 hours in a dose-dependent manner. The increased adhesion was prevented with L-arginine or nitric oxide donors but not an analogue of cGMP. The increased adhesion was inhibited by monoclonal antibodies directed against the β₂-integrin CD18 and endothelial cell adhesion molecule ICAM-1. Platelet-activating factor (PAF) receptor antagonist WEB 2086 also prevented the L-NAME–induced neutrophil adhesion. Intracellular oxygen radical scavengers (dimethyl sulfoxide, butylated hydroxytoluene, and α,α′-dipyridyl), the iron chelator desferrioxamine, and the mitochondrial inhibitor azide inhibited the L-NAME–induced neutrophil adhesion, whereas extracellular oxygen radical scavengers (superoxide dismutase and catalase) had no effect. HUVECs were loaded with 2',7'-dichlorodihydrofluorescein diacetate, and oxidation to the fluorescent dichlorodihydrofluorescein (DCHF) was monitored. Fluorescence was enhanced in the L-NAME–treated HUVECs throughout the 4-hour incubation, an event inhibitable by an antioxidant and azide. The magnitude of the intracellular oxidation of DCHF was equivalent to ≈0.8 μmol/L H₂O₂. These data suggest that prolonged nitric oxide synthesis inhibition in HUVECs causes an oxidant- and PAF-associated rise in adhesion on the surface of these endothelial cells for neutrophils. (Circ Res. 1994;74:1133-1140.)

Key Words: • integrin • neutrophil • N⁶-nitro-l-arginine methyl ester

Endothelium-derived relaxing factor (nitric oxide [NO]) is synthesized by vascular endothelial cells from the terminal guanidino nitrogen atoms of l-arginine.¹ This biosynthetic process is inhibited by analogues of l-arginine such as N⁶-nitro-l-arginine methyl ester (L-NAME) and N⁶-monomethyl-l-arginine (L-NMMA) as described by Rees et al.² NO is a critical homeostatic regulator of vasodilator tone inasmuch as synthesis inhibition of this local autacoid results in a significant rise in vascular resistance in essentially all tissues.³ In addition to its well-described vasodilator properties, endothelium-derived NO regulates other physiological actions within the vasculature, including platelet aggregation⁴ and leukocyte adhesion⁵ in the microcirculation. Clearly, impairment of NO synthesis would, in due time, result in profound circulatory pathologies. Indeed, there is a growing body of evidence indicating that various disease conditions, including hypercholesterolemia, hypertension, and reperfusion injury, are associated with suppressed endothelium-derived NO production.⁶ This is best exemplified by the hyporesponsiveness of both venular and arterial endothelium from hypercholesterolemic, hypertensive, or reperfused vessels to agonist-mediated NO production but not exogenous NO donors.⁷-⁸

A hallmark feature of hypercholesterolemia, hypertension, and ischemia/reperfusion is the adhesion of leukocytes as well as platelets to vascular endothelium.⁹-¹² It remains unclear whether the early alteration in endothelial NO production is a cause of subsequent leukocyte adhesion in these vascular pathologies; however, circumstantial evidence would support this notion. Inhibition of NO synthesis results in increased leukocyte adherence in postcapillary venules, a response that could be prevented by adding exogenous NO¹³ or high concentrations of l-arginine.⁴ Moreover, impairment of basal NO production in postischemic vessels correlated temporally with increased leukocyte adhesion, and the latter was reduced by administering exogenous NO.¹⁴ On the basis of these results, we⁴,¹⁵ and others⁴,¹⁶ proposed that NO was an important endogenous modulator of leukocyte adhesion, although the precise molecular mechanism(s) underlying this observation was not elucidated. One hypothesis derives its basis from the fact that NO is capable of avidly inactivating superoxide,¹⁷,¹⁸ a proadhesive molecule¹⁹ that is ambienly produced within all cells.²⁰ Therefore, after NO synthesis inhibition, one might expect increased oxidative stress, which could presumably activate various endothelial cell adhesive mechanisms, thereby promoting leukocyte adhesion. Previous work has clearly demonstrated that exposure of endothelial cells²¹,²² or isolated vessels²³ to exogenous oxidants promotes neutrophil adhesion via platelet-activating factor (PAF), the β₂-integrin CD18, and the endothelial cell adhesion mole-
cular ICAM-1. The objective of the present study was to determine whether prolonged NO synthesis inhibition in endothelial cells increased surface adherenseness for polymorphonuclear granulocytes (more specifically, neutrophils) via an oxidative stress and to systematically assess the mechanisms involved.

**Materials and Methods**

**Materials**

Monoclonal antibodies (MAbs) RR1/1 (anti–ICAM-1) and R6.5 (anti–ICAM-1) were prepared as previously described.26-27 Briefly, umbilical cords were collected from healthy term neonates delivered at the University Hospital, Linkoping, Sweden. The umbilical cords were kept on ice until the time of processing. Mononuclear leukocytes were isolated by a density gradient centrifugation technique using Lymphoprep (Nycomed, Oslo, Norway) according to the manufacturer’s instructions. The isolated mononuclear leukocytes were washed three times in PBS and resuspended in medium 199 containing 25 mmol/L HEPES, 100 mmol/L sodium bicarbonate, 100 mmol/L sodium lactate, 1% fetal calf serum, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B). The mononuclear leukocytes were incubated with different concentrations of anti–ICAM-1 antibody in the presence of 1 μmol/L L-NAME and 20 μg/mL W6/32 (anti–HLA framework MAb) at 37°C for 1 hour. The endothelial monolayers were washed five times with cold PBS, 1% fetal calf serum was added, and the cells were incubated overnight at 37°C. The next morning, the adherent mononuclear leukocytes were washed again and fixed with 1% formaldehyde in PBS. The cells were then incubated with a 1:200 dilution of a mouse–monoclonal antibody to CD18, followed by a 1:200 dilution of RR1/1 MAb (anti–ICAM-1) overnight at 4°C. The cells were then incubated with a secondary 1:200 dilution of goat–anti–mouse IgG (H+L) at 37°C for 1 hour. The cells were washed again and incubated with fluorescein–conjugated goat–anti–rabbit IgG at 37°C for 1 hour. The cells were then washed extensively with cold PBS and placed in a 96-well plate. The fluorescence was read on a FACScan (Becton Dickinson Systems Inc) with the channel number (by scale) representing the mean fluorescence intensity, as previously described.28 In a final series of experiments, the endothelial monolayers were pretreated with the PAF antagonist WEB 2086 (20 μg/mL). This concentration of WEB 2086 prevents PAF-induced neutrophil adhesion in our assay system.

**Intracellular Oxidant Production**

Oxidative flux within endothelial cells was measured as previously described for myocytes.29 HUVECs were grown to confluence in T25 flasks, detached (Puck’s EDTA solution), washed, and resuspended in 100 μmol/L 2′,7′dichlorodihydrofluorescein diacetate (DCHF; Molecular Probes, Inc) in 10% medium 199 (in HBSS) for 20 minutes at 37°C followed by two washes. Fluorometric studies were performed by monitoring cellular fluorescence with a fluorescence spectrometer (model LS-3B, Perkin-Elmer Ltd; excitation, 488 nm; and emission, 521 nm) over a 4-hour incubation period in control media.
cells and cells exposed to L-NAME, L-NAME plus DMSO, or L-NAME plus azide. In other experiments, endothelial cells were exposed to various concentrations of H₂O₂ for 4 hours. Fluorescence was measured at 0.5, 1, 2, 3, and 4 hours of incubation.

Lytic Injury and Detachment

To ensure that the addition of L-NAME for prolonged periods of time was not causing endothelial cell injury (assessed as lysis and detachment), confluent HUVEC monolayers were incubated overnight with Na³⁵CrO₄. Before the experiments, the monolayers were washed three times to remove unincorporated radioactivity. Cells were incubated for various durations with L-NAME (10⁻³ mol/L). At the end of the incubation periods, washing the wells did not disturb the monolayers of healthy cells but detached the injured cells. NaOH was used to remove the remainder of attached cells. Endothelial cell lysis and detachment were assessed as the percent of ³⁵Cr released into the supernatant and the amount as pellet, respectively.

Statistics

All values are expressed as mean±SEM, and means were compared by ANOVA and a paired Student’s t test with a Bonferroni correction for multiple comparisons. Statistical significance was set at P<.05.

Results

Inhibition of NO Promotes Neutrophil Adhesion

Fig 1 illustrates that exposure of endothelial cells to L-NAME for 1 hour had no effect on neutrophil adhesion, whereas 2- and 4-hour exposure did increase neutrophil adhesion. Maximal increases in leukocyte adhesion were observed at 4 hours. Exposure of endothelial cells to 6 or 8 hours of L-NAME caused a smaller, more variable response (data not shown), which may have been related to some endothelial cell injury (detachment and lysis). Endothelial cell lysis as assessed by ³⁵Cr leakage out of cells increased significantly only at 8 hours of incubation. Since L-NAME–induced adhesion peaked at 4 hours and lysis was not significantly elevated above untreated endothelial monolayers, it is unlikely that the increased adhesiveness was a result of progressive cellular injury. A dose-dependent increase in neutrophil adhesion to HUVECs, reaching peak values at 10⁻³ to 10⁻² mol/L, was observed (Fig 2). Fig 3 demonstrates that the L-NAME–induced increase in neutrophil adhesion to HUVECs was prevented by high doses of L-arginine (10⁻² mol/L). Equimolar concentrations of L-NAME (10⁻² mol/L) and L-arginine (10⁻² mol/L) did not reverse the effects of L-NAME. L-Arginine did not appear to be antiadhesive, per se, inasmuch as this amino acid did not affect neutrophil adhesion induced by PMA or by lipopolysaccharide (LPS) (data not shown). The fact that L-arginine did not reverse LPS-induced adhesion makes it unlikely that the rise in L-NAME–induced adhesion (L-arginine inhibitable) is simply a result of LPS-contaminated L-NAME. D-NAME did not induce neutrophil adhesion to HUVECs. Not shown is that a similar threefold to fourfold increase in neutrophil adhesion was observed with a second NO inhibitor, L-NMMA. Finally, inhibition of NO synthesis in other cells including a rat intestinal epithelial cell line (IEC 18) also caused a significant increase in neutrophil adhesion (2.8±0.4% versus 12.7±0.8%), suggesting that inhibition of NO synthesis in cells could conceivably be a global signal for neutrophil adhesion.
Fig 4. Bar graphs showing the ability of two nitric oxide donors (SIN-1 or nitroprusside) or the cGMP analogue dibutyryl cGMP (Db-cGMP) to reverse L-NAME-induced neutrophil adhesion to human umbilical vein endothelial cells. A concentration of 10^(-3) mol/L L-NAME was used (n=6 each). PMN indicates polymorphonuclear leukocytes. *P<.05 relative to L-NAME value.

Fig 4 demonstrates that the continuous supplementation of exogenous NO via nitroprusside or SIN-1 to L-NAME-treated endothelial cells significantly attenuated L-NAME-induced neutrophil adhesion to HUVECs. Identical concentrations of dibutyryl cGMP did not affect the L-NAME-induced neutrophil adhesion. It should be noted that addition of 1 mmol/L dibutyryl cGMP to endothelial cells increased intracellular cGMP levels more than 100-fold, suggesting that the enhanced endothelial adhesion was independent of alterations in cGMP. Finally, the exogenous NO donors were not directly affecting the ability of neutrophils to adhere, inasmuch as PAF, LTB4, fMLP, or PMA induced similar amounts of neutrophil adhesion in the absence and presence of SIN-1 (1 mmol/L) for 30 minutes (Table 1). Additionally, SIN-1 did not affect polymorphonuclear leukocyte adhesion to TNF-α-treated (4 hours) HUVECs. Identical responses were observed with a second NO donor, CAS 754 (data not shown).

L-NAME–Induced Adhesion Is Cd18/ICAM-1–Mediated

To further elucidate the mechanism underlying the L-NAME–induced neutrophil adhesion to HUVECs, MAbs directed against various adhesion molecules on neutrophils and endothelial cells were tested. Fig 5 indicates that IB4, the anti-CD18 antibody, was most effective at preventing neutrophil adhesion to L-NAME–treated HUVECs. Antibodies directed against ICAM-1 (RR1/1 and R6.5) also attenuated neutrophil adhesion to L-NAME–treated HUVECs (80% and 74%, respectively). An isotype-matched control MAb directed against HLA class I (W6/32) on endothelial cells also did not prevent L-NAME–induced neutrophil adhesion. Flow cytometric analysis revealed that expression of E-selectin was not observed in HUVECs after 4 hours of incubation with L-NAME. Although constitutive ICAM-1 expression was noted on untreated HUVECs, an increase was not observed at 4 hours of L-NAME treatment (data not shown).

The PAF receptor antagonist WEB 2086 greatly attenuated L-NAME–induced neutrophil adhesion (Fig 6). WEB 2086 was as effective as the anti-CD18 antibody at reducing L-NAME–induced adhesion. WEB 2086 at 10 μmol/L inhibits PAF-induced neutrophil adhesion (Fig 6) but does not affect nonspecific PMA-activated neutrophils from adhering to endothelial cells (data not shown).

Table 1. Effect of SIN-1 on Chemotactically Induced Neutrophil Adhesion

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<tr>
<td>Control (n=4)</td>
<td>6.1±1.7</td>
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<td>PAF (n=6)</td>
<td>24.1±2.2</td>
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<td>LTB4 (n=4)</td>
<td>24.0±6.4</td>
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<tr>
<td>fMLP (n=4)</td>
<td>28.0±2.0</td>
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PAF indicates platelet-activating factor; LTB4, leukotriene B4; fMLP, N-formylmethionylleucylphenylalanine; PMA, phorbol myristate acetate; and TNF-α, tumor necrosis factor-α. Values are mean±SEM.

Neutrophils were incubated on endothelial cells for 30 minutes with PAF (10^(-6) mol/L), LTB4 (100 nmol/L), fMLP (10^(-6) mol/L), PMA (3×10^(-7) mol/L), or TNF-α (30 U/mL) in the presence or absence of the nitric oxide donor SIN-1 (1 mmol/L). Each experiment (n value) was run in triplicate.

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Fig 5. Bar graph showing N0^-nitro-l-arginine methyl ester (L-NAME)–induced neutrophil adhesion in the presence of IB4 (anti-CD18 antibody, n=7), RR1/1 or R6.5 (anti-ICAM-1 antibodies, n=7 and n=3, respectively), and the control antibody W6/32 (anti-HLA class l, n=4). HUVEC indicates human umbilical vein endothelial cells. *P<.05 relative to control value (Con). 1P<.05 relative to L-NAME value.
L-NAME–Induced Adhesion Is Oxidant-Mediated

Table 2 summarizes the order of effectiveness of various antioxidants on L-NAME–induced neutrophil adhesion. Although a dose-response effect was observed, only the most effective concentrations for each antioxidant are shown. Three intracellular antioxidants (DMSO, α,α'-dipyridyl, and butylated hydroxytoluene) and desferrioxamine, the iron chelator, significantly attenuated the L-NAME–induced rise in neutrophil adhesion. The antioxidants had to be present for the 4-hour L-NAME incubation to be effective. Administration of these agents at 3 hours with neutrophils did not attenuate the L-NAME–induced adhesion, suggesting that the source of the oxidants was likely the endothelial cells (data not shown). Moreover, none of the intracellular antioxidants had a direct effect on PMA-induced neutrophil adhesion, suggesting that these molecules were unlikely to directly affect neutrophil–endothelial cell adhesive interactions.

TABLE 2. Effect of Antioxidant and Desferrioxamine on N⁵-Nitro-L-arginine Methyl Ester–Induced Neutrophil Adhesion

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>IC₂⁰ concentrations (µmol/L)</th>
<th>% Inhibition</th>
<th>*P &lt; .05 relative to L-NAME (left bars) value</th>
</tr>
</thead>
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<tr>
<td>Azide (0.01 mmol/L)</td>
<td>88.8±4.2*</td>
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<td>*P &lt; .05 relative to L-NAME value</td>
</tr>
<tr>
<td>DMSO (0.02%)</td>
<td>78.6±3.4*</td>
<td>78.6±3.4*</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>α,α'-Dipyridyl (2 mmol/L)</td>
<td>79.3±4.8*</td>
<td>79.3±4.8*</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>Desferrioxamine (0.2 mmol/L)</td>
<td>64.1±6.5*</td>
<td>64.1±6.5*</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (0.1 mmol/L)</td>
<td>49.2±3.8*</td>
<td>49.2±3.8*</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>SOD (60 µg/mL)</td>
<td>9.2±3.5</td>
<td>9.2±3.5</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>Catalase (50 µg/mL)</td>
<td>10.0±4.5</td>
<td>10.0±4.5</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
<tr>
<td>SOD (60 µg/mL) + catalase (50 µg/mL)</td>
<td>−16.0±3.0</td>
<td>−16.0±3.0</td>
<td>*P &lt; .05 relative to L-NAME (left bars) value</td>
</tr>
</tbody>
</table>

L-NAME indicates N⁵-nitro-L-arginine methyl ester; DMSO, dimethyl sulfoxide; and SOD, superoxide dismutase. Values are mean±SEM. Endothelial cells were incubated with L-NAME and each of the antioxidants or desferrioxamine for 4 hours. At 3 hours, neutrophils were added, and adhesion was determined at 4 hours. Data are presented as percent inhibition of L-NAME–induced adhesion. Only the most effective concentration is shown for each agent (n=5).

In contrast, extracellular antioxidants, including SOD, catalase, or a combination of the two, did not attenuate neutrophil adhesion to L-NAME–treated HUVECs (Table 2). To ascertain the source of the intracellular oxidants, HUVECs were treated with sodium azide, the mitochondrial inhibitor, for 3 hours and then washed before the administration of neutrophils. This concentration of azide, which has previously been reported not to affect neutrophil function,33 reduced the L-NAME–induced adhesion by almost 90% but did not affect PMA-stimulated neutrophil adhesion.

Fig 7 summarizes the fluorescence data, indicating that L-NAME–induced oxidation of the DCHF probe within endothelial cells increased during the 4-hour incubation period. In every experiment, there was some spontaneous oxidation of DCHF in untreated endothelial cells. These background values could not be inhibited by azide or DMSO and were therefore subtracted from each of the experimental conditions. In each of five experiments, fluorescence in L-NAME–treated endothelial cells was greater than in control cells, an observation that was inhibited completely by DMSO or sodium azide. L-NAME–induced fluorescence was higher than that induced by 0.5 µmol/L H₂O₂ but lower than that induced by 1.0 µmol/L H₂O₂. When a fluorescence calibration curve was generated by various concentrations of H₂O₂, L-NAME–induced oxidation of DCHF was seen to be equivalent to approximately the same fluorescence induced by 0.8 µmol/L H₂O₂ (Fig 7, inset).

Discussion

We and others have demonstrated that NO synthesis inhibition promotes a rapid (within 30 minutes) leukocyte infiltration into the microcirculation in various organs of the rat,34,35 cat,33 and rabbit.16 In the present study, we demonstrate that prolonged inhibition of NO synthesis in HUVECs will also support neutrophil adhesion, suggesting that the endothelium can increase its adhesion in this human in vitro system. However, the molecular mechanisms involved are at least in part very different from the condition identified to date in vivo. The underlying mechanism for L-NAME–induced leukocyte accumulation in postcapillary venules was dependent on cGMP, because the cGMP analogue (dibutyryl cGMP) prevented the leukocyte influx.20 Moreover, L-NAME superfusion caused mast cell degranulation, and ketotifen, a connective tissue mast cell stabilizer, blocked both the L-NAME–induced mast cell degranulation and leukocyte adhesion.20 Interestingly, SOD, an extracellular superoxide radical scavenger, also prevented the L-NAME–induced mast cell degranulation and leukocyte adhesion.20 These data led us to propose that after NO synthesis inhibition, increased superoxide flux from an unknown cellular source caused mast cell degranulation and the rapid leukocyte adhesion to postcapillary venular endothelium.

It remained unclear whether the endothelial cell, a main source of constitutively produced NO, could also directly contribute to the L-NAME–induced leukocyte adhesion. In the present study, we demonstrate that prolonged exposure of endothelial cells (2 to 4 hours) to inhibitors of NO synthesis induces neutrophil adhesion; however, the adhesion is independent of extracellular oxidants, cGMP, and mast cells. Moreover, the adhe-
sion required at least 2 hours of NO synthesis inhibition and reached peak values at 4 hours.

It is unlikely that NO directly prevents neutrophil adhesion, since delivery of relatively high concentrations of NO donors (1 mmol/L) did not prevent neutrophil adhesion induced by various proinflammatory agents. In fact, the present study strongly supports the view that inhibition of NO synthesis in endothelial cells leads to increased intracellular oxidative stress, which subsequently induces the neutrophil–endothelial cell interaction. Two lines of evidence support this view: (1) L-NAME superfusion of endothelial cells resulted in increased oxidative flux within these cells, as measured by the fluorophore DCHF. (2) Intracellular but not extracellular antioxidant therapy greatly attenuated both oxidation of DCHF and the L-NAME–induced neutrophil adhesion. The precise mechanism and oxidative products involved in the oxidation of the DCHF probe are not entirely clear from the present study, but iron appears to play a role, in light of the fact that desferrioxamine also significantly attenuated the oxidant-mediated leukocyte adhesion. Moreover, DMSO, a hydroxyl radical scavenger,37 was also very effective, invoking the possible involvement of the iron-dependent Haber-Weiss reaction—a reaction that produces the hydroxyl radical. Regardless of the oxidant involved, the fact that inhibition of NO synthesis leads to measurable intracellular oxidative stress suggests that endogenous NO may function to scavenge ambiently produced oxidants (perhaps superoxide) and thereby prevent neutrophil–endothelial cell interactions. In fact, the observation that L-NAME also promoted neutrophil adhesion to an epithelial cell line suggests that inhibition of NO synthesis in any cell might increase surface adhesivity and act as a global signal for phagocytic cells to adhere to that cell.

Our data would implicate mitochondria of the endothelial cell as the primary site of oxidant generation in the absence of NO. This hypothesis is based on the observation that sodium azide, at concentrations that inhibit mitochondrial respiration,33 greatly attenuated L-NAME–induced neutrophil adhesion. Although this source of continuously produced superoxide is an often-ignored component of the oxidants produced by the body, 1% to 4% of all oxygen taken up by mitochondria results in the production of superoxide and/or H2O2.20 Since the DCHF probe can be oxidized by a variety of oxygen species,32 it is difficult to quantify production of a single oxidant; however, our data suggest that in the absence of NO, endothelial cells produced an oxidative flux equivalent to 0.8 μmol/L of H2O2. The ability of NO to inactivate superoxide has previously been suggested by various investigators,17,28 including Rubanyi et al.,39 who demonstrated that superoxide production by activated neutrophils was quickly inactivated by NO donors. The novelty of our observations is that endogenous production of intracellular NO serves to scavenge mitochondrial superoxide oxidant during cell metabolism.

The increased oxidative flux was apparent as early as 30 minutes and persisted throughout the 4 hours of L-NAME exposure. It is likely that the oxidant flux had to reach a critical level before endothelial adhesivity became apparent. This notion is based on the observation that increased neutrophil adhesion occurred predominately at 4 hours and that the membrane-permeable antioxidants and NO donors had to be given simultaneously with L-NAME; addition of these effec-

![Graph showing time course of oxidation of the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate within endothelial cells.](image-url)

**Fig. 7.** Line graph showing time course of oxidation of the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate within endothelial cells. N^6^-Nitro-L-arginine methyl ester (L-NAME)–induced oxidation increased rapidly and persisted for the duration of the 4-hour incubation. This effect was entirely inhibited by azide, the inhibitor of mitochondrial respiration, or the antioxidant dimethyl sulfoxide (DMSO). The L-NAME values were larger than the value observed when endothelial cells were exposed to 0.5 μmol/L H2O2 but not 1.0 μmol/L H2O2. The inset demonstrates the dose-response relation to increasing concentrations of H2O2. Each experiment was run five times.
tors at 3 hours of L-NAME exposure did not abrogate the L-NAME response at 4 hours. The latter suggests that accumulating oxidative stress generated within the endothelial cell ultimately activated proadhesive mechanisms between neutrophils and endothelium.

PAF, a biologically active phospholipid, is a proadhesive molecule expressed within minutes by endothelial cells exposed to various oxidants, thrombin, histamine, and leukotriene C4.21,22,40 The PAF molecule remains endothelial cell associated and acts as a signal to induce adhesiveness of CD11/CD18 on leukocytes. This so-called juxtacrine activation40 plays an important role in producing leukocyte adhesion to vascular endothelium in vitro21,22 and to inflamed vessels in vivo.41 In the present study, we present evidence that the increased oxidant flux within endothelial cells leads to both a PAF- and CD11/CD18-dependent leukocyte adhesion. This notion is based on the fact that both the anti-CD18 antibody IB, and the PAF receptor antagonist WEB 2086 prevented the L-NAME–associated rise in neutrophil adhesion. Although we did not measure PAF synthesis in L-NAME–treated endothelial cells, there is some evidence suggesting a role for NO in PAF production. Recently, nitrovasodilators have been shown to inhibit PAF synthesis by thrombin-activated HUVECs.30 Hence, in addition to inactivating superoxide, the aforementioned data of Heller and colleagues30 would suggest that NO may also affect PAF production within endothelial cells. Our data would suggest that the converse is also true; ie, reduction in NO production stimulates PAF synthesis. However, this requires further investigation.

Although the increase in adhesion was only threefold to fourfold above control, it is likely that chronic impairment of NO synthesis in vivo would likely cause an even more profound accumulation of neutrophils. Moreover, other inflammatory cells, including platelets, monocytes, and the already documented effect of mast cells,46 could also contribute to neutrophil–endothelial cell interactions in vivo. Finally, another important issue to consider is that HUVECs are endothelial cells isolated from a large vein, whereas much of the endothelial cell–leukocyte interactions that take place in vivo transpire in postcapillary venules. Selective isolation of endothelial cells from these venules is technically very difficult but is an important issue to consider whenever using HUVECs. Nevertheless, the data in the present study suggest that prolonged NO synthesis inhibition in endothelial cells characteristic of various pathologies, including ischemia/reperfusion and hypercholesterolemia, causes an oxidant- and PAF-associated CD18-dependent rise in adhesion for neutrophils.

Acknowledgments

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References


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