

Isometric contraction induces the Ca^{2+} -independent activation of the endothelial nitric oxide synthase

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ABSTRACT Shear stress and tyrosine phosphatase inhibitors have been shown to activate the endothelial NO synthase (eNOS) in a Ca^{2+} /calmodulin-independent manner. We report here that isometric contraction of rabbit aorta activates eNOS by a pharmacologically identical pathway. Endothelium-intact aortic rings were precontracted under isometric conditions up to 60% of the maximal phenylephrine-induced tone. The NO synthase inhibitor N^G nitro-L-arginine (L-NA) and the soluble guanylyl cyclase inhibitor NS 2028 induced an additional contraction, the amplitude of which depended on the level of precontraction. The maximal production of NO by isometrically contracted aortic rings (as estimated by the increase in cGMP in detector smooth muscle cells in a superfusion bioassay) was observed during the initial phase of isometric contraction and was greater than that detected following the application of acetylcholine. The supplementary L-NA-induced increase in vascular tone was inhibited by the nonselective kinase inhibitor staurosporine and the tyrosine kinase inhibitors erbstatin A and herbimycin A. Another tyrosine kinase inhibitor, genistein, the calmodulin antagonist calmidazolium, and the selective protein kinase C inhibitor, Ro 31-8220, had no effect. Coincident with the enhanced NO formation during isometric contraction was an increase in the tyrosine phosphorylation of endothelial proteins, which also correlated with the level of precontraction. Thus, isometric contraction activates eNOS via a Ca^{2+} -independent, tyrosine kinase inhibitor-sensitive pathway and, like shear stress, seems to be an independent determinant of mechanically induced NO formation.

The endothelial NO synthase (eNOS) is classified as a constitutively expressed Ca^{2+} /calmodulin-dependent enzyme. However, enhanced enzyme activity does not appear to be strictly dependent on an increase in $[\text{Ca}^{2+}]_i$, at least in response to mechanical stimulation (1). The hypothesis that eNOS can be activated by at least two independent signaling pathways is supported by the observations that disruption of the cytoskeleton attenuates flow-induced NO release in native endothelial cells—but not the agonist-induced production of NO (2)—and that there is an impaired flow-induced dilation in arteries from mice lacking the intermediate filament vimentin (3). Indeed, the endothelial cell can be viewed as a membrane stretched over a frame composed of microtubules, intermediate filaments, and actin fibres that transverse the cells and end in characteristic focal adhesion complexes and cell–cell contacts. Because signaling molecules are concentrated around and are inherent to these sites, it is conceivable that the application of a mechanical stress—transmitted through the cell by the

cytoskeleton—activates signal-transduction cascades without the need of a specific shear stress or stretch receptor (for review, see ref. 4).

Although mechanical stimuli at the endothelial cell surface (shear stress and cyclic strain) can be physically defined, there also are hemodynamically relevant cell–cell-generated forces that cannot be expressed by a simple physical relationship. One example is the contraction of an arterial segment under isometric conditions *in vivo*, in which the development of contractile force within the smooth muscle cell layer counteracts the distending transmural pressure. Although isometric contraction is defined as the development of active tension with no overt shortening, this mode of contraction is accompanied by considerable internal shortening of the contractile apparatus with the concomitant extension of compliant, series-coupled structures. The functional sum of these undamped, series-coupled structures has been termed the “series elastic element.” Cross-bridges within muscle filaments and connections between cells are factors likely to contribute to series elastic-element compliance, which has been reported to be in the range of 4–10% of the optimal tissue length for force development (5, 6). A fluorescent nuclear dye has been used to quantitatively document that, during isometric contraction, circular and diagonal spacing between adjacent smooth muscle cells is decreased, whereas longitudinal spacing is increased. This is as expected from a shortening and widening of the smooth muscle cells on contraction (7). Although the latter study did not actually document movement of endothelium relative to smooth muscle cells, it is clear that under such conditions there is a relative displacement of opposing cell layers within the vascular wall, e.g., smooth muscle cells and elastic lamina vs. endothelial cells. Given the close physical arrangement of endothelial focal-adhesion contacts and the elastic lamina, it is probable that the forces exerted on the abluminal surface of endothelial cells may be even greater than those generated by shear stress on the luminal surface (8). The aim of the present study was to determine whether isometric contraction may initiate a signaling pathway similar to that elicited by fluid shear stress and lead to enhanced endothelial NO production.

MATERIALS AND METHODS

Materials. Genistein was obtained from Calbiochem-Novabiochem, erbstatin A from Biomol (Hamburg, Germany), and Herbimycin A from GIBCO. U46619 was provided by Upjohn, NS 2028 (1H-(1,2,4)oxadiazolo-(4,3- α)-6-bromoquinoxazin-1-one) by NeuroSearch (Glostrup, Denmark), and Ro

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Abbreviations: L-NA, N^G nitro-L-arginine; eNOS, endothelial nitric oxide synthase; Ach, acetylcholine; $\text{PGF}_{2\alpha}$, prostaglandin $\text{F}_{2\alpha}$; PKC, protein kinase C.

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31–8220 by Roche (Gipf-Oberfrick, Switzerland). Unless otherwise indicated, all other chemicals were purchased from Sigma.

Vascular Reactivity Studies. New Zealand White rabbits of either sex (1.5–2.5 kg body weight) were anesthetized with sodium pentobarbital (60 mg/kg i.v.) and exsanguinated by cutting through both the abdominal aorta and vena cava. The descending thoracic aorta was dissected, cleaned of adventitial adipose and connective tissue, and cut into rings (4 mm). Aortic rings were mounted between force transducers (Scaime, Annemasse, France) and a rigid support for measurement of isometric force and incubated in organ baths (10 ml) containing warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs–Henseleit solution (pH 7.4) with the following composition (in mM): NaCl, 118.4; NaHCO₃, 25.0; KCl, 4.7; CaCl₂, 1.6; KH₂PO₄, 1.2; MgSO₄, 1.2; Ca-EDTA, 0.026; glucose, 11.1, and the cyclooxygenase inhibitor diclofenac (1 μM). Passive tension was adjusted over a 30-min equilibration period to 2 g; thereafter, the segments were repeatedly exposed to phenylephrine (10 μM) until stable contractions were obtained. The integrity of the endothelium was assessed in 1 μM phenylephrine-precontracted rings by using acetylcholine (ACh, 1 μM), and vessels exhibiting <70% relaxation were discarded. After a 60-min washout period, aortic rings were contracted to different levels between 5% and 60% of the maximum phenylephrine-induced contraction by PGF_{2α} (0.1–1 μM).

Superfusion Bioassay and the Determination of cGMP Concentration. To assess the production of NO by isometrically contracted rings, a superfusion bioassay was developed in which the Krebs–Henseleit solution (containing 0.1 μM superoxide dismutase) superfusing the aortic rings could be directed onto cultured smooth muscle cells (passage 8–10) pretreated with the phosphodiesterase inhibitor isobutyl methylxanthine (IBMX, 0.1 mM). Briefly, rings were mounted between a force transducer and a rigid support as described above, but instead of being inserted into organ baths, the segments were superfused (1.8 ml/min) with Krebs–Henseleit solution (pH 7.4, 37°C). The orientation of the ring relative to the superfusate was such that no fluid shear stress was generated at the endothelial cell surface. Passive tension was adjusted over a 30-min equilibration period to 2 g; thereafter, the segments were repeatedly exposed to phenylephrine (10 μM) until stable contractions were obtained. Cultured rat aortic vascular smooth muscle cells (24-well plates, supernatant volume 250 μl) were pretreated with IBMX and placed underneath the aortic ring such that the superfusate was directly transferred from the vessel to the cultured cells. Each well was positioned under an aortic ring for 10 sec (300 μl superfusate), and 5 separate wells were assessed at each sample point as described in *Results*. Cells were then incubated with the ring superfusate (total volume 550 μl) for 3 min at 37°C, and the incubation was stopped by the aspiration of the buffer and addition of 300 μl of ice-cold 6% (vol/vol) trichloroacetic acid. Samples were left on ice for 30 min, and cells and supernatants were harvested by scraping and the precipitates centrifuged at 10,000 × *g* (15 min, 4°C). Thereafter, the supernatants were extracted 4 times with 5 vol of water-saturated diethyl ether, and the concentration of cGMP was determined by using a specific radioimmunoassay. cGMP content is expressed as the increase in pmol per mg protein above basal levels (0.72 ± 0.03 pmol of cGMP per mg protein, *n* = 19).

Immunofluorescence Experiments. Aortic segments were fixed under tension with formaldehyde (4% in PBS). After extensive washing, the segments were slit open and permeabilized with Triton X-100 (0.05%), incubated in 100 mM glycine for 10 min, and incubated overnight with a specific phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) followed by a biotinylated secondary antibody, Cy2–streptavidin (BioTrend, Cologne, Germany) for 60 min.

After nuclear staining with 7-amino-actinomycin D (Molecular Probes), the preparations were mounted in Mowiol (Hoechst–Roussel) with the luminal surface upwards between 2 glass coverslips and viewed by using a laser confocal microscope (Leica TCS).

Immunoblotting. Aortic rings mounted in organ baths were rapidly frozen in liquid nitrogen and homogenized with a mortar and pestle. The tissue was recovered and thawed in a buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 25 mM Na₄P₃O₇, 50 mM NaF, 2 mM Na₃VO₄, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, 44 μg/ml phenylmethylsulfonyl fluoride, and 1% (vol/vol) Triton X-100. After centrifugation at 10,000 × *g* for 10 min, the protein concentration in the supernatant was determined, and equal amounts were boiled in SDS/PAGE sample buffer. Proteins in the Triton X-100-insoluble fraction were dissolved by boiling in SDS/PAGE sample buffer. Proteins were separated by 10% or 7% SDS/PAGE as described (9). Tyrosine-phosphorylated proteins were detected by using a specific antibody and were visualized by enhanced chemiluminescence. Prestained marker proteins (Bio-Rad) were used as molecular weight standards for SDS/PAGE.

Statistical Analysis. The concentrations of agonist causing the half-maximal responses were calculated by logit–log regression and are expressed as pD₂ values (–log EC₅₀). Data are expressed as mean ± SEM. Statistical evaluation was performed by using Student's *t* test for unpaired data, one-way ANOVA followed by a Bonferroni adjustment, or repeated-measures ANOVA where appropriate. Values of *P* < 0.05 were considered statistically significant.

RESULTS

Effect of N^G-nitro-L-Arginine (L-NA) on Contractile Responsiveness at Different Levels of Precontraction. Rabbit aortic rings were precontracted to different levels (10–60% of the maximum response) with prostaglandin F_{2α} (PGF_{2α}). Thereafter, L-NA induced a further increase in tone, the amplitude of which depended on the degree of precontraction (Fig. 1A). The maximal effect was observed in rings precontracted with PGF_{2α} to ≈30% of the maximum phenylephrine-induced tone.

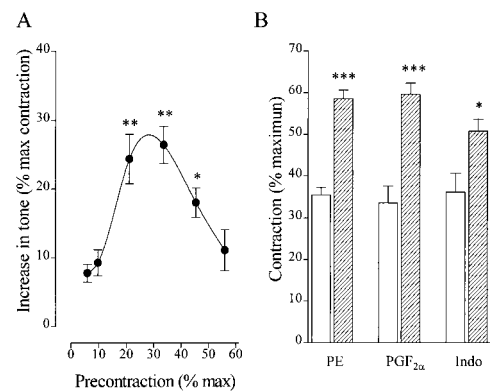


FIG. 1. Influence of the level of precontraction on the vascular response to L-NA. (A) Increase in tone induced by the application of 100 μM L-NA to vessels precontracted under isometric conditions with 0.1–1 μM PGF_{2α} to between 10% and 60% of the maximal 10 μM phenylephrine-induced tone. Data are presented as the mean ± SEM of nine separate experiments. *, *P* < 0.05; **, *P* < 0.01; both indicate a significant L-NA-induced increase in vascular tone. (B) Effect of L-NA (100 μmol/liter, hatched columns) on the tone of endothelium-intact rabbit aortic rings precontracted to ≈35% of the maximum phenylephrine-induced contraction (10 μM) (open bars) with either phenylephrine (PE, 30–100 nM), PGF_{2α} (0.1–1 μM), or indolactam (Indo, 0.1–1 μM). Data are presented as the mean ± SEM of 5–12 separate experiments. *, *P* < 0.05; ***, *P* < 0.001 vs. in the absence of L-NA.

Identical results were obtained when rings were precontracted with phenylephrine or the protein kinase C (PKC) activator, indolactam (Fig. 1B). Oxyhemoglobin (0.1 μ M) also increased tone in vessels precontracted to $\approx 30\%$, but neither oxyhemoglobin nor L-NA had any effect in noncontracted arteries under a passive mechanical stretch of 2 g or in endothelium-denuded preparations (data not shown).

Effect of Inhibition of Soluble Guanylyl Cyclase. NS 2028, a selective soluble guanylyl cyclase inhibitor (10), induced an increase in tone in rings precontracted with $\text{PGF}_{2\alpha}$ to 10–50% of the maximum (Fig. 2A). The relationship between the magnitude of the NS 2028-induced increase in tone and the level of precontraction was similar to that obtained in experiments using L-NA, but the absolute increase in tone observed was markedly smaller. This less pronounced effect of NS 2028 could be attributed to the observation that there is a substantial cGMP-independent component of NO-mediated dilation in rabbit aortic rings. For example, NS 2028 abrogated the 1 μ M sodium nitroprusside (SNP)-induced cGMP increase in aortic rings (cGMP levels were reduced from 0.25 ± 0.01 to 0.15 ± 0.02 pmol/mg protein in phenylephrine-contracted rings and from 2.93 ± 1.54 to 0.35 ± 0.06 pmol/mg protein in SNP-induced rings, $P < 0.001$), whereas it only partially inhibited the NO-mediated relaxant response to ACh (Fig. 2B). The maximal relaxant response to ACh was 86.4 ± 2.95 vs. 51.7 ± 5.19 ($P < 0.01$, $n = 7$) and the pD_2 ($-\log \text{EC}_{50}$) was 7.01 ± 0.44 vs. 6.39 ± 0.97 ($P < 0.05$, $n = 8$) in the absence and presence of NS 2028, respectively.

NO Production in Response to Isometric Contraction. To assess the production of NO by rings subjected to isometric contraction the L-NA-sensitive increase in cGMP was determined in detector smooth muscle cells.

Intracellular cGMP levels in detector cells were assayed at several time points: after the application of ACh to maximally contracted rings; after the return to basal tone after washout; during the initial and plateau phases of the contraction induced by phenylephrine, and following the application of L-NA (Fig. 3, time points A–E, respectively). Under these experimental conditions, the maximal production of NO by the donor segments was detected during the initial phase of isometric contraction. This increase was greater ($P = 0.082$) than that observed following the application of ACh (Fig. 3). Application of L-NA to the donor segments induced a significant increase in tone and decreased intracellular cGMP levels

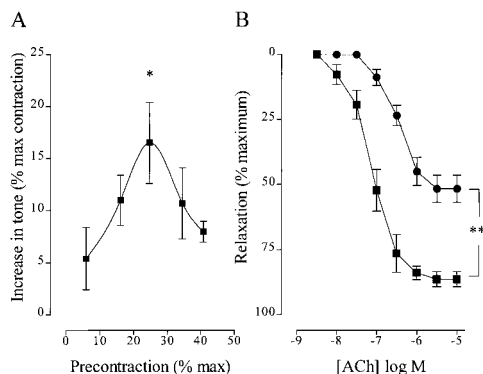


FIG. 2. Influence of the soluble guanylyl cyclase inhibitor, NS 2028, on vascular responsiveness. (A) Increase in tone induced by the application of 1 μ M NS 2028 to vessels precontracted with 0.1–1 μ M $\text{PGF}_{2\alpha}$ to between 10% and 50% of the maximum. Data are presented as the mean \pm SEM of eight separate experiments. *, $P < 0.05$, indicating a significant NS 2028-induced increase in vascular tone. (B) Effect of solvent (■) and 1 μ M NS 2028 (●) on the ACh-induced relaxation of aortic rings maximally contracted with phenylephrine. Data are presented as the mean \pm SEM of eight separate experiments. **, $P < 0.01$, indicating a significant difference between the solvent and NS 2028-treated rings.

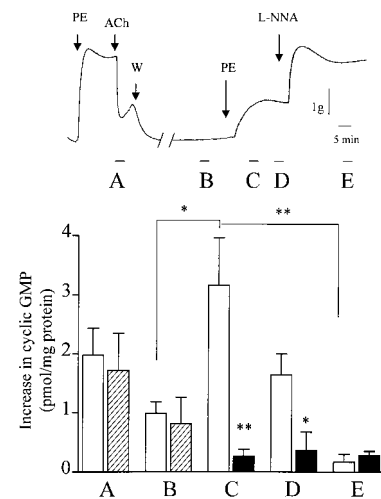


FIG. 3. Determination of NO produced in response to isometric contraction of aortic rings. The intracellular cGMP content was assessed in IBMX-pretreated cultured vascular smooth muscle cells stimulated with the superfusate from aortic rings. cGMP was assessed after the application of 1 μ M ACh to (1 μ M) phenylephrine-contracted rings (A), after the return to basal tone after washout (B), during the initial (C) and plateau (D) phases of the isometric contraction induced by 20 nM phenylephrine resulting in a contraction $\approx 30\%$ of the maximum, and after the application of 100 μ M L-NA (E) as indicated in the upper panel (open bars). In a separate series of experiments, endothelial NO release was determined as described above (hatched bars), but 30 μ M erbstatin A was added after washout of ACh (between time points B and C; filled columns). Results are presented as the increase in cGMP (pmol of cGMP per mg of smooth muscle cell protein) content above basal values, and the data are expressed as the mean \pm SEM of six separate experiments. *, $P < 0.05$.

in detector cells. No increase in detector cell cGMP level was observed when passive stretch in endothelium-intact rings was increased from 2 to 5 g or when endothelium-denuded aortic rings were used as donor (data not shown).

Pharmacological Characterization of the Pathway Resulting in eNOS Activation by Isometric Contraction. The calmodulin antagonist calmidazolium (10 μ M) had no effect on the production of NO induced by isometric contraction as assessed by the lack of effect on the response to L-NA (Fig. 4A), although the concentration used abrogated the vasodilator response to ACh in maximally constricted aortic rings (Fig. 4B).

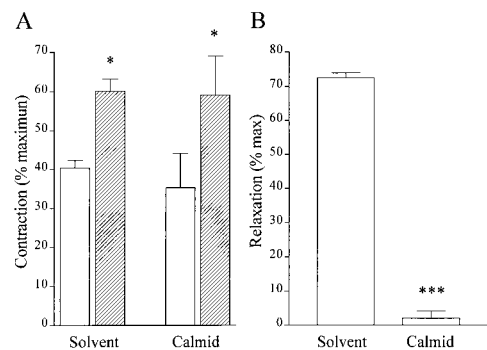


FIG. 4. Effect of calmidazolium on the changes in tone produced by L-NA and by ACh. (A) Effect of 1 μ M calmidazolium on the tone of $\text{PGF}_{2\alpha}$ -contracted rabbit aortic rings before (open bars) and after (hatched bars) the application of 100 mM L-NA. Data are presented as the mean \pm SEM of eight separate experiments. *, $P < 0.05$. (B) Effect of 1 μ M calmidazolium on the relaxant response of 1 μ M phenylephrine-contracted aortic rings to 1 μ M ACh. Data are presented as the mean \pm SEM of eight separate experiments. ***, $P < 0.001$.

Pretreatment of aortic rings with the nonselective PKC inhibitor staurosporine (100 nM) abolished the supplementary contraction elicited by L-NA (Fig. 5A). The more selective PKC inhibitor, Ro 31-8220 (30 nM), which has previously been reported to enhance the production of NO in response to the bolus application of ACh (11), did not affect the L-NA-induced increase in vascular tone in isometrically contracted rings (Fig. 5B).

The effect of tyrosine kinase inhibition on the isometric contraction-induced production of NO was assessed by using the superfusion bioassay. Aortic rings were maximally contracted with phenylephrine and the presence of the endothelium determined by monitoring the response to ACh as described above. After washout (i.e., between time points B and C, Fig. 3), either solvent (0.001% dimethyl sulfoxide) or erbstatin A (30 μ M) was added to the superfusate, and the rings were contracted with phenylephrine to approximately 40% of maximal tone. Under these experimental conditions, erbstatin A abrogated the isometric contraction-induced increase in cGMP in detector cells (Fig. 3). Erbstatin A did not affect basal levels of cGMP or the increase in cGMP elicited by the application of 1 mM SNP to detector cells (data not shown). Erbstatin A only slightly attenuated the ACh-induced production of NO, as previously reported (12).

In organ chamber studies, the tyrosine kinase inhibitors erbstatin A (30 μ M) and herbimycin A (5 μ M) significantly attenuated the NOS inhibitor-induced increase in tone, whereas genistein (100 μ M) had no effect (Fig. 6).

Relationship Between Isometric Contraction and Tyrosine Phosphorylation of Endothelial Proteins *in Situ*. The increase in tyrosine phosphorylation of endothelial proteins in response to isometric contraction was determined by using *en face* confocal microscopy with a specific anti-phosphotyrosine antibody. Under a passive stretch of 2 g, the phosphotyrosine signal in endothelial cells appeared relatively diffuse, with some stronger staining apparent in clusters (Fig. 7A). Phosphotyrosine staining was colocalized with the termini of actin filaments, which were visualized with phalloidin, as well as with vinculin and paxillin, suggesting that these clusters of tyrosine-phosphorylated proteins represent focal adhesion points (data not shown). In aortic rings precontracted with PGF_{2 α} to \approx 15% of the maximum, an increase in protein tyrosine phosphorylation was observed (Fig. 7B). Elevating the level of precontraction to \approx 30% resulted in pronounced changes in phosphotyrosine, an increase in signal intensity, and much larger clusters apparent at the cell-cell boundary (Fig. 7C). A further increase in precontraction (40%) markedly intensified the

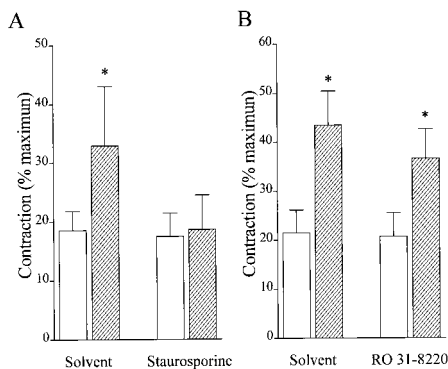


FIG. 5. Effect of the PKC inhibitors staurosporine and Ro 31-8220 on the L-NA-induced increase in tone of rabbit aortic rings. Rabbit aortic rings were precontracted to \approx 20% of the maximal phenylephrine-induced tone with PGF_{2 α} (open bars). Once a stable contraction had been achieved, L-NA was applied (hatched bars). Experiments were performed in the absence (A) and presence (B) of 100 nM staurosporine and 30 nM Ro 31-8220. Data are presented as the mean \pm SEM of four separate experiments. *, $P < 0.05$.

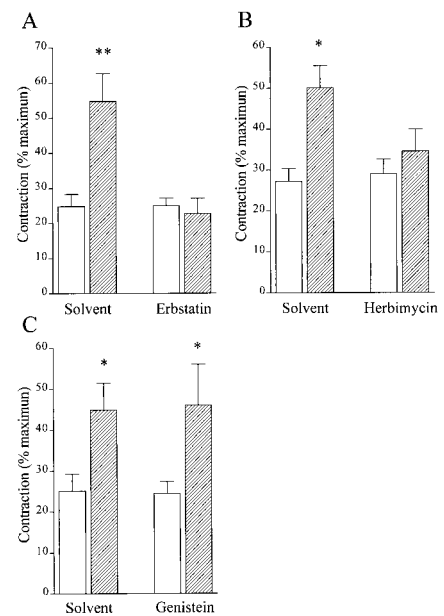


FIG. 6. Effect of tyrosine kinase inhibitors on the L-NA-induced increase in tone of rabbit aortic rings. Rabbit aortic rings were precontracted to \approx 25% of the maximal phenylephrine-induced tone with PGF_{2 α} (open bars). Once a stable contraction had been achieved, 100 μ M L-NA was applied (hatched bars). Experiments were performed in the absence and presence of 30 μ M erbstatin A (A), 5 μ M herbimycin A (B), and 100 μ M genistein (C). Data are presented as the mean \pm SEM of seven separate experiments. *, $P < 0.05$; **, $P < 0.01$.

phosphotyrosine signal such that the cell boundary of each cell was clearly defined (Fig. 7D). Pretreatment of rings with either the tyrosine kinase inhibitor herbimycin A (5 μ M; Fig. 7E) or staurosporine (data not shown) abrogated the stretch-induced increase in cell-boundary tyrosine phosphorylation without affecting the more punctate staining of focal adhesion contacts. Genistein (100 μ M; Fig. 7F), on the other hand, tended to enhance rather than attenuate tyrosine phosphorylation. L-NA did not affect endothelial cell tyrosine phosphorylation in any of the preparations studied (data not shown).

Western blot analysis of aortic rings isometrically contracted with PGF_{2 α} also revealed an increase in the tyrosine phosphorylation of a number of proteins. Precontraction with PGF_{2 α} resulted in an enhanced tyrosine phosphorylation of Triton X-100-soluble proteins recovered from endothelial-intact (Fig. 8A) and endothelium-denuded (Fig. 8B) aortic rings. Whereas an increase in the phosphorylation of an 85-kDa protein was observed independently of the presence of the endothelium, the tyrosine phosphorylation of \approx 79- and 90-kDa proteins in response to isometric contraction was more evident in endothelium-intact preparations. Isometric contraction enhanced the tyrosine phosphorylation of several Triton X-100-insoluble proteins, at least three of which (\approx 80, 110, and $>$ 250 kDa) were pronounced in endothelium-intact preparations (Fig. 8C and D).

DISCUSSION

In this study, we have demonstrated that the application of a NOS inhibitor to endothelium-intact rabbit aortic rings precontracted with PGF_{2 α} elicited a significant additional increase in tension dependent on the level of vascular smooth muscle precontraction. In contrast, the application of L-NA to aortic rings under passive stretch had no effect on vascular tone. The L-NA-induced increase in tone was evident in rings precontracted to between 5% and 10% of the maximum and was most marked in rings precontracted to between 30% and 40%,

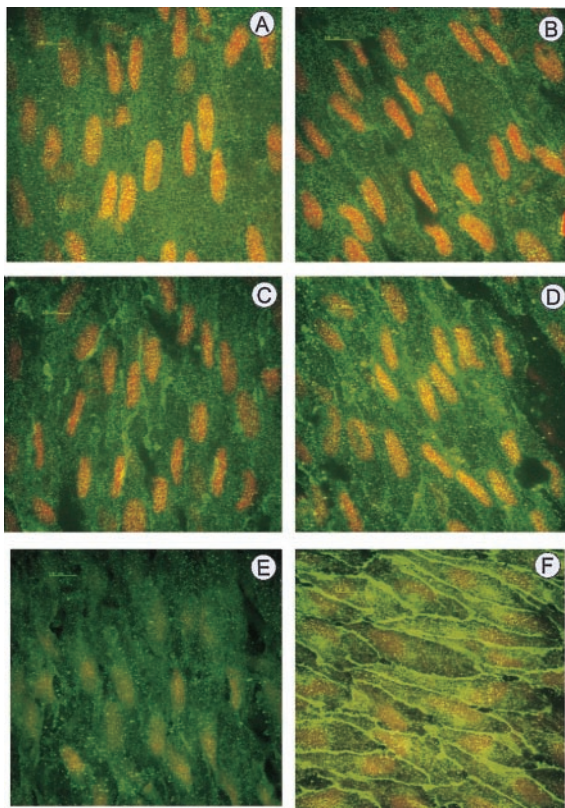


FIG. 7. *En face* confocal microscopy and phosphotyrosine immunolabeling of native rabbit aortic endothelial cells fixed at different levels of vessel precontraction. Rabbit aortic rings under passive tension of 2 g (A) were contracted under isometric conditions with $\text{PGF}_{2\alpha}$ to 15% (B), 30% (C), and 40% (D–F) of the maximum phenylephrine-induced contraction. Experiments were performed in the absence and presence of 5 μM herbimycin A (E) and 100 μM genistein (F). Once a stable contraction was achieved, rings were fixed and prepared as described in *Materials and Methods*. Identical results were obtained in three additional experimental series. (Bar = 10 μm .)

suggesting that endothelial NOS activity is progressively enhanced with increasing vascular tone. Indeed, by using a superfusion bioassay we found that endothelial NO release was increased over basal values during the initial and plateau phases of isometric contraction and was similar to that produced in response to the application of acetylcholine.

The effect of isometric contraction was investigated under shear stress-free conditions in an organ chamber and by using a vessel that synthesizes exclusively NO in response to receptor-dependent agonists (i.e., the component of vasodilation mediated by prostacyclin and the endothelium-derived hyperpolarizing factor is negligible). We found a correlation between the extent of vessel precontraction and the subsequent increase in tone after the application of either an inhibitor of NOS or the soluble guanylyl cyclase. An increase in tone elicited by the application of hemoglobin to rabbit aortic rings precontracted with a variety of agents was initially taken as evidence of the continuous basal formation of NO by the endothelium (13). However, in the latter study the NO scavenger failed to contract passively stretched vessels, suggesting that the production of NO increases with the level of isometric contraction of the vessel studied. Similarly, in this investigation, the application of a NOS inhibitor to passively stretched arterial rings in an organ bath did not induce an increase in tone, although using intracellular levels of cGMP as an index, a basal NOS-inhibitor-sensitive production of NO could be demonstrated. Although hemoglobin has been shown to induce a small increase in tone in passively stretched rat aortic rings (14), we observed that L-NA produced an additional

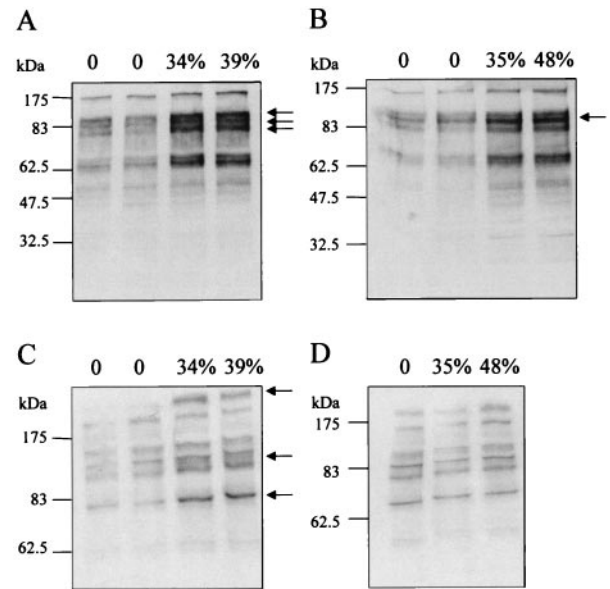


FIG. 8. Western blot analysis comparing the tyrosine phosphorylation of Triton-soluble and Triton-insoluble proteins isolated from aortic rings at different levels of precontraction. Rabbit aortic rings mounted in an organ bath were frozen under either passive tension of 2 g or after contraction with $\text{PGF}_{2\alpha}$ under isometric conditions to 30–50% of the maximum phenylephrine-induced contraction. Triton X-100-soluble (A and B) and Triton X-100-insoluble proteins (C and D) were isolated from endothelium-intact (A and C) and endothelium-denuded (B and D) rings and separated by using SDS/PAGE as described in *Materials and Methods*. Tyrosine-phosphorylated proteins were detected by using a specific antibody. Identical results were obtained in three additional experimental series.

contraction in rat aortic rings and mesenteric segments, the magnitude of which increased progressively with increasing levels of precontraction (J.A., unpublished observation). Taken together, our findings suggest that isometric contraction of an artery *per se* is a stimulus for endothelial NO production that is distinct from, and produced in addition to, the basal formation of NO.

We recently reported that fluid shear stress and receptor-dependent Ca^{2+} -elevating agonists activate eNOS by separate signaling pathways (15, 16). To determine which pathway was activated in endothelial cells by isometric contraction, a number of pharmacological agents were used. The isometric contraction-induced increase in NO release was insensitive to the calmodulin antagonist calmidazolium, although the NO-mediated relaxation of aortic rings to acetylcholine was abrogated. In addition, the L-NA-induced increase in tone was unaffected by the PKC inhibitor Ro 32–8220, which potentiates the relaxant response to receptor-dependent agonists (11). Moreover, the tyrosine kinase inhibitors erbstatin A and herbimycin A prevented the L-NA-induced increase in tone, whereas genistein had no effect. These inhibitors exert differential effects on agonist-induced NO production. Erbstatin A slightly attenuates the relaxation of rabbit aortic rings to high concentrations of ACh (12), whereas genistein almost abolishes Ca^{2+} influx into agonist-stimulated endothelial cells and thus inhibits NO production (9). The marked similarities in the pharmacological profile of eNOS activation elicited by isometric contraction, fluid shear stress, and tyrosine phosphatase inhibitors (11) suggest that all three stimuli activate the same signaling pathway (15).

By using *en face* confocal microscopy we observed that the level of precontraction in aortic rings correlated with the intensity of protein tyrosine phosphorylation in endothelial cells—especially in punctate areas corresponding to points of focal adhesion—and in the area of interendothelial contacts.

Herbimycin and staurosporine reduced, whereas genistein increased, endothelial cell phosphorylation. Because L-NA did not affect the increase in tyrosine phosphorylation in isometrically contracted rings, the activation of tyrosine kinases and/or inactivation of tyrosine phosphatases obviously precedes the activation of eNOS. As shown by using Western blot analysis, the tyrosine phosphorylation of a number of endothelial proteins appeared to be increased in response to isometric contraction. Although eNOS is itself phosphorylated, and phosphorylation on serine and tyrosine residues is increased by shear stress, this phenomenon appears to depend on the presence of extracellular Ca^{2+} (17, 18). A number of alternative candidate cell-cell contact/cytoskeleton-associated proteins exist, including the connexins, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and paxillin, as well as a number of tyrosine phosphatases, kinases, and their substrates. A protein such as PECAM-1 is of special interest in mechanochemical coupling leading to eNOS activation given the colocalization of these two proteins in native endothelial cells (19) and the observation that physical stimuli, but not Ca^{2+} -elevating receptor-dependent agonists, enhance the tyrosine phosphorylation of PECAM-1 (20). The identification of the endothelial proteins tyrosine-phosphorylated during isometric contraction was, however, beyond the scope of this study.

Endothelial NO synthesis may not only be activated by a mechanism related to a shift of tension/stress to focal adhesion points or interendothelial junctions but also may involve the intercellular transport of signaling molecules. Our observation that a modest precontraction elicited endothelial NO production (whereas passive stretch alone did not) may argue in favor of some form of intercellular communication between endothelial cells and the underlying smooth muscle. Indeed, the existence of a bidirectional communication between endothelial and smooth muscle cells has been demonstrated (21), and the application of phenylephrine to isolated, intact arterioles has been reported to induce an increase in endothelial $[\text{Ca}^{2+}]_i$ and activate eNOS (22). Whereas the transfer of Ca^{2+} through such myoendothelial gap junctions may explain the vascular response to L-NA in isolated hamster arterioles, the synthesis of NO elicited by isometric contraction in this study differs from that observed in response to receptor-dependent agonists in that NO synthesis was resistant to both calmidazolium and the tyrosine kinase inhibitor genistein. Moreover, an L-NA-induced increase in tone was observed in aortic rings precontracted with either phenylephrine or the PKC activator indolactam, which does not elicit an increase in endothelial $[\text{Ca}^{2+}]_i$ (22).

In conclusion, we have demonstrated that isometric contraction is a further determinant for the synthesis of NO in arteries. It is tempting to speculate that cell-cell-generated forces, e.g. those elicited by the relative displacement of smooth muscle vs. endothelial cells, play a role in the activation of endothelial cells in much the same manner as the fluid shear stress acting on the luminal surface. Indeed, the intracellular signaling cascade leading to the activation of eNOS (i.e.,

calmidazolium-insensitive, tyrosine kinase inhibitor-sensitive) is pharmacologically identical to that activated by fluid shear stress and tyrosine phosphatase inhibitors but distinct from that elicited by receptor-dependent agonists. Thus our observations imply that in addition to fluid shear stress, the sustained level of neurogenic and/or myogenic tone *in vivo*, which represents a state of isometric contraction, affects endothelial NO production.

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