Nitric oxide enhances hydrogen peroxide-mediated endothelial permeability in vitro

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Okayama, Naotsuka, Christopher G. Kevil, Loisann Correia, David Jourd’heuil, Makoto Itoh, Matthew B. Grisham, and J. Steven Alexander. Nitric oxide enhances hydrogen peroxide-mediated endothelial permeability in vitro. Am. J. Physiol. Cell Physiol. 273 (Cell Physiol. 42): C1581–C1587, 1997.—The objective of this study was to evaluate the effects of nitric oxide (NO) on H2O2-mediated endothelial permeability. H2O2 (0.1 mM) increased permeability at 90 min to 298% of baseline. Spermine NONOate (SNO), an NO donor, at 0.1 or 1 mM did not alter permeability. However, 0.1 mM H2O2 + 1 mM SNO increased permeability to 764%, twice that of 0.1 mM H2O2 alone. These treatments were not directly toxic to endothelial cells. This NO effect was concentration dependent, inasmuch as 0.1 mM SNO did not significantly change H2O2-mediated permeability. The NO-enhanced, H2O2-dependent permeability required the simultaneous presence of NO and H2O2, inasmuch as preincubation with SNO for 30 min followed by 0.1 mM H2O2 did not alter permeability. Staining of endothelial junctions showed widening of the intercellular space only in junctions of cells exposed to H2O2 (0.1 mM) + SNO (1 mM). Furthermore, NO did not affect H2O2 metabolism by endothelial cells but significantly depleted intracellular glutathione. This reduction of cell glutathione produced by NO exposure recovered 15–30 min after removal of the NO donor. NO-enhanced permeability was completely blocked by methionine (1 mM), a scavenger of reactive oxygen species, and by the iron chelator desferrioxamine (0.1 mM). These results suggest that NO may exacerbate the effects of H2O2-dependent increase in endothelial monolayer permeability via the iron-catalyzed formation of reactive oxygen metabolites.

endothelial monolayer; iron; catalase; glutathione peroxidase; spermine NONOate

IT IS WIDELY ACCEPTED that oxidants derived from activated neutrophils and from endothelial cells can increase the permeability of endothelial monolayers in vivo and in vitro (4, 6, 12, 26, 28, 34–36). Among these oxidants, H2O2 is thought to be perhaps the most important in microvascular injury, since changes in endothelial permeability often occur as a result of H2O2 exposure (4, 6, 36). The increased permeability produced by H2O2 is thought to require second messenger activation, e.g., increased cell calcium (6, 35) and protein kinase C activity (36). H2O2 is also directly toxic to endothelial cells (26), inhibits anion transport (12), stimulates sodium-potassium pump activity (28), and can lead to DNA damage (15).

Conversely, the role of nitric oxide (NO) in tissue injury is more controversial. NO is a well-known, endogenously formed second messenger in many biologic systems that regulates a wide variety of physiologic functions (40). NO is reported to protect against peroxide cytotoxicity (39, 42, 43), promote platelet aggregation (30), and promote acute lung injury during hypoxia (32). The question arises whether and when NO is protective or injurious. Recently, McQuaid et al. (27) reported that the permeability produced by H2O2 could be exacerbated when relatively high levels of NO (100 µM sodium nitroprusside) were present. They concluded that the cytotoxic effects of NO on H2O2-induced permeability may be associated with production of singlet oxygen from H2O2 (20), which could overwhelm the cell oxidant defenses. However, their study did not demonstrate the involvement of singlet oxygen, and, furthermore, nitroprusside decomposition yields cyanide and iron as well as NO. Therefore, nitroprusside may be an unsuitable agent for studying the effects of NO on endothelial permeability.

In the present study, we examine the effects of NO generated by the spontaneous decomposition of the spermine NO adduct spermine NONOate (SNO) (24, 25) on H2O2-mediated permeability of cultured endothelial monolayers. We examined the involvement of other secondarily derived oxidants, such as hydroxyl radical (OH·), that are generated from the iron-catalyzed decomposition of H2O2 (9, 19, 41, 44) and evaluated whether altered levels of cell glutathione (GSH) contributed to the increased permeability in this model.

MATERIALS AND METHODS

Cell culture methods. Bovine pulmonary artery endothelial cells were isolated using a modification of the procedure described by Jaffé et al. (16). Endothelial cells were harvested by sterile ablation and digestion in 0.1% collagenase (type II, Worthington Enzymes, Freehold, NJ). Cells were maintained in Dulbecco’s minimum essential medium (Clonetics, San Diego, CA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) and 1% antibiotic-antimycotic at 37°C in 5% CO2 in air. Cultures were passaged weekly in trypsin-EDTA (GIBCO, Grand Island, NY) and seeded onto tissue culture plasticware at 20,000 cells/cm2 for all experiments. Endothelial cultures were used at 1 wk in culture, which is ~3 days after culture confluence was established. The cells used in all protocols were between passages 4 and 10.

Microcarrier bead cultures. Cells were cultured on microcarrier beads as previously described (1, 2, 11). Cells were seeded on Cytopex-3 microcarrier beads (Pharmacia, Uppsal, Sweden) at a density of 2 × 103 cells/cm2. Cell attachment was achieved by intermittent stirring overnight. Microcarrier cultures were maintained at 60 revolutions/min continuously.
and fed three times a week. Cultures were used for these assays 7–30 days after seeding.

Cell column methods. We used a previously reported assay of endothelial monolayer permeability, with minor modifications, to measure changes in barrier produced by stimulation of endothelial monolayers with experimental treatments (1, 2, 11). The method uses a model of the vasculature consisting of a chromatographic cell column filled with endothelial cell-covered microcarrier beads. The permeability of the endothelial monolayers covering the beads is determined from a comparison of the elution curves of tracers injected into the flow at the top of the column. The details of this method are briefly described below.

Chromatographic cell columns were made from water-jacketed glass columns (0.65 cm diameter; Rainin, Emeryville, CA). Cell-covered beads were poured to a column height of ~2 cm, which provides 130 cm² of endothelial cell culture surface or ~1 × 10⁷ cells. The column was washed and equilibrated with Hanks’ balanced salt solution (HBSS; Sigma Chemical, St. Louis, MO) containing 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, pH 7.4) and 0.5% bovine serum albumin. Perfusion through the column was maintained by a peristaltic pump (Minipuls 2, Gilson, Middleton, WI) at 1 ml/min. This flow was chosen to approximate the gravity flow rate observed when the pump was not connected. A bolus of flow tracer and monolayer-permeant tracers was applied by a rotary injection valve (Rainin) using a 50-μl loop. The cell column and all perfusate solutions were kept at 37°C throughout the experiment. Multiple tracer indicator-dilution analysis was used to obtain cell layer permeability from the relative shapes of the elution profiles of tracers simultaneously applied to the top of a cell column. One of the applied tracers (blue dextran, 10 mg/ml, 2,000 kDa) cannot cross endothelial monolayers and follows the mobile phase, i.e., a “flow” tracer. Two other tracers, sodium fluorescein (376 Da) and cyanoecobalamin (1,355 Da), were used as monolayer-permeant tracers. These tracers permeate beneath the cell layer at cell–cell junctions and diffuse into the bead matrix beneath the cells. Importantly, none of these tracers diffuses across cell membranes. Absorbances of 66 samples were used to calculate the fractional recovery per sample of each of the optically absorbing dyes. A modified Marquardt iteration scheme was used to estimate the monolayer permeability that best approximated the experimental data. Best fit was determined by minimization of the coefficient of variation between a computer-generated prediction of the permeant tracer’s elution profile and the experimentally observed elution profile.

Treatment protocols. In all protocols, permeability was measured on the same population of cells in triplicate. Cell columns were initially perfused with a normal perfusate, i.e., HBSS supplemented with 15 mM HEPES (pH 7.4) and 0.5% bovine serum albumin. After the column was equilibrated in this buffer for 15 min, duplicate measurements of cell column permeability were made for baseline. The column perfusate was sequentially switched to the perfusate containing appropriate drugs, and permeability was measured at 10-min intervals for a total of 90 min; then the column was switched to the normal perfusate, and measurements were made after 10-min recovery of permeability.

The drugs used in this study are as follows: 1) peroxide (0.1 mM) alone; 2) peroxide (0.1 mM) + SNO, a donor of NO (0.1 and 1 mM); 3) SNO (1 mM; preincubated for 30 min) and then peroxide (0.1 mM) alone; 4) SNO (0.1 and 1 mM) alone; 5) spermine (1 mM) alone; 6) peroxide (0.1 mM) + SNO (1 mM) + methionine, a scavenger of oxidants (1 mM) (33); 7) methionine (1 mM) alone; 8) peroxide (0.1 mM) + SNO (1 mM) + desferrioxamine, a chelator of iron (100 mM; preincubated for 2 h); 9) desferrioxamine (0.5 mM) alone.

Cell viability assay. Cell viability was quantified by incubating cells cultured on microcarriers and treated with peroxide, NO donor, or combinations or treatments with blockers (see Treatment protocols) in fluorescent diacetate-ethidium bromide for 30 min and then counting the viable (green) and nonviable (red) cells by fluorescence microscopy as previously described (2).

Intracellular GSH assay. Intracellular GSH was measured by a modification of the method described by Beutler et al. (5). Briefly, 0.3 ml of cell-covered microcarrier beads was incubated for 60 min in HBSS, 1 mM SNO in HBSS, 0.1 mM peroxide, or 0.1 mM peroxide + 1 mM SNO as described above. Cells were then lysed in 0.9 ml of water, deproteinized with 400 μl of 5.2 M NaCl-7 mM EDTA-0.3 M H₃PO₄ for 5 min at 25°C, and then centrifuged for 10 min at 12,000 g at 4°C. 5,5'-Dithio-bis-(2-nitrobenzoic acid) (600 μl, 3 mM) in phosphate buffer (pH 7) was added, and the samples were incubated for 5 min at 37°C. Absorbance of the samples at 414 nm was measured, and nanomoles of GSH were calculated as follows: 1.3 × volume × (sample absorbance – blank absorbance)/[0.6 × extinction coefficient (0.0136)]. In some experiments, SNO was removed from cells and GSH recovery was allowed to take place in the presence of HBSS for 60 min after SNO treatment.

Endothelial junctional morphology. To visualize endothelial junctions on microcarrier beads, monolayers were silver-stained as described by Hirata et al. (13) with modifications. Briefly, monolayers were treated with HBSS, 0.1 mM peroxide, 1 mM SNO, 0.1 mM peroxide, and 1 mM SNO and washed with 2% glucose (1 min), then with 1% AgNO₃ (1 min), 2% glucose (1 min), and 1% ammonium bromide-1% cobalt bromide (1 min), and finally with 2% glucose (1 min) and fixed in 3% phosphate-buffered formaldehyde. Monolayers were photographed at ×40 on T-MAX 400 professional film.

Measurement of endothelial peroxide-catabolizing activity. We incubated endothelial cells in HBSS + 1 mM peroxide (60 min) or HBSS + 1 mM SNO (30 min preincubated) + 1 mM peroxide (60 min) to determine whether NO interferes with the total catalase and/or GSH peroxidase activity. The ability of the endothelial cell to remove H₂O₂ was measured using p-hydroxyphenylacetic acid (230 µg/ml) as a fluorescent substrate in the presence of horseradish peroxidase (100 µg/ml). Briefly, 0.3 ml of cell-covered microcarrier beads (4.9 × 10⁶ cells) was incubated in HBSS + 1 mM H₂O₂ with or without 1 mM SNO preincubation. After 1 h, the supernatant was removed and assayed for total peroxide using fluorescent emission at 400 nm (excitation = 323 nm), as described by Hyslop and Sklar (14).

Statistical analysis. Unless otherwise indicated, values are means ± SE. Multiple comparisons of data from all experiments were performed with the Bonferroni test. P ≤ 0.05 was considered significant.

RESULTS

Effects of peroxide and SNO on permeability of endothelial monolayers. H₂O₂ (0.1 mM) significantly increased permeability of endothelial monolayers after 70 min compared with baseline (238 ± 23 and 298 ± 37% of baseline at 70 and 90 min, respectively; Fig. 1, ●). This effect was not recovered when monolayers were perfused for an additional 10 min without peroxide. SNO at 0.1 mM did not alter the increased permeability induced by 0.1 mM peroxide (Fig. 1, □), but 1 mM SNO significantly exacerbated the peroxide-mediated permeability after 60 min (from 195 ± 20 to
and peroxide + SNO data are the same in Figs. 1, 4, 5, and 6. We found that cell viability was not affected by exposure to NO, H₂O₂, or NO + H₂O₂ over 90 min, as measured by fluorescent staining with fluorescein diacetate-ethidium bromide.

Junctional morphology. Monolayers exposed to control buffer and 1 mM SNO show normal linear cell-cell junctions (Fig. 2, A and B). Monolayers treated with 0.1 mM peroxide show some junction disintegration (Fig. 2C). Monolayers treated with 1 mM SNO + 0.1 mM peroxide show extensive retraction and “blebbing” of junctions, with extensive widening of cell borders (Fig. 2D).

Effect of NO on endothelial cell H₂O₂-decomposing activity. p-Hydroxyphenylacetic acid-horseradish peroxidase was used as a sensitive method to measure peroxide in solution in the presence and absence of cells and before and after NO exposure. We found that endothelial cells were able to catabolize 31.5 ± 3.3% of the peroxide in solution (31.5 nmol/ml) in 60 min. Because 1 U/ml of catalase can metabolize 1 µmol/min, our cells have 0.053 U/ml of total catalase or GSH peroxidase activity. This level of activity was only slightly, but not significantly, reduced (25.4 ± 3%) by the addition of 1 mM SNO, an NO donor, during the 60-min incubation period.

NO depletes intracellular GSH. We examined whether incubation with 1 mM SNO for 60 min would alter intracellular GSH levels and whether this would be augmented by 0.1 mM peroxide. We found that 1 mM SNO significantly reduced monolayer GSH (Fig. 3A).
GSH levels were not altered by 0.1 mM peroxide over the course of 90 min. SNO (1 mM) and 0.1 mM peroxide also significantly decreased GSH, but to the same extent as 1 mM SNO alone, indicating that large amounts of NO may predispose the cell to abnormally high oxidant stress levels. After the removal of SNO, cells recovered control levels of GSH 15–30 min after the removal of SNO and incubation in HBSS, which contains glucose as a carbon source (Fig. 3B).

Effect of NO preincubation on peroxide-mediated permeability. We examined whether preincubation with 1 mM SNO for 30 min would significantly alter the permeability produced by treatment of monolayers with 0.1 mM peroxide over the course of 90 min. We found no significant change in endothelial permeability between monolayers treated with 0.1 mM peroxide and those pretreated for 30 min with SNO (Fig. 4), indicating that a simultaneous exposure to both agents is necessary to alter solute permeability.

Effects of methionine on peroxide- and SNO-induced endothelial permeability. To examine the role of hydroxyl-like oxidants in peroxide- and SNO-induced endothelial permeability, we used methionine, a scavenger of oxidants (33). Methionine at 1 mM completely blocked the increased permeability induced by 0.1 mM peroxide and 1 mM SNO after 30 min (from 172 ± 6 to 104 ± 7 and from 764 ± 85 to 125 ± 2% of baseline after 30 and 90 min, respectively; Fig. 5, ). The same concentration of methionine alone did not increase endothelial permeability within 90 min (data not shown).

Effects of desferrioxamine on peroxide- and SNO-induced endothelial permeability. We also examined the need for iron in SNO-enhanced H₂O₂-mediated endothelial permeability. We used 100 µM desferrioxamine, an iron chelator, to limit effects mediated by iron in this system. Preincubation with 100 µM desferrioxamine for 2 h completely blocked the increased permeability induced by 0.1 mM peroxide and 1 mM SNO after 30 min (126 ± 16 and 167 ± 25% at 40 and 90 min, respectively; Fig. 6, ). Desferrioxamine as high as 0.5 mM alone did not alter endothelial permeability within 90 min (data not shown).

**DISCUSSION**

In vivo, levels of NO approach 80 µM; therefore, locally the levels of NO in vivo may be orders of magnitude greater (10). Effects of NO on endothelial permeability could be mediated by at least two possible mechanisms:
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mechanisms. The first possibility is elevated intracellular guanosine 3',5'-cyclic monophosphate (cGMP; through guanylate cyclase activation) (7, 8, 17, 37, 38) and the ability of NO to interact with reactive oxygen metabolites (ROMs) (3). Many studies indicate that endothelial permeability is decreased by activation of guanylate cyclase, apparently through the effects of cGMP on intracellular calcium (7, 8, 17). However, we did not observe changes in the endothelial barrier in response to NO donors; therefore, this first possibility seems unlikely.

A second possibility is that NO interacts with oxidants to modulate the endothelial barrier. Endothelial studies with ROMs show that NO can promote or prevent cytotoxicity (21, 22, 27). This variable nature of NO appears to depend on the fluxes and ratios of NO to ROM in those systems (20, 27, 30, 32, 39, 42, 43). For example, NO will interact with superoxide (O_2^-) to produce peroxynitrite, a highly potent oxidant that may promote oxidant injury (3). NO is also thought to limit inflammatory events dependent on superoxide, including leukocyte rolling and leukocyte-endothelial adhesion and activation (18, 22, 31). It has been suggested that NO synthesis inhibitors promote oxidant injury by decreasing the flux of NO, which would increase superoxide-dependent injury (29).

However, when levels of NO exceed that of superoxide, NO may actually inhibit oxidation reactions by interacting with, and decomposing, peroxynitrite (29). In vivo, large amounts of NO have been shown to be nontoxic to tissues (21) but may somehow exacerbate toxic effects of H_2O_2. For example, relatively low (0.1 mM) levels of NO donors block H_2O_2 effects on the endothelial barrier, but high (1 mM) NO levels enhance H_2O_2-mediated permeability (27). Similar concentration-dependent effects have been reported (23), where injury is reduced by low concentrations of NO donors but is promoted by high NO concentrations.

We found that the NO donor, SNO, itself did not alter permeability at any concentration. However, SNO significantly enhanced the inflammatory effect of 0.1 mM H_2O_2 (from 270% to ~800% of baseline; Fig. 1). This effect was not observed with peroxide plus a low (0.1 mM) concentration of SNO. We have observed that neither 0.1 mM peroxide, 1 mM SNO, nor 0.1 mM peroxide + 1 mM SNO is cytotoxic to endothelial cells. Therefore, although NO is not toxic to the endothelium, it significantly exacerbates H_2O_2-mediated permeability. Endothelial cells exposed to 1 mM SNO + 0.1 mM H_2O_2 display blebbing and even form large intercellular gaps, consistent with the increased permeability as a result of opening cell-cell junctions. This change in morphology was not observed with SNO alone (Fig. 2B), nor was monolayer permeability altered (Fig. 1). Similarly, we have also found that other NO donors, e.g., diethylamine-NO, also fail to alter cell morphology (data not shown) and argue that NO alone does not alter junction structure or function but somehow enhances the effects of peroxide, perhaps via second messenger systems. Because NO is often associated with decreased endothelial permeability (7, 8, 17, 37), this effect is probably not directly related to cGMP.

Cell GSH levels were significantly reduced by 1 mM SNO but were unaffected by 0.1 mM H_2O_2; together, H_2O_2 and SNO did not further deplete cell GSH. After the removal of SNO, cells recover normal levels of intracellular GSH 15–30 min after removal of NO (Fig. 3B). Because NO donors transiently decrease GSH, but not barrier, our data suggest that decreased cell GSH alone does not mediate increased permeability per se.
but renders NO-exposed cells more susceptible to oxidants. Importantly, the capacity of endothelial monolayers to metabolize H$_2$O$_2$ is not altered by SNO, indicating that catalase and GSH peroxidase are not inactivated, as has been suggested in other studies (9). The ability of endothelial monolayers to catabolize peroxide was measured and found to be equivalent to 0.53 mU/ml of catalase or GSH peroxidase per 5 \times 10^6 cells. Incubation with 1 mM SNO did not alter the peroxide-catalyzing capacity of the endothelium. Under control conditions, 31.5 \pm 3.3 \mu M of peroxide were metabolized in 60 min, and monolayers incubated with 1 mM SNO catabolized 25.4 \pm 3 \mu M. Therefore, it is unlikely that H$_2$O$_2$ + NO is a direct result of catalase or GSH peroxidase inhibition. To this end, we also attempted to examine barrier changes as a result of NO-mediated catalase or GSH peroxidase inhibition by incubating cells with SNO before H$_2$O$_2$ exposure. We found that NO preincubation did not enhance peroxide-mediated permeability (Fig. 4). Thus NO + H$_2$O$_2$ is necessary to enhance permeability. Our data suggest that other types of reactions must mediate this response (9, 19, 41, 44).

To examine the participation of other secondarily derived oxidants (e.g., hydroxyl radicals), we used methionine, a potent scavenger, to attenuate H$_2$O$_2$/NO-mediated permeability. Methionine significantly reduced H$_2$O$_2$/NO-mediated permeability (from 800% to baseline levels, Fig. 3), indicating that methionine scavenges a potent oxidant responsible for the enhanced endothelial permeability. Whereas HEPES and albumin may also react with hydroxyl, it is also possible that the unique metal-binding properties of methionine also contribute to its protective effect.

Because it is very unlikely that NO directly reacts with peroxide, we next considered whether metal catalysis might be involved, such that OH$^-$ or an OH$^-$-like species would mediate this response. We investigated a requirement for iron in this system using 100 \mu M desferrioxamine as a potent iron chelator. Desferrioxamine blocked the permeability produced by H$_2$O$_2$/NO (Fig. 6). Alone, desferrioxamine at concentrations as high as 0.5 mM did not alter endothelial permeability. Whereas several possibilities exist, our data are most consistent with the mechanism in which H$_2$O$_2$ and NO cooperate to promote endothelial permeability through the iron-catalyzed formation of a potent oxidant such as OH$^-$, as previously described by Farias-Eisner et al. (9) in the following equations

\[
Fe^{3+} + NO \rightarrow Fe^{2+} + NO^+ \\
H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^- 
\]

and by NO-mediated depletion of intracellular GSH. We propose that Fe$^{3+}$ (presumably located on the cell surface) is reduced by NO to yield Fe$^{2+}$ and NO$^+$. Once produced, Fe$^{2+}$ could then react with H$_2$O$_2$ to form the highly reactive free radical OH$^-$, which could increase endothelial permeability. If this type of oxidant stress occurs simultaneously with a significant decrease in cell GSH, we would predict that permeability would increase in response to an oxidant stress. Therefore, physiological conditions that can substantially elevate H$_2$O$_2$ and NO might result in potential endothelial injury and contribute to increased microvascular permeability in vitro and in vivo.

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REFERENCES


