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Relaxin Activates the L-Arginine–Nitric Oxide Pathway in Vascular Smooth Muscle Cells in Culture

Daniele Bani, Paola Failli, Maria Grazia Bello, Christoph Thiemermann, Tatiana Bani Sacchi, Mario Bigazzi, Emanuela Masini

Abstract—The peptide hormone relaxin (RLX) has been shown to elicit a powerful vasodilatory response in several target organs. This response is mediated by the stimulation of intrinsic nitric oxide (NO) generation. The present study was designed to clarify whether RLX directly promotes the relaxation of vascular smooth muscle cells through stimulation of NO generation. Vascular smooth muscle cells from bovine aortas were incubated with RLX at concentrations ranging from 1 nmol/L to 1 μ mol/L. The expression and activity of NO synthase, production of NO, and the intracellular levels of cGMP and Ca^{2+} were determined. The cell morphology and signal transduction mechanisms of these bovine aortic smooth muscle cells in response to RLX were also studied. RLX stimulated the expression of immunoreactive inducible NO synthase and increased significantly and in a concentration-related fashion inducible NO synthase activity, NO generation, and intracellular cGMP levels. Concurrently, RLX significantly decreased cytosolic Ca^{2+} concentrations and caused changes in cell shape and the actin cytoskeleton that were consistent with cell relaxation. The signal transduction mechanisms leading to the enhanced expression of inducible NO synthase protein and activity caused by RLX involve the activation of tyrosine kinase, phosphatidylcholine–phospholipase C, and the transcription factor nuclear factor- κ B, similar to bacterial endotoxins and proinflammatory cytokines. This study suggests that RLX is an endogenous agent capable of regulating vascular tone by activation of the L-arginine–NO pathway in vascular smooth muscle cells. (*Hypertension*. 1998;31:1240-1247.)

Key Words: muscle, smooth, vascular ■ relaxin ■ nitric oxide

Relaxin is a peptide hormone of ≈ 6 kDa that is predominantly produced by the ovaries and is best known for its actions on the female reproductive system.¹ Recently, evidence has been accumulating that RLX has additional multiple effects on organs other than the reproductive ones. In particular, previous research in our laboratory has shown that RLX exerts a powerful effect on blood vessels, causing vasodilation in the uterus, mammary gland, pigeon crop sac, mesocecum, and coronary system.^{2–8} Our findings fit well with those of other authors that RLX also decreases blood pressure in spontaneously hypertensive rats.^{9,10} All of the above findings support the idea that RLX is effective in reducing vascular tone. Concerning the mechanism of action of RLX on its target organs, our studies of coronary vessels in the isolated, perfused rat and guinea pig heart^{7,8} have shown that the vasodilatory action of RLX is mediated by stimulation of endogenous production of NO, which is a powerful vasorelaxant.^{11,12} It is worth noting that stimulation of intrinsic NO production is also involved in the response to RLX in different cells, such as rat and guinea pig serosal mast cells,¹³ human and rabbit platelets,¹⁴ and mammary adenocarcinoma MCF-7 cells.¹⁵

There is general agreement that the vasodilatory action of NO is primarily an endothelium-dependent process. In fact,

endothelial cells contain the constitutive, Ca^{2+} /calmodulin-dependent isoform of NOS (ie, eNOS) that continuously generates small quantities of NO. In turn, endothelium-derived NO is responsible for the physiological regulation of basal vascular tone, being able to diffuse readily into the SMCs of the vascular wall, where it activates multiple cellular mechanisms that ultimately result in cell relaxation.^{16–18} Other studies have shown that VSMCs are also sites for basal formation of NO due to the presence of the inducible, Ca^{2+} /calmodulin-independent NOS (ie, iNOS) isoform.^{19–24} Indirect evidence for NO production by VSMCs also comes from the results of studies reporting that oxyhemoglobin, a potent inactivator of NO, causes SMC contraction in endothelium-denuded vascular rings^{25–29} and that in vitro incubation of VSMCs together with platelets, which are extremely sensitive to the antiaggregatory action of NO, results in an inhibition of platelet aggregation.³⁰ In VSMCs, iNOS can be upregulated by damage or removal of the endothelium^{31,32} as well as by endotoxins, such as bacterial LPS, and cytokines.^{19,22,33–39}

The intracellular signal transduction mechanisms involved in the expression of iNOS are not completely known. Studies in macrophages, in which the regulation of iNOS expression

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Selected Abbreviations and Acronyms

(BA)SMC	= (bovine aortic) smooth muscle cell
D609	= tricyclodec-9-yl-xanthogenate
I	= inhibitor
i	= inducible
IFN	= interferon
L-NMMA	= <i>N</i> ^G -monomethyl-L-arginine
LPS	= lipopolysaccharide
NF	= nuclear factor
NO(S)	= nitric oxide (synthase)
PC-PLC	= phosphatidylcholine–phospholipase C
PDTC	= pyrrolidine dithiocarbamate
RLX	= relaxin
TPCK	= <i>N</i> -tosylamido-2-phenylethyl chloromethyl ketone
V	= vascular

has been more extensively studied, have shown that induction of iNOS by LPS or cytokines involves the activation of transcription factor NF- κ B,^{40–42} which has been shown to have a binding site on the iNOS gene promoter.⁴² NF- κ B is normally stored in the cytoplasm in an inactivated state by binding with its inhibitor protein I κ B- α . Proinflammatory cytokines promote phosphorylation of I κ B- α and its dissociation from NF- κ B, as well as proteolytic degradation of I κ B- α through a specific I κ B- α protease, thus leading to NF- κ B activation.^{40,43} Of note, NF- κ B activation in response to cytokines has been shown to be under the control of different signal transduction effectors, including tyrosine kinase⁴⁴ and PC-PLC,⁴⁵ both of which are also known to upregulate iNOS expression.^{46,47} Moreover, cytokine-stimulated cells produce reactive oxygen radicals⁴⁰ that are able to activate NF- κ B.^{40,48} Through all of the aforementioned pathways, cells are prompted to express iNOS protein and to generate NO.

The present study was designed to evaluate whether RLX directly promotes the relaxation of SMCs from the vascular wall through activation of the NO pathway, and if so, to verify whether the signal transduction events in response to RLX include the activation of tyrosine kinase or NF- κ B.

Methods**Materials**

Highly purified porcine RLX (2500 to 3000 U/mg), prepared according to Sherwood and O'Byrne,⁴⁹ was the generous gift of Dr Sherwood. Dulbecco's modified essential medium (DMEM) and fetal calf serum (FCS) for cell culture were purchased from Biochrom, and tissue culture plastic ware was from Costar. Antibiotics for cell culture, trypsin solution, HEPES buffer, NADPH⁺, EGTA, EDTA, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, DTT, calmodulin (free base), *Escherichia coli* LPS endotoxin, cycloheximide, D609, PDTC, and TPCK were from Sigma Chemical Co. Tyrphostin AG126 was from Calbiochem Novabiochem. Bovine thrombin was from Boehringer. [³H]L-Arginine and the radioimmunoassay kit for [¹²⁵I]-labeled cGMP were from Amersham. BSA, trichloroacetic acid (TCA), L-NMMA, NaNO₂, and trifluoperazine were from Sigma-Aldrich. Isobutyl methylxanthine (IBMX) was from Aldrich. Stock solutions of IBMX were prepared in 0.1N NaOH and then diluted in Krebs buffer. L-Arginine HCl was from Ultrafine Chemicals Ltd. Fura 2-AM and Pluronic F127 were from Molecular Probes. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was

from Tocris Cookson. Recombinant murine IFN- γ was from PharMingen.

Cell Culture

SMCs from bovine aortas were isolated as described previously.³⁰ The choice of VSMCs of bovine origin was motivated by the fact that these cells retain a distinct muscular phenotype in vitro culture, including the ability to produce NO,³⁰ and that porcine RLX has been found to be active in cattle heifers in vivo.⁵⁰ Smooth muscle tissue strips were explanted from bovine aortas and placed in tissue-culture flasks. The tissue strips were then incubated in DMEM plus 10% FCS until the SMCs, which had spread from the explants, reached confluence. The explants were removed; the SMCs were detached with 0.05% wt/vol trypsin for 20 seconds and then subcultured in DMEM supplemented with 10% FCS (together with 100 IU/mL of penicillin and 100 μ g/mL of streptomycin) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were used between the 4th and 10th passages in culture. The cells were released from the culture plates by treatment with 0.5% trypsin in PBS containing 0.03% EDTA for 5 minutes and allowed to seed for 24 hours before being used for the experiments. At the beginning of the experiments, the medium was replaced with fresh medium alone (controls) or medium with RLX at concentrations ranging from 1 nmol/L to 1 μ mol/L unless specified otherwise. The cells were grown for 24 hours before being processed for functional and morphological assays unless specified otherwise. Cell viability was assayed by the trypan blue exclusion test before and after incubation with either medium alone or medium with 1 μ mol/L RLX. The percentage of viable cells did not differ significantly in the various assays performed (95% before incubation, 97% after 24 hours with medium alone, and 96% after 24 hours of medium with RLX). The absence of endotoxin contamination in the distilled water of the solutions used and the culture medium was evaluated by the *Limulus* amoebocyte lysate assay (E-toxate, Sigma) as described previously.⁵¹

Electron Microscopy

To verify that BASMCs had maintained a distinct SMC phenotype along with successive culture passages, electron microscopy of the cells was performed before and after the experimental period. Cells grown over cellulose discs (Millicell HA, Millipore) placed into the wells of a 24-well plate were fixed in cold 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 3 hours at room temperature and postfixed in 1% OsO₄ in 0.1 mol/L phosphate buffer, pH 7.4, for 1 hour at 4°C. They were then dehydrated in graded acetone, passed through propylene oxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a Siemens Elmiskop 102 electron microscope at 80 kV.

Evaluation of NOS Activity

NOS activity was determined in cell homogenates by measuring the conversion of [³H]L-arginine to [³H]L-citrulline according to Mollace et al⁵² with minor modifications. In brief, 10⁶ BASMCs were seeded into six-well plates, allowed to grow to subconfluence, and then cultured in 2 mL of culture medium in the absence (controls) or presence of RLX at the noted concentrations and times of exposure. In some cultures, the NOS inhibitor L-NMMA (100 μ mol/L) was added to the medium together with RLX (1 μ mol/L). After they were washed, the cells were homogenized with an UltraTurrax Labsonic 1510 homogenizer (Ing Terzano and Co) in HEPES buffer (20 mmol/L, pH 7.2) containing 0.2 mol/L sucrose, 1 mmol/L EDTA, and 1 mmol/L DTT. Part of the cell homogenates was used for determination of total NOS activity. The samples (340 μ L) were added with 60 μ L of a medium of the following composition: 13.2 mmol/L NADPH⁺, 3 mmol/L CaCl₂, 10 μ g/mL calmodulin, 1.3 mol/L L-arginine, and 32 μ Ci/mL [³H]L-arginine. After 60 minutes of incubation at 37°C, the mixture was loaded onto 3-mL Dowex AG 50WX-8 (Na⁺ form, Sigma) columns, eluted with 1 mL of HEPES buffer, and then washed with 5 mL of distilled water. The [³H]L-citrulline obtained by enzyme activity was measured with a

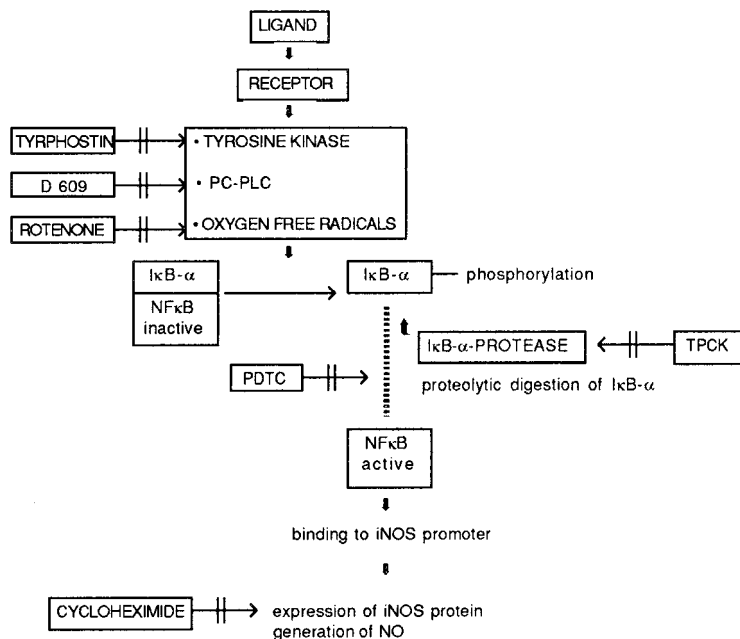


Figure 1. Diagram illustrating the signal transduction pathways involved in iNOS induction and inhibitory drugs that act at different steps (double-slashed arrows).

β -counter (Packard), and the ratio between labeled citrulline and milligrams of protein was taken as NOS activity. The protein concentration was measured spectrophotometrically in the homogenates with the Bradford reagent⁵³ with BSA as the standard. Another portion of the cell homogenates was used to determine the activity of Ca^{2+} /calmodulin-independent iNOS. The homogenates (340 μL) were added to 60 μL of incubation medium as described above but without Ca^{2+} and calmodulin and containing 6.6 mmol/L EGTA and the calmodulin inhibitor trifluoperazine (660 $\mu\text{mol/L}$). The activity of constitutive, Ca^{2+} /calmodulin-dependent NOS (ie, cNOS) was determined from the difference between the values of labeled citrulline produced in the presence of Ca^{2+} and calmodulin and those produced in the absence of Ca^{2+} and calmodulin and in the presence of EGTA and trifluoperazine, as described by Salter et al.⁵⁴

Evaluation of NO Production

This step was performed by measuring the accumulation of nitrite (ie, NO_2^-), a stable end product of NO metabolism, in the supernatant of BASMCs. The amount of nitrite was determined spectrophotometrically by the Griess reaction adapted for a 96-well plate reader.⁵⁵ In brief, 100 μL of sample culture medium was added to 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]ethylenediamine in 5% phosphoric acid). The optical density at a wavelength of 546 nm was measured with a Molecular Devices microplate reader. Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of NaNO_2 dissolved in the culture medium. The values are expressed as nanomoles of nitrite per milligram of protein. The protein concentrations were determined according to Bradford.⁵⁵

A first series of experiments was carried out to evaluate the time-course response of BASMCs to RLX. The cells were seeded into 24-well plates at density of 5×10^4 cells per well, allowed to grow to subconfluence, and then incubated for 4, 24, or 96 hours in 1 mL of medium alone (controls), medium with RLX added (1 $\mu\text{mol/L}$), or medium with RLX (1 $\mu\text{mol/L}$) plus L-NMMA (100 $\mu\text{mol/L}$).

A second series of experiments was carried out to compare the effects of authentic RLX with those of inactivated RLX, obtained according to the method of Büllesbach and Schwabe.⁵⁶ In brief, 1 mg of porcine RLX was dissolved in 0.1 mol/L borate buffer, pH 8.9, with a 10-fold molar excess of 1,2-cyclohexanedione (Sigma), which reacts specifically with arginine residues⁵⁷ and thus modifies the receptor interaction site of the RLX molecule. Excess reagent was removed by dialysis against distilled water. The BASMCs were

seeded into 24-well plates at a density of 5×10^4 cells per well, allowed to grow to subconfluence, and then incubated in 1 mL of medium alone (controls), medium with RLX added at concentrations ranging from 1 nmol/L to 1 $\mu\text{mol/L}$, or medium with inactivated RLX at concentrations ranging from 1 nmol/L to 1 $\mu\text{mol/L}$. Parallel cultures were carried out in medium with L-NMMA (100 $\mu\text{mol/L}$) in the absence or presence of RLX (1 nmol/L and 1 $\mu\text{mol/L}$).

A third series of experiments was carried out to analyze the signal transduction pathway underlying NO production in BASMCs. The cells were seeded into 24-well plates at a density of 5×10^4 cells per well, allowed to grow to subconfluence, and then incubated for 24 hours in 1 mL of culture medium in the absence (controls) or presence of RLX (1 $\mu\text{mol/L}$). For positive controls, parallel experiments were done with LPS (1 $\mu\text{g/mL}$) plus $\text{IFN-}\gamma$ (10 IU/mL) as iNOS inducers. In some of the above cultures, drugs acting at different levels of the signal transduction pathways leading to the induction of iNOS were added to the culture medium together with RLX or LPS plus $\text{IFN-}\gamma$ by following a protocol that had been used previously for similar purposes.⁴⁷ The drugs used were (1) cycloheximide (0.3 $\mu\text{g/mL}$), which prevents the expression of iNOS by interfering with protein synthesis²¹; (2) tyrphostin AG126 (10 $\mu\text{mol/L}$), a tyrosine kinase inhibitor⁵⁸; (3) D609 (30 $\mu\text{g/mL}$), an inhibitor of PC-PLC⁴⁵; (4) rotenone (30 $\mu\text{mol/L}$), an antioxidant drug that prevents the formation of reactive oxygen radicals that are generated by cells after cytokine stimulation and which are able to activate $\text{NF-}\kappa\text{B}$ ⁴⁰; (5) PDTC (25 $\mu\text{mol/L}$), an inhibitor of $\text{NF-}\kappa\text{B}$ activation^{40,41}; and (6) TPCK (30 $\mu\text{mol/L}$), an inhibitor of $\text{I}\kappa\text{B-}\alpha$ protease,⁵⁹ an enzyme that is needed for proteolytic degradation of $\text{I}\kappa\text{B-}\alpha$, which binds to and inactivates $\text{NF-}\kappa\text{B}$.⁶⁰ (A representative diagram of the signal transduction events involved in iNOS induction and the interfering drugs is shown in Figure 1.)

Evaluation of cGMP

cGMP is known to be a mediator of the cell response to NO.¹⁶ To ascertain whether intracellular cGMP undergoes changes in BASMCs after RLX treatment, 5×10^4 cells were seeded into 24-well plates, allowed to grow to subconfluence, and then cultured for 24 hours in 1 mL of culture medium in the absence (controls) or presence of RLX at concentrations ranging from 1 nmol/L to 1 $\mu\text{mol/L}$. In some cultures, L-NMMA (100 $\mu\text{mol/L}$) was added to the culture medium together with RLX (1 $\mu\text{mol/L}$). One hour before the end of the experiments, IBMX (10 $\mu\text{mol/L}$) was added to the cell cultures to inhibit phosphodiesterase activity. On incubation with the

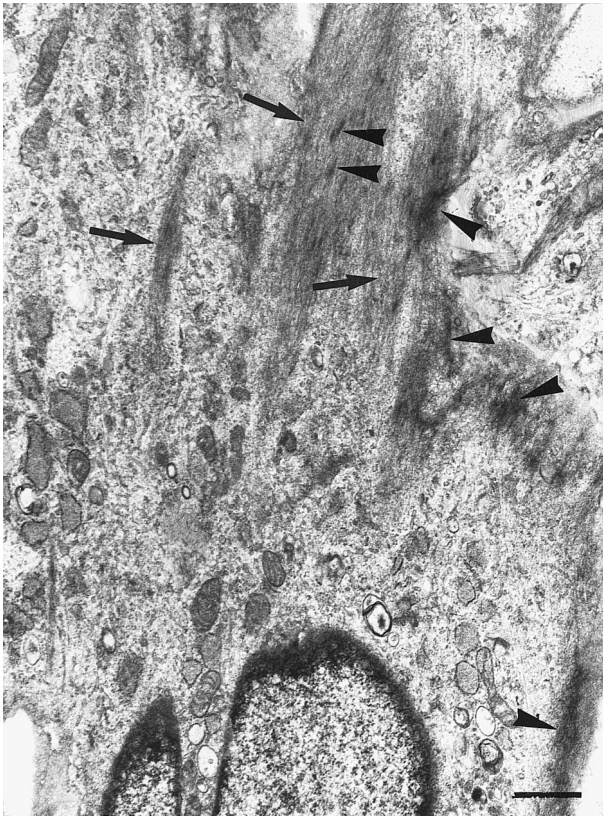


Figure 2. Representative electron photomicrograph of a BASMC showing a distinct muscular phenotype. Cytoplasm contains bundles of myofilaments (arrows). Dense bodies located within bundles and dense plaques at cell boundaries are also shown (arrowheads). Magnification $\times 9000$; bar = 1 μm .

different agents, supernatants were removed and the BASMCs treated with 500 μL of 5% TCA. After extraction of TCA with 0.5 mol/L tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane, the samples were acetylated with acetic anhydride,⁶¹ and the amounts of cGMP in the aqueous phase were measured by radioimmunoassay.⁶² The values are expressed as femtomoles of cGMP per milligram of protein. The protein concentrations were determined according to the method of Bradford.⁵³

Determination of $[\text{Ca}^{2+}]_i$

Because intracellular free Ca^{2+} is known to be involved in the regulation of SMC contraction,⁶³ we aimed at determining whether RLX affects $[\text{Ca}^{2+}]_i$ in BASMC monolayers by using the fluorescent Ca^{2+} indicator fura 2-AM.⁶⁴ In these experiments, $[\text{Ca}^{2+}]_i$ was determined in individual BASMCs by a digital video imaging method, as described previously.⁶⁵ In brief, BASMCs were grown on round glass coverslips to confluence. The cells were then cultured for 24 hours in 2 mL of medium without FCS and in the absence (controls) or presence of RLX at concentrations of 1 nmol/L or 1 $\mu\text{mol/L}$. The cells were incubated with fura 2-AM (10 $\mu\text{mol/L}$) and Pluronic F127 (15%) for 30 minutes at room temperature. $[\text{Ca}^{2+}]_i$ was measured in fura 2-loaded cells in HEPES- NaHCO_3 buffer containing 140 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L NaH_2PO_4 , 12 mmol/L NaHCO_3 , 1.2 mmol/L MgCl_2 , 10 mmol/L HEPES, 1 mmol/L CaCl_2 , and 10 mmol/L glucose, pH 7.4. Fluorescence images were collected with a CCD video camera at two excitation wavelengths (340 and 380 nm) and emission at 510 nm with the Magiscan image analysis system (Applied Imaging). Image ratios (340 nm/380 nm) were obtained every 3 seconds on a pixel-to-pixel basis after subtraction of the background. After recording the basal fluorescence value, thrombin at a final concentration of 3 IU/mL was added directly to the perfusion chamber, and the time course of the

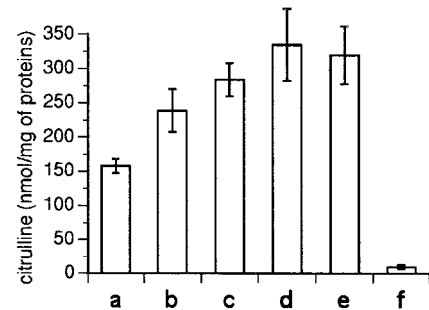


Figure 3. Effects of RLX on Ca^{2+} /calmodulin-independent iNOS activity in BASMC homogenates as evaluated by conversion of $[\text{^3H}]\text{-L-arginine}$ to $[\text{^3H}]\text{-L-citrulline}$. RLX stimulated NOS activity at every concentration assayed. This effect of RLX was abrogated by L-NMMA. a, Untreated cells; b, RLX 1 nmol/L; c, RLX 10 nmol/L; d, RLX 100 nmol/L; e, RLX 1 $\mu\text{mol/L}$; and f, RLX 1 $\mu\text{mol/L}$ + L-NMMA. Data are expressed as mean \pm SEM of 6 wells from 3 independent experiments. Significance of differences (one-way ANOVA): a versus b, NS; a versus c, $P < 0.05$; a versus d and e, $P < 0.01$; and f versus e, $P < 0.001$.

increase in $[\text{Ca}^{2+}]_i$ induced by the agonist was analyzed for at least 6 minutes. Calibration curves were constructed as described.⁶⁵ In some experiments, the NO donor SNAP (100 $\mu\text{mol/L}$) was added to the perfusion chamber and incubated for 5 minutes before the addition of thrombin.

Immunocytochemistry

BASMCs were grown on glass coverslips placed into 24-well plates until subconfluence. The cells were then incubated for 24 hours in 1 mL of culture medium in the absence (controls) or presence of RLX (1 $\mu\text{mol/L}$) or LPS (1 $\mu\text{g/mL}$) plus IFN- γ (10 IU/mL). In some of the above cultures, cycloheximide (0.3 $\mu\text{g/mL}$) was added to the medium. After incubation, some specimens were fixed in 4% formaldehyde in PBS for 10 minutes at room temperature, washed, and then immunolabeled with rabbit polyclonal anti-iNOS antibodies (Calbiochem; working dilution, 1:250). Other specimens were fixed in 50% acetone in ethanol for 10 minutes at 4°C, washed in PBS, and immunolabeled with mouse monoclonal anti-smooth muscle actin antibodies (Sigma; working dilution, 1:500). The immune reaction was revealed by FITC-labeled goat anti-rabbit (Sigma; working dilution, 1:40) or anti-mouse (Sigma; working dilution, 1:30) immunoglobulins. The immunostained sections were mounted in Gel/Mount (Biomed) and then observed and photographed under a Zeiss Axioskop UV-light microscope (Carl Zeiss).

Statistical Analysis

The data are expressed as mean \pm SEM. The distribution of the measured values was assessed to be gaussian. Statistical analysis was performed by either one-way ANOVA followed by the Student-Newman-Keuls multiple-comparison test or two-way ANOVA. Calculations were carried out with the GraphPad Prism 2.0 statistical program (GraphPad Software). A value of $P \geq 0.05$ was considered significant.

Results

Electron microscopic examination of BASMCs showed that these cells have a distinct muscular phenotype. The cells were thicker in the central portion, in which the nucleus and the majority of organelles are located, than at the periphery of the cytoplasm. The organelles consisted mainly of mitochondria and vesicles of smooth endoplasmic reticulum. Glycogen particles were also seen. Bundles of myofilaments were present along the cell periphery beneath the plasma membrane. Myofilaments were intermingled with typical dense bodies and converged toward dense plaques that adhered to

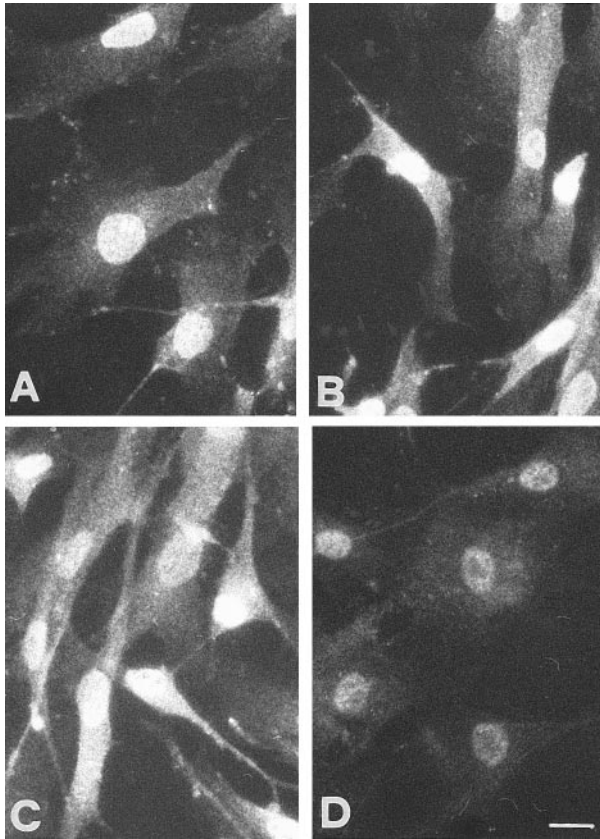


Figure 4. Immunocytochemical expression of iNOS in BASMCs. A, No treatment; B, RLX 1 $\mu\text{mol/L}$; C, LPS 1 $\mu\text{g/mL}$ plus IFN- γ 10 IU/mL; and D, RLX 1 $\mu\text{mol/L}$ plus cycloheximide 0.3 $\mu\text{g/mL}$. In untreated cells, immunoreactive iNOS was mainly located around the nucleus, and the cytoplasm is weakly stained. In both RLX-treated and LPS plus IFN γ -treated cells, iNOS immunoreactivity was intense in the perinuclear area as well as in the remaining cytoplasm. Addition of cycloheximide with RLX strongly reduced iNOS immunoreactivity. Magnification $\times 300$; bar=20 μm .

the inner aspect of the plasma membrane (Figure 2). BASMCs sampled at the beginning and the end of the experimental period showed a similar ultrastructural phenotype. RLX treatment did not cause apparent changes in the electron microscopic features of BASMCs.

The citrulline conversion assay (Figure 3) showed that BASMCs exhibit basal Ca^{2+} /calmodulin-independent NOS activity. With RLX treatment, an increase in Ca^{2+} /calmodulin-independent NOS activity was observed at every RLX concentration assayed. This increase was statistically significant at RLX concentrations of 10 nmol/L ($P < 0.05$) or higher ($P < 0.01$). This effect of RLX was abrogated by the addition of the NOS inhibitor L-NMMA to the cultures ($P < 0.001$). The Ca^{2+} /calmodulin-dependent NOS activity was virtually absent in the BASMCs, as judged by the almost complete correspondence of the values of NOS activities obtained in the presence or absence of Ca^{2+} and calmodulin (data not shown).

Immunocytochemistry also showed that RLX markedly increased the expression of iNOS by BASMCs. A similar effect was obtained by treatment of BASMCs with LPS plus IFN- γ , which are well-known iNOS inducers. These effects were abrogated by cycloheximide (Figure 4).

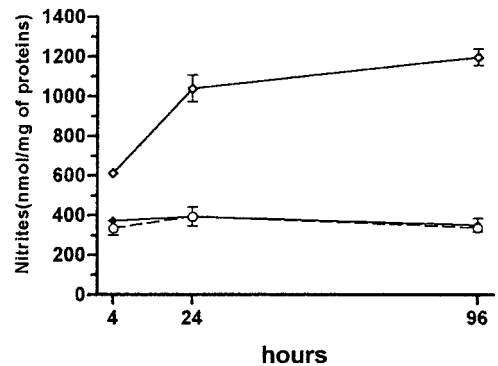


Figure 5. Effects of RLX (1 $\mu\text{mol/L}$) on NO production as evaluated by determination of nitrite in BASMC supernatants after different incubation times. Compared with untreated cells (○), RLX (◇) significantly increased nitrite amounts at every time assayed. This effect of RLX was abrogated by L-NMMA (◆). Data are expressed as mean \pm SEM of 12 wells from 3 independent experiments. Significance of differences (two-way ANOVA): $P < 0.0001$.

In keeping with the above findings, RLX significantly increased the accumulation of nitrite, the stable end product of NO, in BASMC supernatants. Time-course determination of nitrite content (Figure 5) showed that the RLX-induced increase in nitrite was already appreciable after 4 hours of incubation and became even more elevated after 24 and 96 hours of incubation ($P < 0.0001$). Addition of L-NMMA together with RLX abrogated the effect of RLX ($P < 0.0001$). The RLX-induced increase in nitrite in BASMC supernatants, evaluated after a 24-hour incubation (Figure 6), was concentration related ($P < 0.0001$). On the other hand, incubation of BASMCs with inactivated RLX failed to induce any increase in nitrite in the cell supernatants ($P < 0.0001$). Incubation of BASMCs with RLX in the presence of L-NMMA resulted in nearly complete abolition of nitrite formation in the cell supernatants ($P < 0.0001$). The nitrite accumulation induced by RLX was prevented by drugs that interfere with intracel-

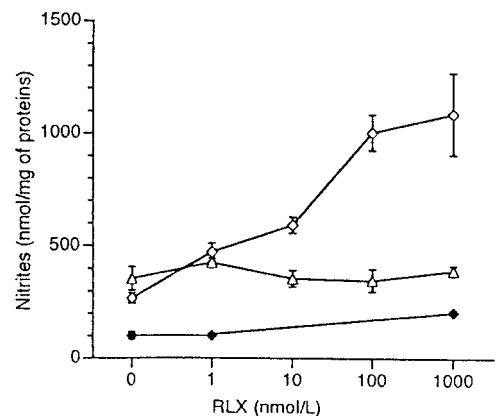


Figure 6. Effects of authentic RLX (◇) and inactivated RLX (△) on NO production as evaluated by determination of nitrite contents in BASMC supernatants after a 24-hour incubation. RLX significantly increased nitrite amounts in a dose-related fashion, whereas inactivated RLX had no effect. L-NMMA (◆) strongly depressed nitrite generation and abolished the effect of RLX. Data are expressed as mean \pm SEM of 12 wells from 3 independent experiments. Significance of differences (two-way ANOVA): $P < 0.0001$.

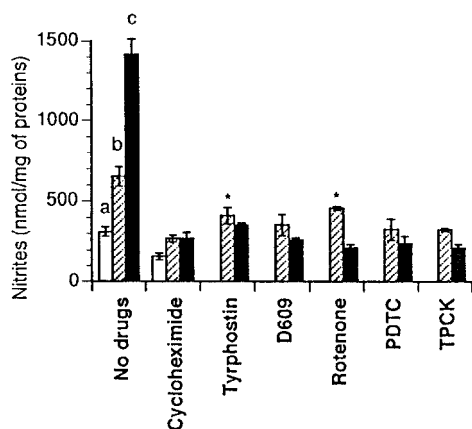


Figure 7. Effects of RLX (1 $\mu\text{mol/L}$) and of LPS (1 $\mu\text{g/mL}$) plus IFN- γ (10 IU/mL), alone or in combination with drugs that interfere with signal transduction, on NO production as evaluated by determination of nitrite contents in BASMC supernatants. Compared with untreated cells (open bars), RLX (hatched bars) as well as LPS plus IFN- γ (filled bars) significantly increased nitrite formation. These effects were abrogated by addition of the different inhibitory drugs assayed. Data are expressed as mean \pm SEM of 12 wells from 3 independent experiments. Significance of differences (one-way ANOVA): b versus a, $P < 0.01$; c versus a, $P < 0.001$; RLX versus RLX+inhibitory drugs, * $P < 0.01$; no asterisk, $P < 0.001$; LPS plus IFN- γ versus LPS plus IFN- γ + inhibitory drugs, $P < 0.001$.

ular signal transduction events (Figure 7), such as the protein synthesis inhibitor cycloheximide ($P < 0.001$), the tyrosine kinase inhibitor tyrphostin AG126 ($P < 0.005$), the PC-PLC inhibitor D609 ($P < 0.005$), or the NF- κB activation inhibitors rotenone ($P < 0.005$), PDTC ($P < 0.001$), and TPCK ($P < 0.001$). The effect of RLX on NO production by BASMCs, as well as the effects of combined administration of RLX and drugs that interfere with signal transduction, were nearly similar to those obtained by using LPS plus IFN- γ in the place of RLX (Figure 7).

Treatment with RLX also caused a concentration-related elevation in intracellular cGMP levels (Figure 8) in a fashion that closely paralleled iNOS activity and NO production. These differences reached statistical significance with an RLX concentration of 10 nmol/L or greater ($P < 0.001$).

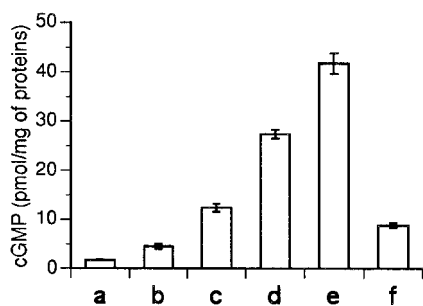


Figure 8. Effects of RLX on intracellular cGMP amounts in BASMCs. RLX causes a concentration-related increase in cGMP levels. This effect of RLX was inhibited by L-NMMA. a, Untreated cells; b, RLX 1 nmol/L; c, RLX 10 nmol/L; d, RLX 100 nmol/L; e, RLX 1 $\mu\text{mol/L}$; and f, RLX 1 $\mu\text{mol/L}$ + L-NMMA. Data are expressed as mean \pm SEM of 12 wells from 3 separate experiments. Significance of differences (one-way ANOVA): a versus b, NS; a versus c, d, and e, $P < 0.001$; and f versus e, $P < 0.001$.

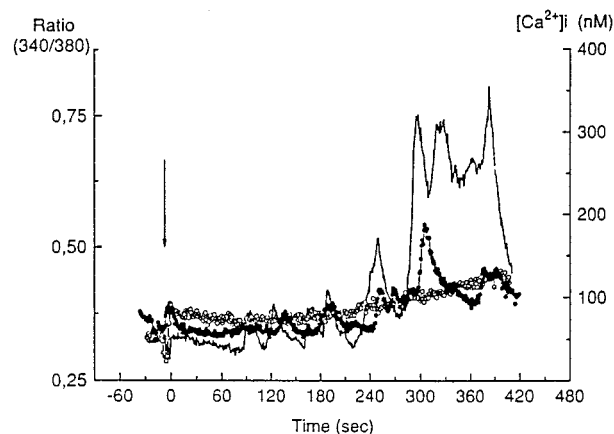


Figure 9. Effects of RLX at different concentrations on the rise in $[\text{Ca}^{2+}]_i$ levels evoked by thrombin in BASMCs as evaluated by digital video imaging. Traces are the mean of at least 20 single responses measured in individual fura 2-loaded BASMCs. Untreated cells, continuous line; 1 nmol/L RLX, filled circles; and 1 $\mu\text{mol/L}$ RLX, open circles. Thrombin (3 U/mL) was administered at the time indicated by the arrow.

Addition of L-NMMA to the culture medium together with RLX resulted in significant inhibition of the RLX-induced rise in cGMP ($P < 0.001$).

Pretreatment of BASMCs with RLX significantly inhibited the rise in $[\text{Ca}^{2+}]_i$ induced by thrombin (Figure 9). In fact, stimulation with thrombin of BASMCs not treated with RLX caused a marked elevation of $[\text{Ca}^{2+}]_i$ that started 250 to 300 seconds after the addition of thrombin. Treatment of the cells with RLX before stimulation with thrombin resulted in a marked reduction of the $[\text{Ca}^{2+}]_i$ rise at an RLX concentration of 1 nmol/L and a complete inhibition of the $[\text{Ca}^{2+}]_i$ rise at a concentration of 1 $\mu\text{mol/L}$, as well as increased latency between the application of thrombin and the $[\text{Ca}^{2+}]_i$ response. Similar inhibition of the $[\text{Ca}^{2+}]_i$ increase was obtained by adding the NO donor SNAP (100 $\mu\text{mol/L}$) just before thrombin (data not shown).

Immunocytochemical staining of BASMCs with anti-actin antibodies showed that the cell shape and distribution pattern

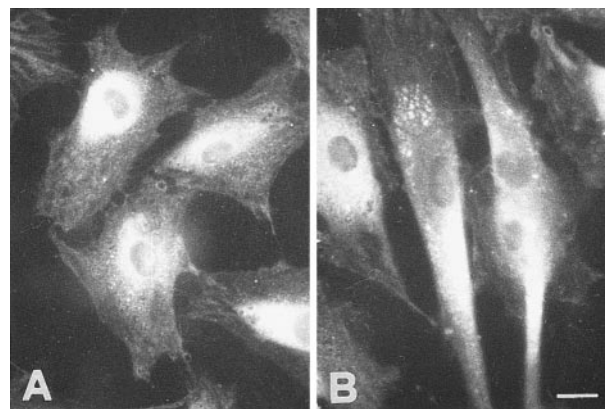


Figure 10. A, Untreated cells are polyhedral and show immunofluorescent actin concentrated mainly around the nucleus. B, Cells treated with RLX (1 $\mu\text{mol/L}$) are mostly fusiform, with long cytoplasmic processes and immunofluorescent actin almost homogeneously distributed throughout the cytoplasm. Magnification $\times 300$; bar = 20 μm .

of the actin cytoskeleton underwent distinct changes after RLX treatment (Figure 10). The BASMCs not treated with RLX were polyhedral, with rare, short cytoplasmic processes. Actin immunoreactivity was concentrated in the central part of the cells, thus masking the nucleus, and in "stress fibers" reaching the cell periphery. Conversely, the RLX-treated cells were mostly elongated, with a spindle or stellate shape and long cytoplasmic processes. Actin immunoreactivity was almost homogeneously distributed throughout the cytoplasm, and the nucleus was easily visible.

Discussion

The present study shows that RLX directly activates the L-arginine-NO pathway in arterial SMCs in culture. Moreover, RLX induces changes in cell shape and the actin cytoskeleton that are consistent with cell relaxation. This finding agrees with our previous reports that RLX is a powerful vasodilatory agent favoring the perfusion of target organs²⁻⁸ and with the findings of other authors that RLX decreases blood pressure in hypertensive rats.^{9,10} The present study also shows that RLX increases intracellular cGMP levels in a concentration-related fashion. This effect of RLX is likely a consequence of the stimulation of NO production by this hormone. In fact, NO binds to the heme iron of soluble guanylate cyclase and thereby activates the synthesis of cGMP.^{16,17,25} In turn, increased production of cGMP plays an important role in vasorelaxation, since it is accompanied by a decrease in $[Ca^{2+}]_i$ in VSMCs, which ultimately results in cell relaxation.¹⁶⁻¹⁸ In BASMCs, RLX inhibits the agonist-activated increase in cytosolic Ca^{2+} and induces changes in cell shape and the actin cytoskeleton that are consistent with cell relaxation. Of note, a similar effect of RLX on cell shape has also been observed in uterine SMCs.⁶⁶ The response of BASMCs to RLX seems to be specific, because substitution of authentic RLX with inactivated RLX failed to result in a cell response in terms of increased NO production.

The present study also shows that the signal transduction mechanisms leading to the induction of iNOS and the enhanced formation of NO in BASMCs stimulated by RLX involve the activation of the transcription factor NF- κ B, likely mediated through tyrosine kinase, PC-PLC, and oxygen free radicals. These mechanisms are similar to those activated in the same cells by well-known iNOS inducers, such as LPS and IFN- γ , and appear to be quite similar to those operative in macrophages on activation by endotoxins and cytokines.^{40-42,45-47}

The concentrations of RLX found by us to be effective in stimulating the response of BASMCs are higher than those measured in bovine plasma.⁶⁷ Nevertheless, the possibility that RLX actually plays a role in the regulation of vascular smooth muscle tone and organ blood perfusion under physiological conditions should not be ruled out. In fact, it should be borne in mind that (1) measurements of plasma levels of RLX performed in the past by radioimmunoassay with heterologous antibodies may have underestimated the actual physiological levels of this hormone and (2) SMCs, after explantation from the arterial wall and adaptation to in vitro culture conditions, may reduce their responsiveness to vasoactive agents, possibly owing to partial dedifferentiation or

reduction of receptors. This view is also strengthened by the results of our experiments on $[Ca^{2+}]_i$ elevations after stimulation of BASMCs with thrombin, a well-known vasoactive agent, which show that very high, supraphysiological concentrations of this agonist are needed to evoke a distinct response by these cells.

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