Endothelium removal induces iNOS in rat aorta in organ culture, leading to tissue damage

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The vascular endothelium is considered to be of crucial importance in maintaining vascular integrity by preventing platelet aggregation and vascular tone by releasing vasodilator substances, such as nitric oxide and prostacyclin, to maintain a vasodilator input into the underlying smooth muscle (25). Damage to the endothelium can occur in many circumstances, including atherosclerosis (5), during ischemia (40), during balloon angioplasty (29), and during coronary bypass surgery (19). Injury to the endothelium is considered to be a detrimental vascular event, characterized initially by constriction of the underlying smooth muscle and increased platelet aggregation at the site of lesion (47). Endothelium damage may also trigger remodeling of the injury site, involving smooth muscle migration and proliferation and the formation of a neointima, which in some cases leads to blood vessel occlusion (11, 26).

One enzyme that is expressed in smooth muscle after balloon angioplasty in vivo is inducible nitric oxide synthase (iNOS) (18, 24, 51). Once induced, iNOS can synthesize nitric oxide at high rates over long periods of time without further stimulus (25). It has been suggested that iNOS may help to ameliorate the effects of endothelial cell removal by allowing the production of nitric oxide in vascular smooth muscle to decrease platelet aggregation and constrictor influences (18, 24, 51). After balloon angioplasty, significant remodeling occurs at the injury site, and this repair process may also be modulated by iNOS. Indeed, when iNOS genes are transferred via various vectors to animals, there is an inhibition of myointimal hyperplasia and an inhibition of restenosis after balloon injury (46). The underlying basis of this response may be a decrease in smooth muscle proliferation (7) and a promotion of reendothelialization (50, 53).

Although nitric oxide is thought to be the main biologically active product of iNOS, nitric oxide can react with superoxide anion to produce peroxynitrite, and this is particularly favored when nitric oxide production is high (3). Peroxynitrite can cause lipid oxidation, deamination, alterations in DNA, protein nitration and oxidation, inhibition of iron-centered enzymes involved in mitochondrial respiration, and inhibition of aconitase activity, all of which can lead to profound cellular disturbances as well as the development of atherosclerotic lesions (38, 43). The induction of iNOS after vessel injury is thought to be secondary to the release of the cytokines tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 (IL-1) from the invading macrophages (28), at least at distant time points from the injury (7 and 14 days) (18).

Our work in this area stemmed from an earlier study, in which we used an organ culture technique in which aortas were cultured for 24 h in vitro in a defined medium with no external growth factors, blood cells, or proteins (4). We found that the endothelium had to remain intact during the culture; otherwise the tissue became unresponsive to the α-adrenoceptor agonist phenylephrine. Our hypothesis to explain these results was that events within the vessel wall were set in train after endothelial cell removal to trigger iNOS production, and the resulting nitric oxide diminished constrictor effects. This may explain why, after balloon angioplasty, induction of iNOS is seen within 24 h when there are hardly any infiltrating macrophages present (8, 18). Although NO inhibition partially restored the responsiveness, it was clear that there was an irreversible alteration in vessel contractility. This current

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study was conducted to explore this finding further, and in particular to determine whether this endogenous induction of iNOS led to peroxynitrite-induced tissue damage.

METHODS

Preparation of rat aortas. Male Sprague-Dawley rats (250–350 g) were killed by decapitation. The thoracic aorta was removed under sterile conditions and placed in ice-cold physiological salt solution (PSS), which was bubbled with 95% O2-5% CO2. Each aorta was either cut into 4-mm rings (contractile studies) or left intact (Western blotting and NOS assay). In some cases the endothelial cells were removed by gentle rubbing of the inner lumen with a stainless steel wire. In contractile studies wherever possible, the removal of the endothelium was verified by examining acetylcholine-induced vasodilation in phenylephrine-constricted aortas, abolition of which was taken to indicate effective removal of the endothelium (see Vasoconstriction in rat aortic rings). In all tests, acetylcholine had no vasodilator effect. In some aortas, the removal of the endothelium was verified histochemically by use of silver nitrate staining, and in all cases the removal of the endothelium was complete.

Rat aorta in 24-h organ culture. Rat thoracic aortas were isolated under sterile conditions as just described and incubated with 3 ml PSS at 37°C in a sterile incubator with a 95% O2-5% CO2 atmosphere for 24 h and with the drug under investigation. The bathing solution also contained penicillin (10 IU/ml), streptomycin (10 µg/ml), fungizone (25 ng/ml), and Dextran 70 (5% wt/vol, average mol wt = 70,000) to maintain oncotnic pressure.

Vasoconstriction in rat aortic rings. Aortic rings, either freshly prepared or after 24-h organ culture, were placed in organ baths containing 1 ml PSS at 37°C and were bubbled with 95% O2-5% CO2. The rings were mounted between two stainless steel hooks that were passed through the lumen. Contractile force was measured by an isometric force displacement transducer connected to a MacLab recorder. The aortas were allowed to equilibrate for a period of 45 min with several washes of prewarmed PSS, and the resting tension was adjusted to 2 g. When a steady baseline was achieved, all rings were then contracted to 80% maximum with phenylephrine (100 nM), and the absence of functional endothelial cells was confirmed at this stage by observing a lack of relaxation to acetylcholine (10 µM). The rings were then washed and allowed to equilibrate a further 45 min, after which time a cumulative concentration-response curve to phenylephrine (0.001–10 µM) was performed. In all experiments L-arginine (100 µM) was added to the bathing solution from 15 min before the phenylephrine curve to ensure sufficient substrate for NOS.

NOS activity in rat aorta. At the completion of the 24-h organ culture, the aorta was placed in 3 ml PSS containing [3H]arginine (final concentration 30 µM; 3.3 µCi/ml) for 30 min. The aorta was then removed, frozen in liquid nitrogen, and smashed in a metal capsule. The resulting particles were placed in 0.5 ml of buffer (100 mM HEPES and 10 mM EGTA, pH 5.5). The tissue was subjected to five cycles of freeze/thaw (contractile studies) or left intact (Western blotting and NOS assay). In some cases the endothelial cells were removed by gentle rubbing of the inner lumen with a stainless steel wire. In contractile studies wherever possible, the removal of the endothelium was verified by examining acetylcholine-induced vasodilation in phenylephrine-constricted aortas, abolition of which was taken to indicate effective removal of the endothelium (see Vasoconstriction in rat aortic rings). In all tests, acetylcholine had no vasodilator effect. In some aortas, the removal of the endothelium was verified histochemically by use of silver nitrate staining, and in all cases the removal of the endothelium was complete.

Western blot analysis of iNOS after endothelial damage. Thoracic aortas were isolated and prepared, and in some cases the endothelial cells were removed as described in Preparation of rat aortas. They were then homogenized and subjected to Western blotting analysis, according to Binko and Majewski (4), by use of a specific antibody for iNOS (Transduction Laboratories, Lexington, KY). iNOS corresponding to a single 130-kDa band was visualized with enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) and exposure to Kodak chemiluminescence film. Molecular weight markers were run in parallel to all samples for verification of molecular weight size, and iNOS was verified by comparing blots of samples with blots of cell lysate isolated from mouse macrophages (Transduction Laboratories). LPS was used as a positive control for aortic tissues.

Western blot analysis of nitration of tyrosine residues after endothelial damage. Thoracic aortas were isolated and kept in organ culture for 24 h. They were then chpped and homogenized in 300 µl of homogenizing buffer (0.9% NaCl, 20 mM Tris base, 10 mM EDTA, 10 mM sodium vanadate, and 1% sodium dodecyl sulfate (SDS), pH 7.4). The homogenate was removed and centrifuged for 20 min at 10,000 g at 4°C. The supernatant was removed and used for Western blot analysis.

Protein concentrations were adjusted in all samples to 0.87 mg/ml. Samples were boiled at 100°C for 3 min before running on gels. Fifteen microliters of sample (13 µg of protein) were loaded per well in 2× reducing buffer (10% bromophenol blue marker, 20% glycerol, 10% 2-mercaptoethanol, and 2% SDS). Proteins and molecular weight markers were electrophoresed for 45 min at 200 V in running buffer (0.25 M Tris base, 1.92 M glycine, and 1% SDS) on a 7.5% reducing SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane in transfer buffer (25 mM Tris base, 0.19 M glycine, and 20% methanol) at 4°C, 100 V for 75 min.

Nonspecific binding sites were blocked for 1 h with 1% bovine serum albumin (BSA) solution in Tris buffer-saline (TBS). Membranes were then incubated at room temperature for 20–24 h with a polyclonal mouse anti-rabbit nitrotyrosine antibody (diluted in 1 µg/ml TBS; Upstate Biotechnologies, Lake Placid, NY). Membranes were then washed twice with TBS for 10 min, once with 0.1% Tween-20 in TBS for 15 min, and then twice with TBS for 10 min. Membranes were then incubated for 1 h with swine anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; diluted 1:3,000; DAKO, Carpinteria, CA). The membranes were then washed again by following the procedure just described. Nitrotyrosine bands were visualized with ECL and exposure to Kodak chemiluminescence film. Molecular weight markers and nitrotyrosine molecular weight markers were run in parallel to all samples.

Detection of apoptosis smooth muscle I. DNA damage and apoptosis were assessed in aortic ring preparations after 24 h of organ culture by detecting DNA strand breaks by means of free 3'-OH groups with a modification of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method, as previously described (14). The rings were fixed with Bouin's fluid (saturated aqueous picric acid, formaldehyde 40% (wt/vol), and glacial acetic acid in a final ratio of 15:5:1 for 5 h and then stored in 70% ethanol before being processed for routine paraffin embedding. Paraffin sections (5 µm) were placed on slides pretreated with
0.01% aqueous solution of poly-L-lysine (300,000 mol wt; Sigma Chemical, St. Louis, MO). Deparaffinization [twice in a bath of Histosol (Fromme, Sydney, Australia) for 5 min] and hydration were performed by placing the slides through a series of decreasing concentrations of ethanol (twice for 3 min in a bath of 100, 90, and 70% ethanol) and then finally in distilled water. The access of TdT to fragmented DNA was improved by pretreating tissue sections with 20 µg/ml of proteinase K (Sigma Chemical) for 7 min at room temperature, and the digestion was stopped by washing twice in TBB buffer (Tris 0.5 M and 0.15 M NaCl, pH 7.5). 3'-End labeling of fragmented DNA was carried out in a reaction mixture containing 5× terminal transferase buffer (1 M potassium cacodylate, 125 mM Tris-HCl, and 1.25 mg/ml BSA, pH 6.6, at 25°C), CoCl₂ (25 mM), digoxigenin-labeled dideoxy-dUTP (5 µM), TdT (25 IU/µl), and H₂O (50:10:5:1:185; all reagents from Boehringer Mannheim, Mannheim, Germany). Background effects were minimized by coverslipping and sealing edges with art cement (National Art Materials, Bayswater, Australia). The transferase reaction was allowed to proceed for 1 h at room temperature. The reaction was terminated by washing slides in TB (twice for 3 min), and nonspecific binding of the detection antibody was eliminated by preblocking the sections with 10% normal goat serum and 10% normal fetal calf serum in TB for 20 min at room temperature. Incorporated digoxigenin was detected with an anti-digoxigenin peroxidase-linked antibody (1:1,000 in 10% normal sheep serum in TB). Excess antibody was removed by washing in TB (twice for 3 min). Apoptotic cells were visualized with an alkaline phosphatase substrate by following the manufacturer’s instructions (Fast Red TR/Naphthol AS-MX, Sigma Chemical) for 13 min, and the reaction was stopped by washing in TB. Sections were mounted under glass with 0.01% aqueous solution of poly-L-lysine (300,000 mol wt; Sigma Chemical, St. Louis, MO). The transferase reaction was allowed to proceed for 1 h at room temperature. The reaction was terminated by washing slides in TB (twice for 3 min), and nonspecific binding of the detection antibody was eliminated by preblocking the sections with 10% normal goat serum and 10% normal fetal calf serum in TB for 20 min at room temperature. Incorporated digoxigenin was detected with an anti-digoxigenin peroxidase-linked antibody (1:1,000 in 10% normal sheep serum in TB). Excess antibody was removed by washing in TB (twice for 3 min). Apoptotic cells were visualized with an alkaline phosphatase substrate by following the manufacturer’s instructions (Fast Red TR/Naphthol AS-MX, Sigma Chemical) for 13 min, and the reaction was stopped by washing in TB. Sections were mounted under glass with gelatine glycol (Sigma Chemical). Control sections were processed in an identical manner except that the TdT enzyme was substituted by the same volume of distilled water. The access of TdT to fragmented DNA was improved by pretreating tissue sections with 20 µg/ml of proteinase K (Sigma Chemical) for 7 min at room temperature, and the digestion was stopped by washing twice in TBB buffer (Tris 0.5 M and 0.15 M NaCl, pH 7.5). 3'-End labeling of fragmented DNA was carried out in a reaction mixture containing 5× terminal transferase buffer (1 M potassium cacodylate, 125 mM Tris-HCl, and 1.25 mg/ml BSA, pH 6.6, at 25°C), CoCl₂ (25 mM), digoxigenin-labeled dideoxy-dUTP (5 µM), TdT (25 IU/µl), and H₂O (50:10:5:1:185; all reagents from Boehringer Mannheim, Mannheim, Germany). Background effects were minimized by coverslipping and sealing edges with art cement (National Art Materials, Bayswater, Australia). The transferase reaction was allowed to proceed for 1 h at room temperature. The reaction was terminated by washing slides in TB (twice for 3 min), and nonspecific binding of the detection antibody was eliminated by preblocking the sections with 10% normal goat serum and 10% normal fetal calf serum in TB for 20 min at room temperature. Incorporated digoxigenin was detected with an anti-digoxigenin peroxidase-linked antibody (1:1,000 in 10% normal sheep serum in TB). Excess antibody was removed by washing in TB (twice for 3 min). Apoptotic cells were visualized with an alkaline phosphatase substrate by following the manufacturer’s instructions (Fast Red TR/Naphthol AS-MX, Sigma Chemical) for 13 min, and the reaction was stopped by washing in TB. Sections were mounted under glass with gelatine glycol (Sigma Chemical). Control sections were processed in an identical manner except that the TdT enzyme was substituted by the same volume of distilled water. Photomicrographs were taken with an Olympus camera coupled to an Olympus microscope BX-50 and with Kodak Ektachrome film.

Drugs and materials. Calpain inhibitor 1, chelerythrine, tyrphostin A25, PDTC, 1-pyrrolidinecarbodithioic acid; PD-98059: 2-amino-3-(9H-purin-6-yl)fluavone, and TPCK; Ntosyl-Phe chloromethyl ketone were obtained from Calbiochem-Novabiochem, Alexandria, Australia. L-Arginine, BSA, cycloheximide, Dextran 70, glycine, gluteraldehyde solution 25%, indomethacin, LPS (from Escherichia coli serotype 0127:B8), phenylephrine hydrochloride, SDS, silver nitrate, vanadyl sodium, and Tween-20 were from Sigma Chemical. Reduced glutathione was from Aldrich Chemical (St. Louis, MO); acetylcholine perchlorate, saturated aqueous picric acid, formaldehyde, and glacial acetic acid came from BDH (Sydney, Australia); nitrocellulose membrane and ECL reagents were from Amersham; L-Arg (L-arginine) and L-NIO (L-5-(1-iminoethyl)ornithine hydrochloride) were from Cayman Chemicals; [⁢H]arginine (36.8 Ci/mmol) and [¹⁴C]citrulline (58.8 Ci/mmol) were from Du Pont-NEN (Boston, MA); penicillin, streptomycin, and fungizone were from CSL (Victoria, Australia); swine anti-rabbit secondary HRP antibody was from DAKO; INOS rabbit anti-mouse polyclonal antibody and mouse activated macrophage lysate came from Transduction Laboratories. All Western blot reagents were purchased from Bio-Rad (Hercules, CA).

LPS was dissolved in sterile water to a concentration of 1 mg/ml to yield a final concentration of 10 µg/ml. A stock solution of L-NIO was made in sterile filtered water and stored frozen until the day of the experiment. All other drugs were made up daily in fresh PSS: (in mM) 118 NaCl, 4.7 KCl, 1.03 KH₂PO₄, 25 NaHCO₃, 11.1 D(-)-glucose, 1.2 MgSO₄, 1.6 CaCl₂, 0.067 Na₂EDTA, and 0.14 L-ascorbic acid, pH 7.4.

Three centimeters of Dowex (50W × 8–200–400, H⁺ form) were layered onto a 40-ml glass column and washed with 3 ml of 0.8 N NaOH to convert the Dowex to Na⁺ form. After extensive washing with deionized water (~ 50 ml), the column was equilibrated with 400 µl of buffer (100 mM HEPES and 10 mM EGTA, pH 5.5).

Statistical analyses. All results are expressed as means ± SE; n indicates the number of observations. The differences in constriction to phenylephrine between treatments were analyzed by two-way analysis of variance (ANOVA) with repeated measures (the repeated-measures factor was the concentration of phenylephrine). Citrulline formation was analyzed by Dunnett’s test after a one-way ANOVA. In all statistical tests, a probability value of P < 0.05 was taken as significant. The GB-STAT computer package (Dynamic Microsystems, Silver Spring, MD) was used. Although multiple rings were taken from each animal, each ring from an individual animal was used for a different experiment.

RESULTS

The effect of 24-h organ culture on the vasosonstrictor effect of the α-adrenoceptor agonist phenylephrine was measured in rat aortic rings. There were three types of aortas: freshly excised aortas denuded of endothelium (FRE), aortas after 24-h organ culture with the endothelium removed before culture (ERB), and aortas after 24-h organ culture with the endothelium removed after culture (ERA). In FRE aortas, phenylephrine (0.001–10 µM) produced a concentration-dependent constriction that was similar to the constriction produced in ERA aortas (Fig. 1A). However, phenylephrine failed to constrict ERB aortas (Fig. 1A).

The lack of responsiveness of the ERB aortas appears due in part to the release of nitric oxide, because the acute application of the nitric oxide synthesis inhibitor L-NIO (100 µM) in the presence of phenylephrine had a marked constrictor effect in an aortic ring unresponsive to phenylephrine alone (Fig. 2A). In the absence of phenylephrine, L-NIO had no constrictor effect in either ERA or ERB aortas (not shown). When L-NIO was present from just before the phenylephrine concentration-response curve in ERB aortas, there was a partial restoration of the constrictor effect, but this was still significantly below that of ERA aortas (Fig. 2B). This suggests that there was some additional event during the incubation that diminished the constrictor potential during the incubation in ERB aortas. Alternatively, some factor other than nitric oxide was involved in the diminished response. Prostaglandins do not appear to be involved, as indomethacin (10 µM) present acutely after the incubation did not affect the diminished response to phenylephrine in ERB aortas (Fig. 1B).

The protein synthesis inhibitor cycloheximide (1 µM for 24 h) completely prevented the diminution in the response to phenylephrine in ERB aortas (Fig. 1C). At least part of this effect is due to the blockade of synthesis of NOS protein, because when NOS enzyme activity was measured by the conversion of [⁢H]arginine to [⁢H]citrulline, the NOS activity was far greater in ERB aortas compared with ERA aortas, and this effect was also prevented by cycloheximide (1 µM for...
Furthermore, in ERB aortas iNOS protein was produced, and this was prevented by cycloheximide and was not seen in ERA aortas (Fig. 4). The increase in NOS activity in ERB aortas was similar to that produced by LPS (10 µg/ml for 24 h) in ERA aortas (Fig. 3). In FRE aortas we did not observe any NOS activity (not shown).

The possibility that events triggered by nitric oxide during the incubation were responsible for the inability of acute l-NIO to restore fully the response of ERB aortas to phenylephrine (Fig. 2B) was tested by having L-NIO present during and after the organ culture. In this case, the response to phenylephrine in ERB aortas was restored to the same level as in ERA aortas (Fig. 1D). There are indications that peroxynitrite, a product of nitric oxide and superoxide radicals, was formed during the incubation, because there were significant nitrated tyrosine residues on proteins in ERB but not ERA aortas (Fig. 5). The antioxidant reduced glutathione (GSH) is reported to prevent peroxynitrite formation, and when present during the 24-h incubation also altered the response of ERB to phenylephrine, in that acute application of l-NIO now fully restored the vasoconstrictor response (Fig. 1E) compared with a partial effect in the absence of GSH (Fig. 2B).

There were also indications of cell damage from use of a TUNEL assay, which measures DNA damage and is an indicator of apoptosis. In this case, there were many TUNEL-positive cells in ERB but not ERA aortas (Fig. 6). Furthermore, both GSH and l-NIO markedly reduced the number of TUNEL-positive cells, indicating a protective effect (Fig. 6).

The signaling pathways that link induction of iNOS were also investigated by using inhibitors of various transduction pathways during the 24-h culture (Fig. 7). We investigated this in two ways, first in ERA aortas to determine whether the inhibitor had a direct effect on phenylephrine constriction, and second, in ERB aortas to determine whether it could prevent the iNOS-mediated suppression of the phenylephrine constriction. The tyrosine kinase inhibitor tyrphostin A25 (30 µM; see Ref. 15) present during the 24-h culture had a slight inhibitory effect on the constritor response to...
phenylephrine in ERA aortas (Fig. 7A), but in the ERB aortas it almost completely prevented the fall in the constrictor effect of phenylephrine. This indicates that tyrosine kinases are probably implicated in the induction of iNOS. Indeed, in ERB aortas, induction of iNOS protein was prevented by tyrphostin A25 (Fig. 7A). The selective protein kinase C inhibitor chelerythrine (3 µM; see Ref. 21) markedly inhibited the constrictor effect of phenylephrine in ERA aortas, but in ERB aortas it did not restore constriction (Fig. 7B), which indicates that protein kinase C was not involved in the decreased response to phenylephrine in ERB aortas. On the other hand, the mitogen-activated protein (MAP) kinase inhibitor PD-98059 (30 µM) (9) inhibited the constrictor effect of phenylephrine in ERA aortas and brought the diminished constrictor effect of phenylephrine in ERB aortas up to the level seen in ERA aortas in the presence of PD-98059 (Fig. 7C), which is indicative that MAP kinase is involved in the diminished response in ERB aortas. Calpain inhibitor 1 (3 µM), which blocks the activation of nuclear factor κB (NFκB) (17), had a slight inhibitory effect on the constrictor response to phenylephrine in ERA aortas (Fig. 7D), but in the ERB aortas it almost completely prevented the fall in the constrictor effect of phenylephrine, indicating that NFκB is probably implicated in the induction of iNOS. Indeed, the proteasome inhibitor TPCK (100 µM), which also interferes with NFκB processing (16), also restored the constrictor effect of phenylephrine in ERB aortas, but it should be noted that TPCK also slightly reduced the constrictor effect of phenylephrine in ERA aortas (Fig. 7E). Finally, PDTC (1 mM), an inhibitor of NFκB activation (41), partially restored the constrictor effect of phenylephrine in ERB aortas (Fig. 7F).

DISCUSSION

In the present study, in vitro removal of the endothelium and subsequent organ culture of the aortas in a chemically defined medium for 24 h (ERB aortas) resulted in the complete abolition of constrictor responses to the α-adrenoceptor agonist phenylephrine. Similar results in organ culture of denuded aortas in chemically defined medium have been previously obtained (30), where it was postulated that endotoxin contamination of the bathing medium led to a diminished constrictor effect through the expression of iNOS.
and the release of nitric oxide. Endotoxin contamination is an unlikely explanation in the present study, because culture of aortas with an intact endothelium and then acute endothelium removal after 24-h culture, ERA aortas, resulted in vasoconstriction similar to that of freshly excised aortas, FRE. This suggests that the response of the aortas during culture to the endothelium removal, and not the culture conditions per se, was the critical event. Nitric oxide is implicitly involved in the decreased aortic constrictor responses in ERB aortas, because both NOS enzyme activity and iNOS protein immunoreactivity were markedly elevated. These data agree with findings in which balloon angioplasty in vivo has been shown to cause the induction of iNOS protein in the underlying smooth muscle (18, 51). Our data indicate that simple endothelial damage is enough to induce iNOS without the stretching of the tissue as in balloon angioplasty. It was unclear from previous in vivo balloon angioplasty studies whether the induction of iNOS was due to cytokines released by infiltrating macrophages, blood borne elements, or some other event (18). The current observation of iNOS induction in vitro in chemically defined medium suggests that factors intrinsic to the blood vessel wall are involved.

The effect of endothelium removal on vascular responsivity has some similarity to the effects of bacterial LPS on smooth muscle function, where LPS induces iNOS and causes hyporeactivity to a wide range of constrictors including K⁺ (25, 31, 32). Both LPS (12) and smooth muscle damage (39) have been suggested to induce cyclooxygenase, which may alter vascular responsiveness through increased prostaglandin production. However, the cyclooxygenase inhibitor indomethacin had no effect on the diminished response to phenylephrine in ERB aortas, indicating no involvement of cyclooxygenase under these conditions.

The observation that acute application of the NOS inhibitor L-NIO only partially restored α-adrenoceptor vasoconstriction in ERB aortas suggests that factors

Fig. 5. Western blot analysis of nitrotyrosine protein in ERB (lane 3) or ERA (lane 5) aortic rings. L-NIO (100 µM for 24 h, lane 6) or reduced glutathione (3 mM for 24 h, lane 7) was present in some experiments in ERB aortas. Lane 4 represents freshly excised endothelium-denuded aortas (FRE). Lane 1 is standard molecular mass markers; lane 2 is nitrotyrosine molecular mass markers. In all lanes with aortas, protein load was adjusted to 13 µg. Blot is representative of 3 blots.

Fig. 6. TUNEL assay of transverse section of rat aorta placed in organ culture for 24 h. A: ERA; B: ERB; C: ERB with L-NIO (100 µM for 24 h); D: ERB with GSH (3 mM for 24 h). Pictures are representative of ≥3 different aortas.
other than the immediate release of nitric oxide are important. It should be noted that L-NIO has been reported to be superior to other NOS inhibitors N\textsubscript{\text{\texttext{-}}}-monomethyl-L-arginine, N\textsubscript{\text{\texttext{-}}}-nitro-L-arginine, and nitro-L-arginine methyl ester in inhibiting immune complex-induced vascular injury that is mediated by NOS (33). It is unlikely that there was an immediate significant physical damage to the underlying smooth muscle, because when endothelium was removed after culture (ERA aortas), the phenylephrine vasoconstrictor effect was maintained. Treatment with the protein synthesis inhibitor cycloheximide during culture completely protected the ERB aortas from having a diminished constrictor response. This is difficult to interpret definitively, because it would be expected that cycloheximide would prevent the synthesis of many elements in the tissue repair-tissue destruction process. However, it is clear that cycloheximide completely abolished both the enhancement in NOS activity in ERB aortas and the expression of iNOS. When this is coupled with the observation that 24-h cotreatment with L-NIO also fully restored responsiveness, the primary involvement of iNOS seems likely. The partial restorative effect of acute L-NIO compared with the full restoration of vasoconstriction by 24-h cotreatment with L-NIO suggests that the nitric oxide released during the 24-h organ culture has a role in determining the long-term constrictor function of the tissue, either directly or indirectly. One possibility is that the nitric oxide generated could react with superoxide ions to produce peroxynitrite, which has been demonstrated to occur when NOS is induced by LPS (44). Peroxynitrite leads to decreased mitochondrial respiration, contractile dysfunction, and DNA damage (3). Nitration of tyrosine residues on proteins is one indicator of cellular effects of peroxynitrite (3), and we found marked increases in nitrotyrosine immunoreactivity over a wide range of protein bands in ERB aortas, and this was prevented by treatment with either L-NIO or the antioxidant GSH, which has been shown to protect against peroxynitrite damage (1). Consistent with this, in ERB aortas treated with GSH for 24 h, L-NIO given acutely was now able to restore completely the constrictor effect of phenylephrine, which contrasts with the partial restoration in the absence of GSH treatment. Finally, we assessed DNA damage and apoptosis by use of a TUNEL assay and

![Graphs showing effect of inhibitors on phenylephrine-induced constriction](http://ajpendo.physiology.org/)

**Fig. 7.** Effect of inhibitors of signaling pathways on phenylephrine-induced constriction of aortas after removal of endothelium before 24-h organ culture of rat aortas. Constrictor effects of phenylephrine were determined in ERB or ERA aortic rings. Values are means ± SE. In some experiments, either tyrphostin A25 (T-25, 30 µM; A), chelerythrine (CHEL, 3 µM; B), PD-98059 (30 µM, C), calpain inhibitor 1 (Calpi, 3 µM, D), the proteosome inhibitor TPCK (100 µM, E), and PDTC, an inhibitor of NF\textsubscript{\text{\text{k}}B activation (1 mM, F) were present during organ culture. Significant differences (all P < 0.05, ANOVA with repeated measures): in A, ERB + T-25 responses were significantly different from ERB and not different from ERA + T-25 responses; in B, ERB + CHEL were not significantly different from ERB responses, but ERA + CHEL was different from ERA responses; in C, ERB + PD-98059 responses were significantly different from ERB responses and not different from ERA + PD-98059 responses, and ERA + PD-98059 responses were significantly different from ERA responses; in D, ERB + Calpi responses were significantly different from ERB and not different from ERA + Calpi responses; in E, ERB + TPCK responses were significantly different from ERB and not different from ERA + TPCK responses; ERA + TPCK responses were significantly different from ERA responses; in F, ERB + PDTC responses were significantly different from ERB and also different from ERA + PDTC responses.
found that, in ERB aortas, there were significant numbers of TUNEL-positive cells and these were decreased by either GSH or l-N1O. Whereas the protein nitrosylation in ERB aortas suggests that peroxynitrite is generated, the protective effects of GSH against diminished constriction and increased apoptosis are not definitive in implicating peroxynitrite in these events, because GSH can also prevent the formation or activity of other reactive species. Furthermore, hydroxyl radical, a breakdown product of peroxynitrite (34), is also able to induce apoptosis of smooth muscle cells (27) and may therefore be implicated. Further experiments are required to pinpoint the exact molecular species involved in the cellular damage.

A characteristic of both the response to blood vessel damage and culture of vascular smooth muscle is a change in the phenotype of the smooth muscle cells from a contractile to a proliferative phenotype (35, 45), and it is possible that this may be involved in the diminished contractile response in ERB aortas in the present study. We did not measure phenotype changes; however, the observation that chronic NOS inhibition completely prevented the diminution of contraction to phenylephrine suggests that if this process is involved, then nitric oxide or some event associated with nitric oxide must be involved. It should be noted, however, that nitric oxide suppresses smooth muscle proliferation in culture (7), which argues against the phenotype change hypothesis.

In the present study, the activator of iNOS production after endothelium removal is essentially unknown, and it is unclear whether cytokines or local growth factors may be involved. Various signaling pathways, including tyrosine kinases (23), protein kinase C (10), MAP kinases (42), and NFkB (20), have been reported to be involved in the induction of iNOS by cytokines, growth factors, and bacterial LPS in a variety of cell types; knowledge about these pathways may give us clues about the nature of the endogenous activator. The lack of constrictor effect of phenylephrine in ERB aortas was reversed by the tyrosine kinase inhibitor tyrphostin A25, which also prevented the induction of iNOS protein. Similar results were also found by use of the MAP kinase inhibitor PD-98059, as well as drugs acting on the NFkB cascade, calpain inhibitor 1, TPCK, and PDTC. The protein kinase C inhibitor chelerythrine did not restore the constrictor effect of phenylephrine. These data suggest a scheme whereby a tyrosine kinase is activated by some factor that sets in train a MAP kinase cascade, which in turn acts through NFkB to induce iNOS production. Indeed, it is well established that tyrosine kinases are linked to MAP kinase activation (49) and that the MAP kinase pathway can interact with the NFkB pathway (22). Of course it cannot be ruled out that each of these systems is independent, but in that case it would be surprising that blockade of each individually could restore full constrictor function of the ERB aortas. The similarity of the signaling pathway for endogenous iNOS induction to that for exogenous cytokines and growth factors suggests that local production of growth factors or cytokines may be involved.

The organ culture method, in which we observed that endothelium removal induces iNOS, does not represent the in vivo situation, where it is likely that the smooth muscle cells are bathed in a variety of factors that may influence iNOS expression and activity. Potential in vivo modulators include inducers of iNOS, such as IFN-γ, TNF-α, and IL-1, and also suppressors of induction, such as transforming growth factor-β, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor (13, 48). What our data do show, however, is that in the absence of exogenous suppressors of iNOS induction, endothelial cell removal is sufficient in itself to cause enough iNOS expression that peroxynitrite damage is evident.

These results may be relevant to the pathophysiology of vascular remodeling and restenosis, which is a major problem associated with balloon angioplasty (2, 37). After balloon angioplasty, apoptosis of the smooth muscle cells is seen within 30 min (36); therefore, this is likely a response to the direct physical damage. Our data indicate that an additional factor in the apoptosis of smooth muscle cells is peroxynitrite-induced damage, and this can occur in the first 24 h. Therefore, reducing the potential for peroxynitrite-induced damage by using antioxidants may be of benefit. There are several studies showing that antioxidant therapy may indeed have benefits in vascular remodeling of animals as well as humans (52), although this has been attributed to other effects such as inhibition of lipid oxidation (6). It is interesting to note that the induction of iNOS after balloon angioplasty, although observed over 14 days (18), appears to have functional significance in being able to inhibit platelet aggregation only over the first 3 days (51). Thus there may be an initial window when nitric oxide and perhaps peroxynitrite are relevant, which may allow specific targeting of high-dose antioxidants in this early period to prevent peroxynitrite damage.

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Received 11 May 1998; accepted in final form 14 September 1998.

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