Shear stress induces iNOS expression in cultured smooth muscle cells: role of oxidative stress

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Shear stress induces iNOS expression in cultured smooth muscle cells: role of oxidative stress. Am J Physiol Cell Physiol 279: C1880–C1888, 2000.—After deendothelialization, the most luminal smooth muscle cells of the neointima are in contact with blood flow and express inducible nitric oxide synthase (iNOS) in vivo. We hypothesized that shear stress may be a stimulus for this iNOS overexpression. We have thus submitted smooth muscle cells to laminar shear stress and measured the iNOS expression. Shear stress (20 dyn/cm²) induced iNOS mRNA and protein expression, whereas brain NOS mRNA expression was decreased. Conversely, nitrite production was increased. This production was blocked by a selective iNOS inhibitor. Pyrrolidine dithiocarbamate, an antioxidant molecule, and BXT-51072, a glutathione peroxidase mimic, both inhibited the shear-induced iNOS expression. Shear stress also increased the expression of both membrane subunits of NADPH oxidase p22phox and Mox-1. Shear stress activated the redox-sensitive nuclear translocation of the transcription nuclear factor-κB (NF-κB) and stimulated the degradation of both cytosolic inhibitors α and β. These results show that shear stress can induce iNOS expression and nitrite production in smooth muscle cells and suggest that this regulation is probably mediated by oxidative stress-induced NF-κB activation.

nuclear factor-κB; nitric oxide synthase; NADPH oxidase

THE VASCULAR WALL of large arteries is organized into three different functional and structural compartments. This specific organization (compartmentalization of the vascular wall) modulates the response of the vascular wall to different stimuli. Smooth muscle cells constitute the media layer, whereas endothelial cells constitute the intima and physiologically perceive the shear stress at the interface between the flowing blood and the fixed arterial wall. In these endothelial cells, shear stress regulates the expression of numerous genes [angiotensin converting enzyme, platelet-derived growth factor, vascular cell adhesion molecule-1 (VCAM-1), etc.] (see Ref. 17 for review). In particular, increased shear stress intensity upregulates endothelial nitric oxide synthase (NOS) activity and leads to nitric oxide (NO) overproduction in endothelial cells (23, 24). In response to deendothelialization, smooth muscle cells migrate from the media to the lumen and form the neointima. During this process, the most luminal smooth muscle cells are in close contact with the blood flow and thus sense the shear stress. Therefore, smooth muscle cells undergo the shear stress that could modify the pattern of protein expression within the most luminal smooth muscle cells. In vivo, a gradient of inducible protein expression including inducible NO synthase (iNOS) (12) is usually observed from the most luminal to the deeper part of the neointimal proliferation (28). We have recently shown that shear stress inducible angiotensin converting enzyme expression in smooth muscle cells (9). It would permit the shift of a constitutive endothelial function to smooth muscle cells in an intimal position. In this way, it has been demonstrated that iNOS expression is induced in the most luminal smooth muscle cells of the intimal layer in vivo (12).

The formation of the neointima is inhibited by high flow rate (35), and this phenomenon is partially mediated by NO production (6). Administration of L-arginine, the NOS substrate, was associated with a reduced neointimal hyperplasia (11) independent of any endothelial process (32), suggesting that NO may be produced by smooth muscle cells. Given that smooth muscle cells are exposed to flow in neointima formation, we hypothesized that shear could participate in iNOS induction and NO production in smooth muscle cells. The antiproliferative effects of NO on smooth muscle cells and its ability to induce expansive remodeling would be of interest to limit intimal proliferation and lumen stenosis in different pathophysiological situations.

A key component of the induction of iNOS is the nuclear factor-κB (NF-κB). NF-κB is a redox-sensitive factor that is activated by the cytosolic release of the inhibitor κB (IκB) proteins and the translocation of the active p50/p65 heterodimer to the nucleus. Increase in the production of radical oxygen species is a common pathway to a wide variety of NF-κB inducers (1).
though several lines of evidence suggest that shear stress is an inducer of NF-κB activation in endothelial cells (4, 19), there is no data available about the induction of NF-κB in smooth muscle cells submitted to shear stress. The aim of our present study was to evaluate whether shear stress regulates the expression of iNOS in smooth muscle cells and whether the redox-sensitive factor NF-κB was involved in this regulation.

MATERIALS AND METHODS

Animals. Normotensive male Wistar rats (160–180 g) were obtained from Ifla Credo (Labresle, France). The procedure followed for the care and euthanasia of the studied animals was in accordance with the European Community standards on the care and use of laboratory animals (Ministère de l’Agriculture, France; authorization no. 00577).

Cell isolation and culture. The smooth muscle cells from the aortic media were isolated and cultured as described previously (2) and were used at passage 3. The purity of the cultures was assessed by using morphological and immunohistological criteria. Smooth muscle cells were characterized with antibodies raised against smooth muscle cell α-actin (8).

Shear stress device. The cells were seeded on a rectangular plastic (cell culture-treated) plate previously coated with collagen (0.1% in hydroxy chloride, Sigma). Collagen was chosen as a substrate to increase the adherence forces of the cells submitted to shear stress. Cells were used 2 to 4 days after reaching confluence.

Cells were then exposed to a fluid-imposed shear stress with the use of a parallel plate channel flow device derived from the one described by Levesque and Nerem (15). The cell culture flow chamber was designed to provide a steady, uniform laminar flow. It was positioned in a closed continuous flow loop. The flow loop consisted of an elevated reservoir that provided the required pressure drop across the chamber and a roller pump to return the outflow from the collecting reservoir back to the feeding reservoir. The flow chamber and the entire apparatus were sonicated and sterilized before each experiment to avoid lipopolysaccharide (LPS) contamination. The upper reservoir was filled with 350 ml of Dulbecco’s modified Eagle’s medium (DMEM, Biomedia) at 37°C (equilibrated with 95% air–5% CO2) supplemented with 10% heat-inactivated fetal calf serum (Bio-Rad), with its pH, temperature, and flow rate monitored continuously. The experimental surface of shear-subjected cells was 18 cm2. As a control, unstimred cells were seeded in the same conditions without insertion in the flow chamber device and were incubated with shear stress-conditioned medium to test LPS contamination. After being submitted to shear, the cell plates were removed from the flow chambers under sterile conditions.

Shear stress values. The shear stress intensity Y (dyn/cm²) was calculated as follows (15): 

\[
Y = \frac{6 \mu Q}{h^2}
\]

where \( \mu \) is the media viscosity (DMEM 0.0084 ± 0.08 poise at 37°C), Q the flow rate (ml/s), p the flow path (1.8 cm), and h the gap height over the cell layer (0.025 cm). The viscosity of the media and the flow chamber cross section were constant in the device. To change the intensity of shear stress, the induction flow rate had to be modified. For pharmacological experiments, cells were preincubated with the blocker for 15 min before being submitted to shear stress. The experiments were performed with the same concentration of compound in the medium.

Endotoxin measurement. The level of endotoxins (i.e., LPS) was measured in the supernatant of control cells, in the supernatant conditioned by a 20-dyn/cm² shear stress, and in the freshly prepared culture medium using a commercial semiquantitative assay kit (Sigma) (22). One hundred microliters of the sample were incubated for 1 h at 37°C with 100 μl of amoebocyte lysate from Limulus polyphemus. The results were evaluated by comparison of the gelation of the sample and compared with a standard curve from 400 to 0.06 endotoxin units (EU/ml) of commercial endotoxin solution (Sigma).

Nitrite production. To measure nitrite production, each cell plate was put into 10 ml of DMEM without phenol red and 20 mM HEPES with or without 2.10⁻⁵ M of a selective iNOS inhibitor [L-NAME(1-iminoethyl)lysine (L-NIL)] (20) for 4 h. The medium was then removed and nitrates were detected with the use of a fluorescent assay (18). Nitrite production was measured with the use of a fluorescent assay as described previously (18). In this assay, nitrates, a degradation product of NO, interact with a nonfluorescent substrate (diaminophthalene, Fluka) to form a fluorescent component that is detectable at 450 nm (1-[H]naphthotriazole). A standard curve from 10 nM to 10 μM was made using a commercial nitrite solution (Merck) and treated in the same conditions to test the proportionality of the method. One hundred microliters of diaminophthalene (0.05 mg/ml in HCl 0.62 N) was added to 1 ml of the sample. After 10 min of incubation in a dark room, 50 μl of NaOH 2.8 N was added to stop the reaction. Fluorescence was read in a spectrofluorometer (Hitachi F2000). The cells were scrapped off and the total proteins were assayed using the protein assay system (Bio-Rad). Results were expressed as nanomoles of nitrates per milligram of proteins. Nitrite levels have been shown to reflect >75% of the total NO produced by vascular smooth muscle cells (31).

RT-PCR. For RT-PCR analysis, cells were scraped from each plate into 1 ml of TRIzol solution (GIBCO BRL). Total RNA was prepared using the manufacturer’s instructions. One microgram of total mRNA was reverse transcribed using an oligo (dT) primer. PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), S14, iNOS, p22phox, and Mox-1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; bNOS, brain nitric oxide synthase.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Length, bp</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5′-GTGAAAGTGACGACTCAAGC-3′</td>
<td>5′-GGTGAAAGCAGCGATGGC-3′</td>
<td>299</td>
</tr>
<tr>
<td>S14</td>
<td>5′-ATGAGACCGCCGACAGCG-3′</td>
<td>5′-CGACGTGCTGTCAGAGGGA-3′</td>
<td>127</td>
</tr>
<tr>
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<td>5′-GGTGGTGGGTCGGGAGCG-3′</td>
<td>5′-CGACGTGCTGTCAGAGGGA-3′</td>
<td>227</td>
</tr>
<tr>
<td>bNOS</td>
<td>5′-CTGGCTCAAGAAGATACAGGGT-3′</td>
<td>5′-GGACGTGCTGTCAGAGGGA-3′</td>
<td>293</td>
</tr>
<tr>
<td>p22phox</td>
<td>5′-GGGACGTGCTGTCAGAGGGA-3′</td>
<td>5′-GATGGTGGTGGGAGCG-3′</td>
<td>306</td>
</tr>
<tr>
<td>Mox-1</td>
<td>5′-GATGGTGGGAGCG-3′</td>
<td>5′-CATAAGAACAAAAACACCCAC-3′</td>
<td>578</td>
</tr>
</tbody>
</table>

PCR was carried out to detect mRNAs for rat GAPDH, S14, iNOS, bNOS, p22phox, and Mox-1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; bNOS, brain nitric oxide synthase.

Table 1. Nucleotide sequence of primers used for PCR with expected size of amplicons
brain NOS (bNOS), p22phox, and Mox-1 mRNA were performed using the primers presented in Table 1. 32P- radiolabeled primers were added to the PCR mixture, and the PCR products were separated on an 8% acrylamide/ N,N'-dihydroxyethylene-bis-acrylamide gel in 1× Tris-borate-EDTA buffer. After ethidium bromide staining, the gel was dried and radioactivity was counted using an instant imager. iNOS, bNOS, p22phox, and Mox-1 mRNA levels were normalized to GAPDH mRNA, and results were expressed in arbitrary units.

Western blot. Cells were scraped off into lysis buffer that contained protease inhibitors for Western blot experiments and total protein measurement. Cell proteins were denatured using Laemmli reagent.

For the IkB Western blot, 25 μg of the protein samples were electrophoresed in a 4% SDS-PAGE gel for 15 min at 100 V and then in a 12% SDS-PAGE gel for 45 min at 200 V. The samples were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 1 h. After being blocked for 1 h in 5% fat dry milk, the membrane was then incubated with monoclonal antibodies against α- and β-isofoms of IkB (Tebu) diluted 1:3,000 in 1% PBS-BSA. The membrane was washed four times with PBS-Tween, 0.5% fat dry milk, and incubated with the second antibody against mouse IgG labeled with peroxidase diluted 1:2,000 in 1% PBS-BSA. The membrane was washed again four times, and the protein bands were visualized by enhanced chemiluminescence (Amersham).

For iNOS detection, 50 μg of the protein sample were electrophoresed in a 4% SDS-PAGE stacking gel and an 8% SDS-PAGE running gel. Samples were transferred to a PVDF membrane at 300 mV for 2 h, and, after being blocked for 1 h with 1% BSA, incubated overnight at 4°C with a polyclonal antibody against iNOS (Transduction Laboratories) diluted 1:500 in 1% PBS-BSA. The incubation with the second antibody and the revelation were performed using the same protocol as for IkB Western blot.

Electrophoretic mobility shift assay. Nuclear proteins from cells were prepared as previously described (26). Gel-shift assays were performed with a commercial kit according to the manufacturer’s instructions (Promega). The NF-κB oligonucleotide probe used (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with [γ-32P]ATP by using T4 polynucleotide kinase. Nuclear proteins (15 μg) were incubated for 20 min with the labeled probe and migrated in a 4% polyacrylamide gel. The specificity of the binding reaction was determined by coincubating duplicate samples with 100-fold molar excess of unlabeled oligonucleotide probe (competition).

Pharmacology signaling. To test the role of NF-κB in shear-induced iNOS overexpression, we incubated smooth muscle cells with 10−5 M pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor (19), for 1 h. The cells were then submitted to a 20-dyn/cm2 shear stress for 24 h. To test the role of oxidative stress in the observed phenomenon, the same experiments were performed with 10−5 M of BXT-50172 (21), a potent antioxidant that mimics the glutathione-peroxidase activity.

Statistical method. Results were expressed as means ± SE. Significance was estimated by analysis of variance and the Bonferroni test or by the Student’s t-test. P < 0.05 was considered significant.

RESULTS

Cell viability and endotoxin measurement. Shear-stressed cells in primary culture for 24 h is a difficult experimental condition that can lead to cell suffering and death. To estimate the cell density, we assayed the levels of total proteins in both control and treated plates. A 24-h exposure to shear stress (20 dyn/cm²) did not significantly change the cell density on plates submitted to shear compared with the control, since the amount of total proteins was not modified in the plates treated with L-NIL and shear stress (Fig. 1A). Furthermore, cell integrity was tested by estimating both the GAPDH and S14 (2 housekeeping genes) mRNA levels by RT-PCR. Neither shear stress nor pharmacological

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**Fig. 1.** Shear stress did not modify cell viability. A: effect of shear stress and inducible nitric oxide synthase (iNOS) inhibition on total protein expression in cultured smooth muscle cells. Cells were submitted to 24 h of a 20-dyn/cm² shear stress in culture medium (shear stress) or in culture medium + 2.10−8 M of L-NIL (L-NIL), a selective inhibitor of iNOS activity (shear stress + L-NIL). Control cells were cultured in the same conditions without insertion into the flow chamber. OD, optical density. B: effect of shear stress and different inhibitors on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and S14 mRNA expression in cultured smooth muscle cells. Cells were submitted to a 20-dyn/cm² shear stress with or without inhibitors for 24 h. Pyrrolidine dithiocarbamate (PDTC) is an inhibitor of the nuclear factor-κB (NF-κB) system, and BXT-51072 is an antioxidant with a glutathione peroxidase mimicking activity. The GAPDH and S14 mRNA levels were estimated by RT-PCR from 1 μg of total mRNA. cpm, Counts/min.
treatments influenced the GAPDH nor the S14 mRNA signal (Fig. 1B).

Because iNOS expression and NF-κB activation can be induced by LPS in smooth muscle cells, we verified that no LPS contamination was present in the medium conditioned by a 20-dyn/cm² shear stress for 24 h. Exposure of the cells to shear stress did not modify the level of endotoxin in the conditioned medium, compared with the supernatant of the control cells. This level is lower than 4 EU/ml and comparable to the level estimated in the unused commercially available culture medium.

Shear stress induced the expression of iNOS. The expression of iNOS was dependent on the shear stress intensity (Fig. 2A) and its duration (Fig. 2B). The iNOS/GAPDH mRNA level was 0.02 ± 0.004 in control cells. No induction was observed at 10 dyn/cm² for 24 h (0.05 ± 0.02). A shear value of 20 dyn/cm² for 24 h induced a significant response (3.42 ± 0.95, P < 0.001). Six hours of a 20-dyn/cm² shear rate was insufficient to induce any iNOS expression (0.02 ± 0.006). After a 20-dyn/cm² shear stress for 24 h, the level of iNOS protein was also significantly increased in smooth muscle cells (+170 ± 73%, P < 0.05; Fig. 2C).

Because smooth muscle cells could also express the brain isoform of the NOS, we tested the effect of shear stress on this isoform mRNA expression. A 24-h shear stress at 20 dyn/cm² reduced the bNOS mRNA level in smooth muscle cells (from 7.8 ± 1.4 in controls to 0.5 ± 0.12, P < 0.0001; Fig. 3A).

Shear stress-induced nitrite production. We estimated the production of NO of the stressed smooth muscle cells by measuring the amount of nitrite, its main metabolite. The nitrite production was enhanced in the medium of smooth muscle cells submitted to a 20-dyn/cm² shear stress for 24 h. Nitrite production in control cells was 0.26 ± 0.017 nmol/mg protein for 4 h, whereas stressed cells synthesized 0.69 ± 0.09 nmol/mg protein for 4 h (P < 0.001). This increase in nitrite production was inhibited by 2 × 10⁻⁵ M L-NIL, a selective inhibitor for iNOS (0.37 ± 0.08 nmol/mg protein for 4 h, P < 0.001), confirming that this production is mainly due to iNOS expression (Fig. 3B).

Fig. 2. iNOS expression increased in response to shear stress. iNOS mRNA expression corresponded to the ratio between the iNOS and the GAPDH PCR product radioactivity. Similar results were obtained when S14 mRNAs were used as a reference (data not shown). A: dependency on shear intensity of iNOS mRNA expression in cultured smooth muscle cells. Cells were submitted to different levels of shear stress for 24 h. ***P < 0.001. B: iNOS mRNA expression of cultured smooth muscle cells submitted to different durations of exposure to shear stress (20 dyn/cm²). ***P < 0.001. C: iNOS protein level in cultured smooth muscle cells submitted to shear stress (20 dyn/cm² for 24 h). Western blot gel (top) and the densitometric quantification of the bands (bottom). *P < 0.05. NS, not significant.
Role of NF-κB and oxidative stress on shear stress-induced iNOS expression. To test the implication that NF-κB plays a role in the shear stress-induced iNOS expression, we incubated smooth muscle cells with PDTC, an NF-κB blocker, and an antioxidant drug. PDTC at the concentration of 10^{-5} M completely prevented the induction of iNOS by 24-h shear stress at 20 dyn/cm² (0.01 ± 0.003 vs. 3.4 ± 0.9, P < 0.001; Fig. 4A). Furthermore, BXT-50172 (10^{-5} M), an antioxidant that mimics glutathion peroxidase activity, suppressed the shear-induced iNOS induction in smooth muscle cells (Fig. 4B). These results suggest that oxidative stress and NF-κB are involved in the response of smooth muscle cells to shear stress. We then tested the effect of shear stress on both NF-κB translocation to the nucleus and the expression of its 1kB. In smooth muscle cells submitted to shear stress, the amount of NF-κB translocated to the nucleus was increased by 400 ± 30% (P < 0.001; Fig. 5A). The coincubation of the sample with an excess of unlabeled oligonucleotide probe suppressed the gel shift, which showed the specificity of the binding (Fig. 5A, lane competition). Furthermore, in the cytoplasm of the stressed cells, 1kB α- and β-proteins were reduced, respectively, by 86.56 ± 10.3% and 64.21 ± 10.2% of control values (P < 0.001; Fig. 5B).

Shear stress increased the NADPH oxidase expression. The enzyme implicated in the production of reactive oxygen species and thus in generation of oxidative stress in smooth muscle cells is the NADPH oxidase. Because our experiments with BXT-51072 have suggested that oxidative stress was implicated in the response to shear stress, we estimated the expression of the two active membrane-associated subunits of the NADPH oxidase: p22^{phox} and Mox-1. Shear stress (20 dyn/cm², 24 h) significantly increased the p22^{phox} and Mox-1 mRNA levels in smooth muscle cells (from 5.47 ± 0.12 in the control cells to 7.03 ± 0.38 in the stressed ones, P < 0.01 for p22^{phox}; and from 0.331 ± 0.04 in the control cells to 0.879 ± 0.19 in the stressed ones, P < 0.05 for Mox-1; Fig. 6).

DISCUSSION

The present study shows that shear stress increased both iNOS mRNA and protein expression in rat aortic smooth muscle cells. This induction occurred only after prolonged exposure (24 h) to shear stress and was accompanied by a rise in nitrite production in the conditioned medium. This nitrite overproduction (+160 ± 26%) was more important than that which we observed in smooth muscle cells treated for 24 h with 40 μg/ml LPS (i.e., 20,000 EU/ml) plus 100 U/ml interferon-γ (+75 ± 22%; data not shown), suggesting that this production was physiologically relevant.

A preferential inhibitor of iNOS blocked this shear stress-induced nitrite production. Because this inhibitor is 30-fold more specific to iNOS than to bNOS (20), it is unlikely that the observed NO overproduction also resulted from the activity of the brain isoform. Furthermore, shear stress significantly decreased bNOS mRNA levels, confirming that bNOS was not involved in the observed phenomenon. The decrease in bNOS expression was not due to cell death during the experiment because the amounts of total proteins and housekeeping gene expression (GAPDH and S14) were not significantly diminished in stressed plates. These data confirm the observation reported by Papadaki and co-workers (25). In their study, they showed that two stages in nitrite production rate can be discerned in
cultured smooth muscle cells exposed to flow. In the first stage (in the first hour of exposure to shear stress), nitrite production was quickly increased because of an upregulation of the calcium/calmodulin-sensitive bNOS activity. In the second stage (from 1 to 24 h of exposure to high shear stress levels), shear stress induced a stable production of nitrites that was not sensitive to a calmodulin inhibitor (25). These data suggest that the mechanism implicated in this NO overproduction in the late stage (enzyme expression) is different from that implicated in the first stage (enzyme activity). Our results confirm that although the initial burst in nitrite production in stressed cells could depend on the activity of constitutive NOS, a prolonged exposure to a physiological level of shear stress induces the expression of the iNOS. Its activity is not directly regulated and produces a large amount of NO (13, 24). A small quantity of protein is able to produce significant levels of NO, and this induction leads to a stable and long-term production of NO (12).

This expression can be regulated by numerous extracellular factors, including LPS from bacteria (10, 31) that could be present in our apparatus and then interfere with the effect of shear stress. The amount of LPS in the medium conditioned by a 20-dyn/cm² shear stress for 24 h was not different from that which was measured in the supernatant of the control cells and was lower than 4 EU/ml. Furthermore, 10 dyn/cm²

![Fig. 4. Role of NF-κB and oxidative stress in iNOS expression.](image)
iNOS mRNA expression corresponded to the ratio between the iNOS and the GAPDH PCR product radioactivity. A: inhibition of shear stress-induced iNOS expression by an NF-κB blocker in cultured smooth muscle cells. Cells were submitted to a 20-dyn/cm² shear stress for 24 h in the presence or absence of 10⁻⁶ M PDTC. ***P < 0.001. B: inhibition of shear stress-induced iNOS expression by an antioxidant in cultured smooth muscle cells. Cells were submitted to a 20-dyn/cm² shear stress for 24 h in the presence or absence of 10⁻⁵ M BXT-51072, an antioxidant that mimics the glutathione peroxidase activity. ***P < 0.001, **P < 0.01.

![Fig. 5. Effect of shear stress on NF-κB activation.](image)
A: the electrophoretic mobility shift assay gel (top); the densitometric quantification of the NF-κB nuclear level (bottom). The competition lane corresponded to coincubation of the sheared sample with an excess of unlabeled oligonucleotide probe. B: Western blot of inhibitors IκB (IκB) on SDS-PAGE gel (top). Densitometric quantification of IκB levels of cultured smooth muscle cells exposed to shear stress compared with control cells (bottom), ***P < 0.001.
shear stress and passive transfer of shear-conditioned medium failed to induce any change in iNOS expression. Thus LPS contamination could not be responsible for the induction we observed.

Our results clearly demonstrate that shear stress is a potent inductor of iNOS expression in smooth muscle cells. They agree with the in vivo data reported by Yan and coworkers (38) showing that iNOS expression is induced in the innermost layers of the neointima in rat carotid arteries after balloon-induced deendothelialization and intimal smooth muscle cell migration. In response to mechanical injury, smooth muscle cells migrate in the intima and proliferate in contact with the blood flow and are thus submitted to shear stress ($\sim$20 dyn/cm$^2$) (38). The in vivo expression of the enzyme is localized in the smooth muscle cells that are closest to the lumen, suggesting that the blood flow contact could be necessary for the induction of iNOS expression. Furthermore, in this study, the phenomenon is delayed, occurring only 24 h after deendothelialization. The intensity of the applied shear stress and the delay necessary for iNOS induction in smooth muscle cells correspond to what we have observed in vitro and are consistent with an important role of shear stress in iNOS overexpression in vivo. Recently, Fukuda and coworkers (7) have demonstrated that iNOS expression is involved in the media layer of shear-induced cerebral aneurysm in rats. Their results strongly suggest that the increase in wall shear stress is responsible for the iNOS induction because the reduction of shear attenuated the iNOS immunoreactivity in the artery.

The cytokine-induced iNOS expression is under the control of the NF-κB (33). Because shear stress is able to induce NF-κB activation in endothelial cells (14), we wondered whether shear stress-induced iNOS expression in smooth muscle cells could be mediated by NF-κB activation. One part of NF-κB activation is due to the phosphorylation of IκB by IκB kinase. IκB kinase activity is increased by shear stress in endothelial cells (4) and leads to the dissociation of the cytosolic IκB-NF-κB complex that is accompanied by the degradation of IκB (37). Thus the degradation of IκB is considered to be a marker of NF-κB translocation. Our results show that shear stress caused a significant decrease in the cytosolic IκB α and β levels and an increase in NF-κB translocation to the nucleus of smooth muscle cells. Moreover, PDTC, a potent inhibitor of NF-κB translocation that acts by scavenging the intracellular reactive oxygen intermediates (30), abolished the iNOS induction in response to shear stress. Thus the effect of shear stress on iNOS expression was, at least in part, mediated by NF-κB.

Translocated NF-κB is able to bind both the promoter sequence (called shear stress responsive element) that is present in many genes in which expression is modulated by shear stress (29) and an NF-κB-specific responsive element. The rat iNOS gene promoter has been cloned (30). No shear stress responsive element has been found, but several functional NF-κB responsive elements have been described and are implicated in the cytokine-induced iNOS expression. Thus shear stress-induced iNOS expression seems to be due, in part, to the binding of NF-κB on its specific responsive element in the iNOS gene promoter than to a direct shear-dependent response. These data fit well with the delayed character of the response.

The intracellular signaling pathway mechanisms that lead to NF-κB activation in response to shear are not yet identified. However, regulation of the IκB-NF-κB system is considered to be, in part, under the dependence of the redox state of the cell. Shear stress is able to increase superoxide anion production in endothelial cells by increasing NADPH oxidase activity (5). A similar phenomenon could occur in stressed smooth muscle cells. It has been shown that rat aortic smooth muscle cells use a NADH/NADPH oxidase to generate superoxide anions (36). This enzyme is com-
posed of two membrane-bound [p22\text{phox} (36) and Mox-1 (34)] and three cytosolic subunits (p40, p47, and p67) in smooth muscle cells. The two membrane-associated subunits (p22\text{phox} and Mox-1) have been shown to be important for free radical production. Transfection of smooth muscle cells with antisense-p22\text{phox} inhibits angiotensin II-stimulated \text{O}_2⁻ production, showing that p22\text{phox} is essential in free radical synthesis in these cells (36). Recently, Mox-1 has been described as the active subunit of the NADH/NADPH oxidase in smooth muscle cells (34). In our experiments, 24 h of a 20-dyn/cm² shear stress increased both p22\text{phox} and Mox-1 mRNA expression in smooth muscle cells. Because the transcriptional regulation of these genes has not yet been described, the mechanism involved in this induction remains to be determined. These results suggest that oxidative stress is increased in smooth muscle cells submitted to shear stress. We then tested the effect of BXT-51072, an efficient antioxidant with glutathione peroxidase mimicking activity (21), on the iNOS expression. We have recently shown that BXT-51072 inhibits oxidative stress-induced VCAM-1 expression in endothelial cells (26). Because \(10^{-5} \text{ M}\) of BXT-51072 abolished the shear stress-induced iNOS expression, we can conclude that oxidative stress is implicated in the delayed response of smooth muscle cells to shear stress.

Our results clearly demonstrate that shear stress is one of the inducers of iNOS expression in aortic smooth muscle cells. This conclusion fits with numerous studies showing that flow inhibits neointima formation after angioplasty (6, 16) and that NO plays a crucial role in this phenomenon (6). Nevertheless, as NO production appeared to be accompanied by an increase in free radical production, it is likely that a part of the synthesized NO was converted into peroxynitrites (3). Because peroxynitrites have been shown to lead to cell disturbances, as well as to the development of atherosclerotic lesions (27), it is difficult to know whether or not iNOS induction in intimal smooth muscle cells is beneficial.

In conclusion, our study shows that shear stress increases oxidative stress in smooth muscle cells and induces NF-\(\kappa\)B-dependent gene expression such as iNOS. Therefore, shear stress could be responsible for luminal iNOS expression in the neointima in vivo (38). Conversely, our data suggest that in the response of smooth muscle cells to arterial wall injury, the shear stress induced by blood flow could be an important stimulus that follows their intimal migration and proliferation. Nevertheless, further experiments need to be performed to better understand the direct or indirect signaling pathways that lead to the increase in oxidative stress in response to shear stress.

REFERENCES

3. Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624, 1990.
22. Nadan R and Brown D. An improved in vitro pyrogen test to detect picograms of endotoxin contamination in intravenous


