Exogenous NO enhances hydrogen peroxide-mediated neutrophil adherence to cultured endothelial cells

Okayama, Naotsuka, Hiroshi Ichikawa, Laura Coe, Makoto Itoh, and J. Steven Alexander. Exogenous NO enhances hydrogen peroxide-mediated neutrophil adherence to cultured endothelial cells. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L820–L826, 1998.—One important aspect of oxidant injury is the enhancement of neutrophil-endothelial adhesion by oxidants. Recent studies suggest that nitric oxide (NO) can limit oxidant-mediated tissue injury, since inhibitors of endogenous NO synthesis often promote neutrophil-endothelial adhesion. However, less is known about the direct role of exogenous NO in modulating proadhesive effects of oxidants. The objective of this study was to examine how an NO donor modifies hydrogen peroxide-mediated adhesion of neutrophils to cultured endothelial cells. Human umbilical vein endothelial cell monolayers were exposed for 30 min to 0–0.1 mM hydrogen peroxide with or without the NO donor spermine-NONOate (SNO; 0–0.5 mM), and the adhesion of $^{51}$Cr-labeled polymorphonuclear neutrophils (PMNs) was measured in a static adhesion assay. PMN adherence was not altered by either peroxide (up to 0.1 mM) or SNO (up to 0.5 mM) alone but was significantly increased by over 300% by coadministration of both 0.1 mM peroxide and 0.5 mM SNO. This increase in adhesion with these two agents was correlated with an increase in the presentation of surface P-selectin but not intercellular adhesion molecule-1. Both PMN adhesion and P-selectin presentation were blocked by 0.1 mM deferoxamine (an iron chelator) and 1 mM methionine (an oxy-radical scavenger). WEB-2086, a platelet-activating factor-receptor antagonist (10 µM), also prevented PMN adhesion but not P-selectin expression. An antibody directed against either P-selectin or intercellular adhesion molecule-1 also blocked adhesion. These data indicate that NO may actually exacerbate rather than protect against the inflammatory effects of peroxide in some models of inflammation through the synthesis of platelet-activating factor and the mobilization of P-selectin.

Oxygen radicals; iron; platelet-activating factor; P-selectin; intercellular adhesion molecule-1; spermine-NONOate

MATERIALS AND METHODS

Subjects. The procedures used to obtain human endothelial cells were approved by the Institutional Review Board for Human Research at the Louisiana State University Medical Center. Each subject provided written consent and was paid for participating in the study.

Cell culture and treatment protocols. Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment as previously described (35). The cells were plated in endothelial cell growth medium (EGM; Clonetics, San Diego, CA) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), thymidine (2.4 mg/l; Sigma, St. Louis, MO), glutamine (230 mg/l; Gibco BRL, Gaithersburg, MD), heparin sodium (10 IU/ml; Sigma), antibiotics (100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 0.125 mg of amphotericin B), and endothelial cell growth factor (80 µg/ml; Biomedical Technologies, Stoughton, MA). All other tissue culture reagents were obtained from Gibco BRL. The cell cultures were incubated at

Copyright © 1998 the American Physiological Society

L820 1040-0605/98 $5.00
37°C in a humidified atmosphere with 5% CO₂ and expanded by brief trypsinization (0.25% trypsin in PBS containing 0.02% ethylenediamine tetraacetic acid). Primary passage HUVEC were seeded into gelatin- (0.1%) and fibronectin-coated (25 µg/ml) 11-mm 48-well tissue culture plates and used when confluent. Culture medium was replaced every second day. Only primary cultures were used for these studies. Cells were identified as endothelial cells by their cobblestone appearance at confluency and positive labeling with 1) low-density lipoprotein labeled with 1,1'-diodoacetyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Biomedical Technologies) and 2) mouse anti-human factor VIII (Caltbiochem, San Diego, CA).

For PMN adhesion and adhesion molecule expression assays, HUVEC monolayers were exposed to 0–0.5 mM SNO with or without 0.1 mM hydrogen peroxide or to 0–0.1 mM peroxide with or without 0.5 mM SNO (Cayman, Ann Arbor, MI) for 30 min at 37°C in Hank’s balanced salt solution (HBSS). We also examined the effect of 0.5 mM spermine (Sigma), a by-product of SNO, plus 0.1 mM hydrogen peroxide on PMN adhesion. To evaluate the contributions of iron, oxy radicals, and PAF on peroxide/SNO-induced events, we coincubated monolayers with 0.1 mM desferrioxamine (DF; Sigma), an iron chelator, preincubated for 2 h; 1 mM methionine (Sigma), an oxy-radical scavenger; and 10 µM WEB-2086 (preincubation for 15 min; Boehringer, Ingelheim, Germany), a PAF-receptor antagonist.

In one set of experiments, an anti-P-selectin antibody (clone PB 1.3, 20 µg/ml) or an anti-ICAM-1 antibody (CD54, 20 µg/ml) was added after the addition of SNO (0.5 mM) and hydrogen peroxide (0.1 mM) to monolayers to block PMN binding mediated by endothelial P-selectin and ICAM-1 to demonstrate the dependence of adhesion on the upregulation of endothelial P-selectin and the presence of ICAM-1. (These antibodies were provided by Dr. Donald Anderson from Pharmacia-Upjohn Laboratories, Kalamazoo, MI.)

PMN isolation. Human PMNs were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma) (35). This procedure yields a PMN population 95–98% viable (by trypan blue exclusion) and 98% pure (by acetic acid-cryocrystal violet staining).

Static adhesion assay. Isolated PMNs were suspended in HBSS and radiolabeled by incubation (at a concentration of 2 × 10⁷ cells/ml) with 30 Ci of Na⁵¹CrO₄/ml of PMN suspension at 37°C for 1 h. The cells were washed twice with 4°C HBSS, centrifuged at 250 g for 4 min to remove unincorporated radioactivity, and resuspended in HBSS. Monolayers were treated with SNO, hydrogen peroxide, or a combination of both of the agents with and without DF, methionine, and WEB-2086. After 30 min, these treatments were removed, and labeled PMNs were allowed to adhere for 30 min in HBSS. Labeled PMNs were added to HUVEC monolayers at a PMN-to-HUVEC ratio of 10:1. In one set of experiments, an anti-P-selectin or ICAM-1 antibody was coincubated with PMNs. After coincubation, the percentage of added PMNs that adhered to the HUVEC monolayers was quantified (35).

Endothelial adhesion molecule surface expression assay. Surface expression of P-selectin and ICAM-1 was assayed using the method of Khan et al. (7). HUVEC monolayers grown in 48-well plates were fixed with 0.25 ml of 1% paraformaldehyde in PBS for 10 min at room temperature after exposure to chemical agents. After being washed three times with 1 ml of HBSS-PBS (1:1) solution, monolayers were incubated with an anti-P-selectin antibody (10 µg/ml) or an anti-ICAM-1 antibody (10 µg/ml) in HBSS-PBS+5% FCS at 37°C for 30 min. Monolayers were washed twice with 0.5 ml of HBSS-PBS solution and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 diluted; Sigma) in HBSS-PBS+5% FCS at 37°C for 30 min. Monolayers were washed four times with 0.5 ml of HBSS-PBS, followed by incubation with 0.25 ml of 0.003% hydrogen peroxide plus 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine (Sigma) in acetone at 37°C for 60 min in the dark. The color reaction was stopped by adding 75 µl of 8 N H₄SO₄, and the samples were transferred to 96-well plates that were read on a plate reader at 450 nm, blanking on monolayers stained only with a secondary antibody.

Statistical analysis. All values are expressed as means ± SE. Data were analyzed using one-way ANOVA with Bonferroni corrections for multiple comparisons. P < 0.05 was considered significant.

RESULTS

Effect of hydrogen peroxide and SNO on PMN adherence. Adhesion of PMNs to HUVEC monolayers was not affected by a 30-min incubation with 0–0.5 mM SNO alone (Fig. 1A) or with 0–0.1 mM hydrogen peroxide alone (Fig. 1B). However, up to 0.1 mM SNO significantly increased PMN adherence when 0.1 mM peroxide was coincubated (Fig. 1A; 0.1 mM SNO, P < 0.05; 0.5 mM SNO, P < 0.01 compared with monolayers...
treated with 0.1 mM peroxide alone). Furthermore, up to 0.01 mM peroxide also significantly increased PMN adherence when 0.5 mM SNO was coincubated (Fig. 1B; 0.01 and 0.1 mM peroxide, \( P < 0.01 \) compared with monolayers treated with 0.5 mM SNO alone). Neither spermine (0.5 mM) plus peroxide (0.1 mM) nor spermine (1 mM) itself changed PMN adherence within 30 min (data not shown).

Effect of DF and methionine on PMN adherence induced by peroxide+SNO. Figure 2 illustrates that PMN adherence induced by 0.1 mM peroxide plus 0.5 mM SNO involves cell-associated iron and an oxy radical-dependent mechanism, since both DF (0.1 mM), a chelator of cell-associated iron, and methionine (1 mM), an oxy-radical scavenger, significantly attenuated this increase in PMN adhesion within 30 min. In experiments using DF, cells were preincubated for 2 h before the addition of SNO and peroxide. Neither DF nor methionine alone at these concentrations significantly affected PMN adhesion (data not shown).

Effect of peroxide and SNO on surface expression of adhesion molecules. Endothelial P-selectin surface expression was not affected by 0–0.5 mM SNO alone; however, P-selectin was significantly increased by the coincubation with both 0.1 mM peroxide and 0.5 mM SNO within 30 min (Fig. 3A; \( P < 0.05 \) compared with monolayers treated with 0.1 mM peroxide alone). Similarly, 0–0.1 mM peroxide alone did not change P-selectin expression; however, when 0.5 mM SNO was coincubated, up to 0.01 mM peroxide significantly increased the expression within 30 min (Fig. 3B; \( P < 0.01 \) compared with monolayers treated with 0.5 mM SNO alone). Conversely, levels of surface ICAM-1 were not affected by 0.1 mM peroxide alone, 0.5 mM SNO alone, or the combination of both peroxide and SNO (Fig. 4), consistent with the lack of an acute (<30 min) effect of oxidants on ICAM-1 surface expression.

Effect of DF and methionine on surface expression of P-selectin increased by peroxide+SNO. The mobilization of P-selectin produced by 0.1 mM peroxide plus 0.5 mM SNO for 30 min was significantly attenuated by 0.1 mM DF or 1 mM methionine (Fig. 5). These data suggest the importance of P-selectin expression in eliciting this adhesive response. In this experiment, DF was also preincubated with cells for 2 h before the SNO+peroxide treatment. Neither DF nor methionine alone affected P-selectin expression (data not shown).

Effect of anti-P-selectin and ICAM-1 antibodies on PMN adherence induced by peroxide+SNO. PMN adhesion to HUVEC monolayers increased by coadministra-
tion of 0.1 mM peroxide plus 0.5 mM SNO for 30 min was blocked by an anti-P-selectin antibody, an anti-ICAM-1 antibody, and the combination of both antibodies (Fig. 6). These data indicate that this form of PMN adhesion is likely to be dependent on the presence of both P-selectin and ICAM-1 on the endothelial cell surface. Interestingly, the combination of both antibodies was not additive (compared with each antibody alone), suggesting that P-selectin and ICAM-1 may cooperatively modulate PMN adhesion in this model.

Effect of WEB-2086 on PMN adherence and P-selectin surface expression induced by peroxide+SNO. PMN adhesion to HUVEC monolayers increased by coadministration of 0.1 mM hydrogen peroxide plus 0.5 mM SNO for 30 min was also significantly prevented by a PAF-receptor antagonist, WEB-2086 (10 µM; Fig. 7). These data suggest that PAF synthesis is an important step in the increased PMN-endothelial adhesion in this model. However, 10 µM WEB-2086 did not change surface expression of P-selectin increased by coadministration of 0.1 mM peroxide plus 0.5 mM SNO for 30 min (Fig. 8), suggesting that endothelium-derived PAF helps trigger PMN adhesion not by modulating endothelial P-selectin expression but rather through mobilization of PMN pools of CD18.

**DISCUSSION**

Under some circumstances, NO has been demonstrated to protect endothelial cells from the effects of endothelium-derived oxidants produced by ischemia-reperfusion (1, 8, 10, 14, 22) as well as from the effects of neutrophil-derived oxidants (2, 15, 16, 24, 26, 30). Much attention has recently been focused on the ability of NO (which is itself a free radical) to interact with and block oxidants and/or intermediates in the formation of toxic oxygen species. Therefore NO may limit ROM-mediated injury (12, 13, 18). However, several other recent studies also indicate that these protective effects are critically dependent on both the identity and the
concentration of the oxidant and NO in these models (19, 23, 32).

Here, we have attempted to examine PMN-endothelial adhesion as an important index of oxidant-mediated endothelial stress in a model of tissue oxidant injury using concentrations of NO and hydrogen peroxide found in several similar studies (2, 5, 31). We observed that neither 0–0.1 mM hydrogen peroxide nor 0–0.5 mM SNO (an NO donor) induced any change in neutrophil adhesion; however, in combination (0.01 or 0.1 mM peroxide plus 0.5 mM SNO and 0.1 mM SNO plus 0.1 mM peroxide), both agents significantly increased the adhesion of neutrophils to cultured endothelium (Fig. 1). These data indicate that NO and peroxide are in fact the important chemical mediators in this model.

The increased adhesion promoted by NO + peroxide was significantly attenuated by 0.1 mM DF as well as by 1 mM methionine (Fig. 2), suggesting that hydroxyl radicals are formed by the interaction of hydrogen peroxide with surface-bound iron through the "Fenton"-type chemical reactions. Our data also implicate hydroxyl radicals, since methionine, an oxy-radical scavenger, also blocks these events.

An important question in this study is why we documented an increase in PMN adhesion with peroxide + SNO, whereas several other studies indicated that NO protects against the deleterious effects of oxidants. As previously reported, we found that NO donors themselves are not cytotoxic and do not promote inflammation (9). Wink and co-workers (33, 34) have shown that NO protects several cell types against hydrogen peroxide-mediated cytotoxicity; this involves 1) NO chelation of metals that can block the Fenton chemistry and 2) NO scavenging of heme protein radicals, preventing the oxidation of DNA. NO may also scavenge superoxide, which would limit the formation of both peroxide and hydroxyl radicals, which are highly cytotoxic. However, in previous studies on exogenous peroxide and NO, it has been observed that exposure of endothelial cells to modest levels of NO donors, e.g., SNO, results in a reversible and significant reduction in intracellular glutathione, making these cells particularly susceptible to an additional oxidant stress like hydrogen peroxide (23). We observed that the stress induced by SNO and hydrogen peroxide requires the simultaneous presence of both agents to promote increased solute permeability (23). In that report, the data also suggested that DF and methionine could modify that response, which supported a role for both iron and hydroxyl radicals. Further studies that implicate the participation of endogenous NO using NO synthesis inhibitors will provide insights into the role of endogenously formed NO. Future studies may determine which strategy most closely resembles specific in vivo phenomena.

One important potential mechanism for this adhesive response is through the upregulation of P-selectin by the mobilization of Weibel-Palade bodies (4, 36), which also mediate injury in vivo (20). The increased adhesion of neutrophils to SNO and/or hydrogen peroxide-challenged HUVEC monolayers is significantly blocked by either an anti-P-selectin antibody or an anti-ICAM-1 antibody (Fig. 6) and strongly suggests that the final result of the combined exposure is the presentation of both endothelial P-selectin and ICAM-1. P-selectin is an important endothelial adhesive determinant in the acute adhesion of neutrophils to the endo-
endothelial P-selectin. It has been previously demonstrated that P-selectin is in fact mobilized to the endothelial cell surface in response to oxidant exposure (17). ICAM-1, however, is not rapidly mobilized from a preformed pool and must be synthesized to be upregulated, which takes longer than 2 h. Thus our data also suggest that adhesion in this model is very similar to the adhesive mechanism for neutrophils proposed in vivo. In this scheme, endothelial P-selectin tethers neutrophils, permitting them to interact with neutrophil CD18 to form firm adhesive interactions with endothelial ICAM-1.

PAF synthesis appears to play an important role in this process. We saw that WEB-2086, a PAF-receptor antagonist, also significantly blocked the SNO and/or hydrogen peroxide-induced neutrophil hyperadhesiveness to HUVEC monolayers (Fig. 7). We have previously observed that the acute oxidant-mediated neutrophil adhesion in vitro depends on the presence of PAF (3). Based on these results, our data indicate that the sequence of events in this form of adhesion is as follows: 1) NO + hydrogen peroxide mobilizes existing pools of endothelial P-selectin, 2) NO + hydrogen peroxide also stimulates PAF production by endothelial cells, and 3) binding of endothelial cell-derived PAF to the neutrophil then stimulates the presentation of CD18 on the neutrophil surface. In this model, PAF stimulates neutrophils to become competent for firm endothelial ICAM-1 binding, which is significantly accelerated by neutrophil "tethering" to the endothelial surface, mediated by the upregulated presentation of endothelial P-selectin. Although it has been reported that under some circumstances PAF stimulates endothelial P-selectin expression (29), our data suggest that PAF probably only mediates neutrophil-dependent events in this model, the most likely being mobilization of CD18, since WEB-2086 did not block P-selectin expression on endothelial cells (Fig. 8).

Therefore, adhered neutrophils in our model appear to be bound via a two-step P-selectin- and ICAM-1-dependent mechanism in which only tightly adhered neutrophils remain bound during the washing steps to remove "unbound" neutrophils. We have previously demonstrated that in "static" adhesion systems like this one, P-selectin will promote PMN-endothelial adhesion (3) despite the lack of continuous fluid shear.

In this study, it is observed that NO and hydrogen peroxide can promote the adhesion of neutrophils to endothelial cells by the iron-dependent formation of potent oxidant species (possibly hydroxyl radicals) that trigger the release of PAF. PAF presented on the endothelial surface then appears to trigger CD18 presentation by the neutrophil. These oxidants also mobilize P-selectin to the endothelial cell surface and cooperatively augment the adhesive capacity of constitutively expressed ICAM-1. One other possibility that is not examined in this study is that hydrogen peroxide may indirectly lead to the inhibition of mitochondrial function, with an accompanying increase in superoxide in the cells. Superoxide generated indirectly in this manner could lead to the formation of peroxynitrite, which might possibly mediate these effects. However, we are unaware of data in the literature that would support such a model. Future studies may address this possibility.

Our data indicate that those conditions that duplicate these levels of NO and hydrogen peroxide in vivo may actually promote some forms of inflammation, such as neutrophil-endothelial adhesion, rather than diminish it. These data also indicate that therapies using NO donors may need to carefully evaluate the concentrations of NO in experimental models to optimize the beneficial effects of NO against oxidant injury.

We thank Dawn Powell for dedicated technical help in the preparation of this manuscript.

This study was supported in part by National Institutes of Health Grants HL-47615 and PO1-DK-43785.

Address for reprint requests: J. S. Alexander, Dept. of Molecular and Cellular Physiology, Louisiana State Univ. Medical Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932.

Received 15 July 1997; accepted in final form 6 February 1998.

REFERENCES