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Relaxin potentiates the expression of inducible nitric oxide synthase by endothelial cells from human umbilical vein in *in vitro* culture

S.Quattrone[1], L.Chiappini[1], G.Scapagnini[2], B.Bigazzi[1] and D.Bani[1,3]

[1]Department Anatomy, Histology & Forensic Medicine, Section of Histology, University of Florence, Florence and [2]Department Experimental & Clinical Pharmacology, University of Catania, Catania, Italy

[3]To whom correspondence should be addressed at: Dipartimento di Anatomia, Istologia e Medicina Legale. Sezione di Istologia, Università di Firenze, V.le G.Pieraccini, 6, I-50139 Florence, Italy. E-mail: daniele.bani@unifi.it

The hormone relaxin (RLX), which can be detected in human venous cord blood, has been shown to be a potent vasodilator, acting through increased expression of inducible nitric oxide synthase (NOS II) and nitric oxide (NO) generation. This study aims at clarifying whether RLX, at concentrations of 100 and 1000 ng/ml for 6 or 12 h of exposure, can influence the expression of NOS isoforms in human umbilical vein endothelial cells (HUVEC) cultured *in vitro*. NOS mRNA expression was studied by quantitative real-time RT–PCR, NOS protein expression and activity was studied by Western blot and nitrite assay, and immunoreactive NOS localization was performed by confocal microscopy. Untreated HUVEC expressed all the NOS isoforms, especially the constitutive, endothelial-type NOS III and, to a lesser extent, NOS II and NOS I. RLX-treated cells showed an increased expression of NOS II, attaining a maximum with 1000 ng/ml RLX, which gave rise to increased NO generation, as shown by nitrite assay. This effect of RLX appears to be mediated by activation of NOS II transcription factor NF-kappaB, since it was abolished by the NF-kappaB inhibitors curcumin-95 and dexamethasone. These findings suggest that RLX in the umbilical vein might contribute to the NO-dependent regulation of vascular tone.

Key words: human umbilical vein endothelial cells/relaxin/nitric oxide synthase I/NOS II/NOS III

Introduction

In recent years, evidence has been provided that the peptide hormone relaxin (RLX), a member of the insulin/insulin growth factor (IGF) superfamily best known for its effect on the female reproductive system, is a potent vasodilator (Bani, 1997). RLX-induced vasodilatation has been repeatedly observed in experimental animals of both sexes and in several organs, including the uterus, mesocaecum, mammary gland, pigeon crop sac, heart, kidney and liver (Bani *et al*., 1988, 1995, 1998b, 2001; Bigazzi *et al*., 1986, 1988; Lee *et al*., 1992; Bani Sacchi *et al*., 1995; Masini *et al*., 1997; Danielson *et al*., 1999). From these studies, vasodilatation emerges as a specific, physiological effect of RLX, for it is exerted at concentrations that are in the range of those measured in blood of pregnant animals (O'Byrne and Steinetz, 1976). Furthermore, specific RLX-binding sites have been identified in the blood vessels of target organs for RLX (Kohsaka *et al*., 1998).

The mechanism of action of RLX on blood vessels appears to involve the stimulation of the biosynthesis of nitric oxide (NO), a gaseous free radical and potent endogenous vasodilator (Palmer *et al*., 1987), by cells of the vascular wall (Bani Sacchi *et al*., 1995; Masini *et al*., 1997). In particular, our previous studies on coronary endothelial cells and vascular smooth muscle cells in *in vitro* culture have demonstrated that RLX increases the expression of the inducible, high-yield NO synthase isoform (NOS II), thereby promoting intrinsic NO generation (Bani *et al*., 1998a; Failli *et al*., 2001). Under physiological conditions, the vasodilatatory action of NO is thought to be primarily an endothelium-dependent process. Endothelial cells constitutively express a Ca^{2+}/calmodulin-dependent isoform of NOS (NOS III) that continuously produces small amounts of NO, involved in a moment-to-moment regulation of the vascular tone (Moncada *et al*., 1991). Endothelial cells also express NOS II, which synthesizes greater amounts of NO than NOS III and can be up-regulated by different stimuli, especially inflammatory cytokines and mediators (Kaku *et al*., 1997).

Of note, RLX has been reported to cross the placental barrier from the mother to the fetus in rhesus monkeys (Cossum *et al*., 1991) and has been detected in the blood of human umbilical vein at delivery, although at very low concentrations as compared with maternal blood (Petersen *et al*., 1995). It is known that a reduced blood flow in the umbilical vein is a major cause of fetal growth restriction (Rigano *et al*., 2001) and is assumed to be a negative predictor for perinatal outcome (Baschat *et al*., 2000). On these grounds, it is possible that RLX in the umbilical vein may contribute to regulation of the vascular tone to sustain fetal perfusion. The present study was designed to clarify whether relaxin can influence the pattern of expression of the different NOS isoforms in human umbilical vein endothelial cells (HUVEC).

Materials and methods

Cell culture

Human umbilical cords from normal pregnancies at term were kindly provided by the Obstetric Unit, Careggi General Hospital, Florence. HUVEC were isolated by perfusion/incubation with 0.2% w/v collagenase I (Sigma, USA) using a method currently employed in our laboratory (Cozzolino *et al*., 1990).

Once isolated, the cells were seeded in gelatin-coated flasks and grown in MCDB 131 culture medium supplemented with 20% fetal bovine serum, 10 mmol/l L-glutamine, 50 µg/ml heparin (Sigma), and 50 µg/ml endothelial cell growth factor (ECGF) (Roche, Italy). Unless otherwise specified, reagents for cell culture were from Gibco BRL (UK). Cell culture plastic ware was from Falcon (Becton Dickinson Europe, France). HUVEC at the 1st–3rd culture passage were grown to subconfluence and then incubated for 6 and 12 h in the absence (controls) and presence of highly purified porcine RLX (2500–3000 IU/mg, generously given by Dr O.D.Sherwood) at the final concentrations of 100 and 1000 ng/ml. In preliminary experiments, lower RLX concentrations (10, 30 and 60 ng/ml) were also assayed but they were found ineffective (data not shown). To clarify whether NF-kappa B could be involved in the biological effects of RLX on HUVEC, as previously described in vascular smooth muscle and endothelial cells (Bani *et al*., 1998a; Failli *et al*., 2001), in selected experiments, 30 µmol/l curcumin-95 (Advanced Orthomolecular Research, Canada) was added to the HUVEC together with RLX. Curcumin is a plant-derived polyphenol and potent antiinflammatory agent that specifically inhibits the nuclear importation of transcription factor NF-kappa B (Singh and Aggarwal, 1995), which is known to play a key role in NOS II expression (Xie *et al*., 1994). In other experiments, 8 µmol/l dexamethasone (Sigma), a widely used, potent NF-kappa B inhibitor (Yamamoto and Gaynor, 2001), was substituted for curcumin-95. At least three independent experiments, each in triplicate, were carried out.

Evaluation of NOS mRNA expression by RT–PCR

The expression of mRNA for the different NOS isoforms, i.e. the constitutive, Ca^{2+}/calmodulin-dependent NOS I and NOS III, and the inducible, Ca^{2+}/calmodulin-independent NOS II, was evaluated by RT–PCR. Briefly, HUVEC plated in 6-well multiplates and treated or not with RLX at the noted concentrations and exposure times underwent extraction of total RNA using Trizol reagent (Gibco BRL). Reverse transcription was performed with 5 µg of total RNA, 200 IU of Superscript RNase H^- reverse transcriptase (Gibco BRL), and 2 µmol/l of random hexamers (Promega, USA) and running the reaction at 37°C for 1 h and then at 94° C for 5 min in a final volume of 50 µl. The resulting cDNA samples were PCR-amplified using 2 IU of *Taq* DNA polymerase (Pharmacia Biotech, Italy) and 0.4 µmol/l each of sense and antisense primers, which were designed over the 3′untranslated region (UTR) of the gene to emphasize differences among the NOS isoforms. Four microlitres of cDNA were used for amplification of NOS I, II and III. To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for human β-actin as housekeeping gene were used in separate PCR reactions. The following amplification cycles were used: for NOS I: initial denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s for a total of 40 cycles; for NOS II: initial denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s for a total of 30 cycles; for NOS III: initial denaturation at 94°C for 30 s, primer annealing at 59°C for 30 s, and extension at 72°C for 30 s for a total of 30 cycles. The following sets of primers were used: NOS I (GenBank accession number: NM_000620) sense 5′-CCCTCCTGGGTTTACTCCT-3′, antisense 5′-GCCTAGTTCCTGCAGCCTTT-3′; NOS II (GenBank accession number: NM_000625) sense 5′-CCCCATCAAGCCCTTTACTT-3′, antisense 5′-CACCTCCTGGTGGTCACTT-3′; NOS III (GenBank accession number: NM_000603) sense 5′-CCTCCAGGAAGGAGCAAAAC-3′, antisense 5′- TCCTGAGAGAGAGGCAAGAGGA-3′; β-actin (GenBank accession number: NM_001101) sense 5′-AACTGGAACGGTGAAGGTG-3′, antisense 5′-CTGTGTGGACTTGGGAGAGG-3′.

Quantification of NOS mRNA by real-time PCR

This was carried out using the Light Cycler instrument (Roche Molecular Biochemicals) and the same primers and cDNA described above. Aliquots of the above obtained cDNA (0.1 µg) and known amounts of external standard (purified PCR product diluted in RT mix, 10^2–10^8 molecules) were amplified in parallel reactions (final volume 20 µl). Each PCR reaction contained 0.5 µmol/l of primers, 2.5 mmol/l Mg^{2+} and 1×FastStart SYBR Green master mix (Roche Molecular Biochemicals). PCR amplifications were performed using the following four cycle program: (i) denaturation of cDNA (1 cycle: 95° C for 10 min); (ii) amplification (40 cycles: 95°C for 0 s, 58°C or 59°C for 5 s, 72°C for 10 s); (iii) melting curve analysis (1 cycle: 95°C for 0 s, 70° C for 10 s, 95°C for 0 s), and (iv) cooling (1 cycle: 40°C for 3 min). Temperature transition rate was 20°C/s except for the third segment of the melting curve analysis where it was 0.2°C/s. The fluorimetric gain value was 6. Real-time detection of fluorimetric intensity of SYBR Green I, indicating the amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. Only fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining, and DNA sequencing. For each single determination the PCR were run in triplicate and gave an average variability of <10%.

Western blot analysis for NOS proteins

HUVEC seeded into 75 cm^2 flasks were grown for 12 h in the absence or the presence of RLX (1000 ng/ml), alone or with added curcumin-95 (30 µmol/l) or dexamethasone (8 µmol/l), two inhibitors of the NOS II transcription factor NF-kappa B (Singh and Aggarwal, 1995; Yamamoto and Gaynor, 2001). Cells were then detached in trypsin/EDTA, washed thoroughly and placed in cold lysis buffer of the following composition: 20 mmol/l Tris–HCl pH 7.4, 10 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1.3 mmol/l Na_2EDTA, 1 mmol/l dithiothreitol (DTT), 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 0.25% Triton X-100, 20 µg/ml Leupeptin, 1 µg/ml Pepstatin, 1 mg/ml Pefabloc SC, 2.5 µg/ml Aprotinin. Upon centrifugation at 15 000 *g* at 4° C, the supernatants were collected and the total protein content was measured spectrophotometrically using the bicinchonic acid method. The samples, each containing 70 µg of total proteins, were electrophoresed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (200 V, 1 h) using a denaturating 7.6% polyacrylamide gel with molecular weight markers (Bio-Rad, USA) and blotted (150 V, 1 h) onto nitrocellulose membranes (Amersham). After thorough washings in phosphate-buffered saline (PBS) with 0.1% Tween 20 (T-PBS) added, the membranes were treated with albumin 5% in T-PBS to block aspecific binding sites and incubated overnight at 4°C with stirring with rabbit polyclonal antibodies against NOS I (Calbiochem, USA), NOS II (Alexis, Switzerland) or NOS III (Alexis), diluted 1:50 000 in T-PBS with 1% bovine serum albumin (BSA) added. Immune reaction was revealed by peroxidase-labelled goat anti-rabbit antibodies (Vector, USA), diluted 1:10 000 in T-PBS with 1% BSA and applied to the membranes for 1 h at room temperature under stirring, followed by 1 min incubation with the chemiluminescent substrate ECL (Amersham) and exposure to high-sensitivity photographic film (Biomax ML; Kodak, USA).

Evaluation of NO production

This step was performed by measuring the accumulation of nitrites, a stable end product of NO metabolism, in the supernatant of HUVEC. Cells were seeded into 24-well plates at a density of 5×10^4 cells/well, allowed to grow to subconfluence and then incubated for 12 h in medium alone (controls) or medium added with RLX at the noted concentrations. The amount of nitrites was determined spectrophotometrically by the Griess reaction adapted for a 96-well plate reader (Bani *et al*., 1998a), by conversion of nitrates to nitrites by a 30 min incubation at 37°C with 100 mIU/ml nitrate reductase (Sigma) and 20 µg/ml NADPH (Sigma). In brief, 100 µl of sample was added to 100 µl of Griess reagent (1% sulphanilamide and 0.1% *N*-[1-naphtyl]ethylendiamine in 5% phosphoric acid). The optical density at a wavelength of 546 nm was measured with a Bio Rad 550 micro plate reader. Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of $NaNO_2$ dissolved in culture medium and expressed as nmol/mg of proteins, the latter measured spectrophotometrically by the bicinchonic acid method. The reported values are the mean (± SEM) of three separate experiments, each performed in duplicate.

Detection of immunoreactive NOS

HUVEC were grown on glass coverslips placed into 24-well plates until subconfluence. The cells were then incubated for 12 h in the absence (controls) or presence of RLX (1000 ng/ml). After incubation, the specimens were fixed in cold 4% formaldehyde in phosphate-buffered saline for 30 min at room temperature, washed and then incubated with rabbit polyclonal antibodies against NOS I (Calbiochem; diluted 1:200 in PBS), NOS II (Alexis; diluted

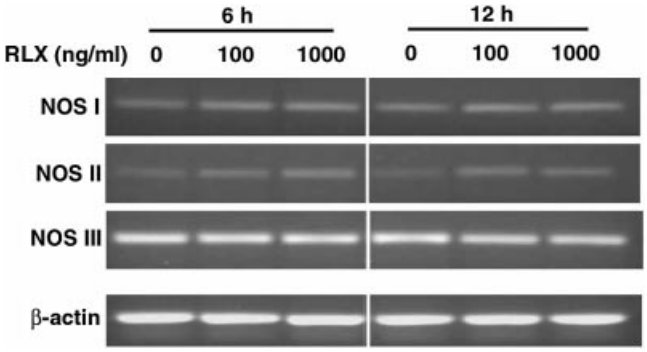

Figure 1. Expression of nitric oxide synthase (NOS) transcripts in human umbilical vein endothelial cells (HUVEC) evaluated by qualitative RT–PCR. The amplified fragments for NOS I, NOS II, NOS III and the control gene β-actin were run on 2% agarose gel.

1:400 in PBS) or NOS III (Alexis; diluted 1:200 in PBS). To evaluate the specificity of the staining for the different NOS isoforms, preadsorption tests were carried out with the specific epitopes of the reported antisera, using rat recombinant neuronal NOS I, NOS II blocking peptide (1131–1144) and NOS III blocking peptide (599–613) (Calbiochem), as described previously (Vannucchi *et al*., 2002). The immune reaction was revealed by Alexa 488 nm-labelled goat anti-rabbit antibodies (Molecular Probes; diluted 1:200 in PBS). To achieve a precise intracellular localization of NOS isoforms, the immunostained sections were examined on a Bio-Rad 1024 ES confocal laser scanning microscope (Bio-Rad, UK) with laser beam excitation at 488 nm wavelength. In selected experiments, prior to fixation, HUVEC were incubated for 30 min at room temperature with the supravital dye MitoTracker Red (Molecular Probes, USA; 100 nmol/l) to identify mitochondria (Macho *et al*., 1996).

Calculations and statistical analysis

For each time-point (e.g. 6 and 12 h), statistical comparison of differences between HUVEC undergoing the different treatments was carried out using one-way analysis of variance followed by Student–Newman–Keuls multiple comparison test. $P \leqslant 0.05$ was considered significant. Calculations were done using a GraphPad Prism 2.0 statistical program (GraphPad Software, USA).

Results

In untreated HUVEC, RT–PCR allowed us to detect the expression of mRNA for all the different NOS isoforms (Figure 1). The presence of NOS I mRNA in HUVEC is an unprecedented finding. RLX, at both concentrations and exposure time assayed, appeared to increase NOS II mRNA expression. Quantitative analysis of NOS isoform transcripts by real-time PCR showed that RLX treatment resulted in a dose-dependent increase in NOS II mRNA (Figure 2). Analysis of the standard curves for the tested NOS showed a fair linearity, whose parameters were consistent with an efficient and sensitive PCR reaction (Figure 3). The effect of RLX on NOS II mRNA was greater after 6 h than 12 h of incubation, thus indicating a high rate of NOS II expression in response to RLX. In particular, a 6 h exposure of HUVEC to 1000 ng/ml RLX resulted in a 15-fold increase in the amount of NOS II mRNA compared to the untreated cells. On the other hand, RLX treatment did not cause appreciable changes in the expression of NOS I or NOS III mRNA, regardless of the dose and the exposure time to the hormone. Co-addition of curcumin-95 (30 μmol/l) together with RLX (1000 ng/ml) to HUVEC for 6 h caused a complete inhibition of NOS II mRNA up-regulation (Figure 2).

Western blot analysis allowed us to confirm that mRNA expression actually resulted in NOS protein synthesis (Figure 4). Indeed, untreated HUVEC showed a clear-cut expression of NOS III and a weak expression of NOS I, whereas NOS II was barely detectable or

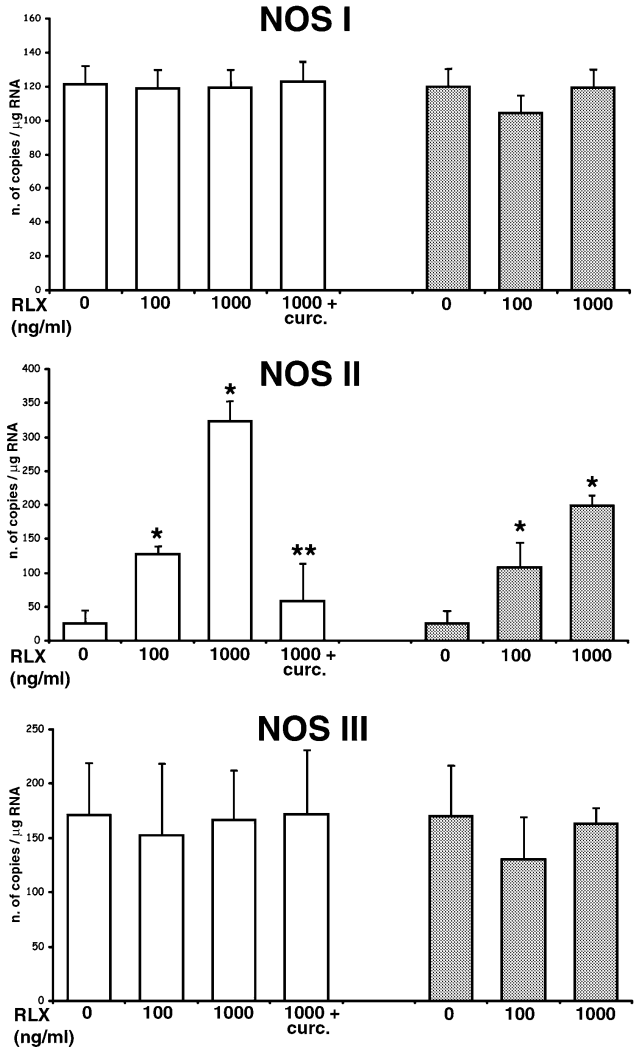

Figure 2. Quantitative evaluation of nitric oxide synthase (NOS) isoform transcripts in human umbilical vein endothelial cells (HUVEC) by real-time PCR after 6 h (open columns) and 12 h (gray columns) of incubation in the absence and presence of relaxin (RLX) at the noted concentrations. curc. = curcumin-95 (30 μmol/l). Significance of differences (one-way ANOVA, n = 3): *$P < 0.001$ versus untreated cells; **$P < 0.001$ versus RLX = relaxin at 1000 ng/ml.

undetectable. After a 12 h incubation with RLX (1000 ng/ml), NOS II protein underwent a marked increase, whereas NOS I and NOS III were substantially unchanged. Co-addition of curcumin-95 (30 μmol/l) or dexamethasone (8 μmol/l) abolished the RLX-induced rise of NOS II expression. This NOS II protein was functional for it gave rise to increased generation of NO, as judged by the increased amount of nitrites, the end products of NO metabolism, in HUVEC supernatants after a 12 h incubation with 100 and 1000 ng/ml RLX (Figure 5).

Immunocytochemical localization of NOS proteins by means of confocal laser scanning microscopy revealed that the different NOS isoforms have distinct localization patterns within HUVEC (Figure 6). In particular, NOS I appeared to be mainly located in the cytoplasmic matrix (Figure 6A), with no apparent relationship with plasma membrane. In HUVEC preparations stained simultaneously with MitoTracker Red, there was no co-localization of the two fluorescent labels, thus indicating that NOS I was not associated with mitochondria (Figure 6B). NOS II also showed a diffuse intracytoplasmic location (Figure 6C and D) which did not co-localize with

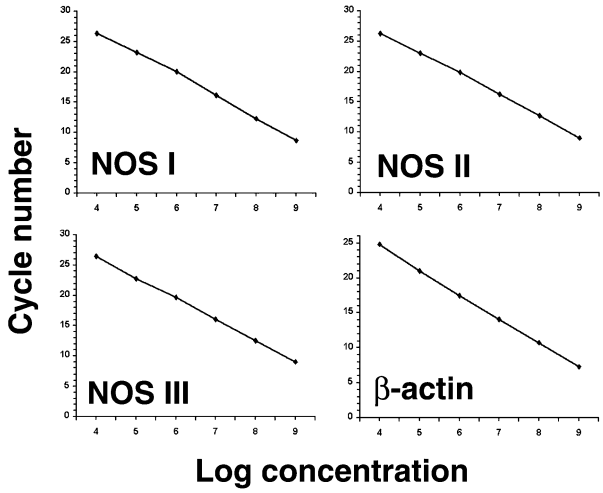

Figure 3. Quantitative evaluation of nitric oxide synthase (NOS) isoform transcripts in human umbilical vein endothelial cells (HUVEC) by real-time PCR. Representative examples of standard curves for NOS I, II, III and β-actin after 6 h of incubation (range: 10^2–10^8 molecules).

MitoTracker Red-stained mitochondria (not shown), whereas NOS III was mainly distributed at the cell periphery, thus suggesting a relationship with the plasma membrane or the cortical cytoplasm (Figure 6E and F). On a visual examination, RLX treatment (1000 ng/ml) caused an increase in the staining intensity of NOS II (compare Figure 6C and D) but no apparent changes in the staining intensity of NOS I or NOS III (not shown). As expected, preadsorption of the anti-NOS antisera with the respective epitopes resulted in no immunostaining (not shown).

Discussion

The current *in vitro* study provides evidence that the peptide hormone RLX can influence endothelial cells from the human umbilical vein by up-regulating the expression of inducible NOS II mRNA and protein. In this respect, HUVEC behave similarly to bovine vascular smooth muscle cells and rat coronary endothelial cells, in which RLX has been previously demonstrated to potentiate the intrinsic NO biosynthetic pathway through increased NOS II expression (Bani *et al*., 1988a; Failli *et al*., 2001). As judged by quantitative analysis of NOS II mRNA, the effect of RLX on HUVEC reported here is dose-related and is more prominent at 6 h than at 12 h, thus suggesting that the mechanism of induction of NOS II gene expression is activated promptly upon stimulation with RLX. This mechanism appears to involve activation of the transcription factor NF-kappa B, which is known to mediate NOS II gene expression in response to different stimuli (Xie *et al*., 1994). Unlike other transcription factors, the NF-kappa B proteins reside in the cytoplasm in an inactive state bound to the inhibitor subunit IkappaB but, upon activation, IkappaB is removed and NF-kappa B is translocated to the nucleus. The nuclear translocation of NF-kappaB is induced by many agents, including inflammatory cytokines, mitogens, bacterial products, oxidative stress and hormones. Upon activation of NF-kappa B, many genes are induced including various inflammatory cytokines, adhesion molecules, and also NOS II (reviewed in Baeuerle, 1991; Grilli *et al*., 1993; Baeuerle and Henkel, 1994). In our study, co-incubation of RLX-treated HUVEC with curcumin-95, a natural polyphenol that inhibits the nuclear translocation of activated NF-kappa B (Singh and Aggarwal, 1995), or dexamethasone, which also enhances the cytosolic retention of NF-kappa B (Yamamoto and Gaynor, 2001),

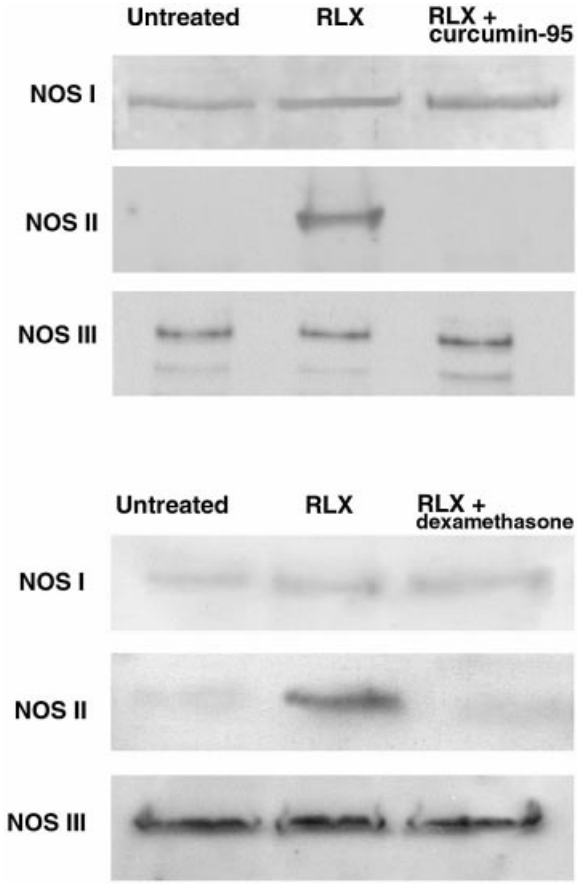

Figure 4. Western blot analysis of nitric oxide synthase (NOS) isoforms in HUVEC after 12 h of culture in the absence and the presence of relaxin (RLX) (1000 ng/ml). At a visual examination, RLX treatment induces the expression of NOS II protein, an effect that is abolished by 30 μmol/l curcumin-95 (upper panel) or 8 μmol/l dexamethasone (lower panel), whereas no apparent changes are induced on NOS I and NOS III protein expression.

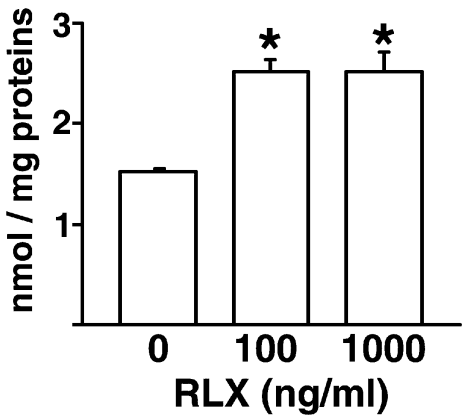

Figure 5. Evaluation of nitric oxide (NO) production by measurement of nitrites in the supernatant of human umbilical vein endothelial cells (HUVEC). Compared with the untreated cultures, relaxin (RLX) added to the culture medium for 12 h caused a clear-cut increase of nitrites. Significance of differences (one-way ANOVA, $n = 3$): $^*P < 0.05$ versus untreated cells.

reversed the effect of RLX. This finding is in keeping with our previous studies on vascular smooth muscle and endothelial cells in culture, in which NOS II induction due to RLX also appears to involve NF-kappaB activation (Bani *et al*., 1988a; Failli *et al*., 2001).

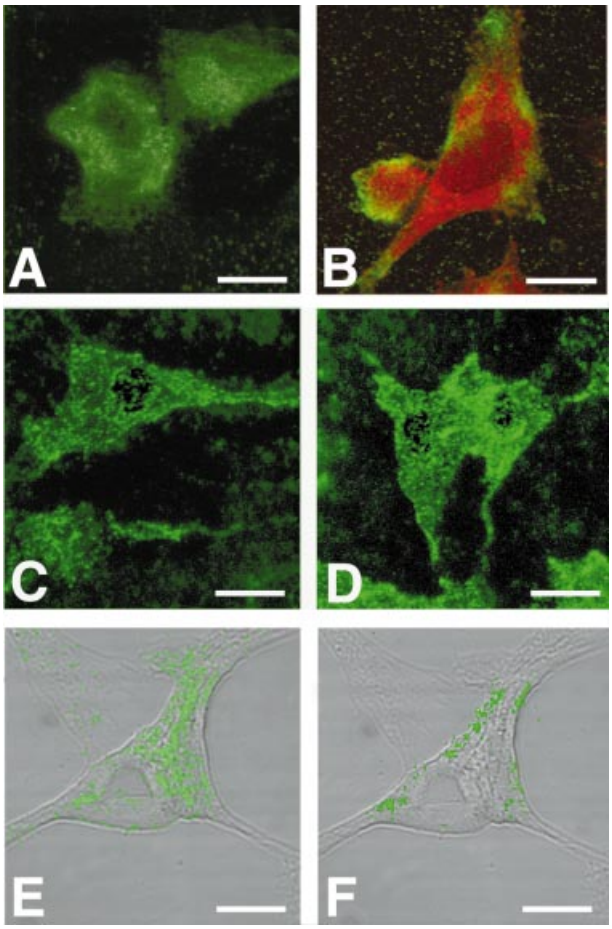

Figure 6. Confocal laser scanning microscopy of immunoreactive nitric oxide synthase (NOS) isoforms in human umbilical vein endothelial cells (HUVEC) after 12 h of culture. NOS I shows a diffuse cytoplasmic distribution (**A**) and does not co-localize with mitochondria, stained in red by MitoTracker Red dye (**B**). NOS II also shows a cytoplasmic distribution (**C**) and its staining intensity increases upon treatment with 1000 ng/ml relaxin (**D**). NOS III appears to be mainly located at the cell periphery, an impression reinforced by higher intensity of the immunostaining in surface optical sections (**E**) than in in-depth optical sections (**F**) of the same cell (in **E** and **F**, green fluorescence images are superimposed on phase-contrast images of the same cell). Magnification: ×750; bar = 20 µm.

In the present study, the RLX concentrations that caused NOS II overexpression in HUVEC are far higher than those measured in human venous cord blood (Petersen *et al*., 1995). Nonetheless, the possibility that endogenous RLX may play a physiological role in umbilical vein function should not be ruled out *a priori*. In fact, the responsiveness to RLX of HUVEC cultured *in vitro* is likely to be lower than that of the corresponding endothelial cells of the umbilical vein *in situ*. Moreover, during pregnancy, endothelial cells of the umbilical vein are exposed to RLX for long periods of time, especially during the first trimester when maternal RLX attains the highest circulating levels (Bell *et al*., 1987; Petersen *et al*., 1995), and it cannot be excluded that prolonged exposure to endogenous RLX, albeit at far lower concentrations than those used in the current *in vitro* study, could also result in a biological response. On these grounds, the hypothesis can be put forward that cord blood RLX could be involved in the endothelium-dependent regulation of the vascular tone of the umbilical vein, likely directed to optimize fetal perfusion and to sustain fetal growth.

At variance with NOS II, the constitutive NOS I and III isoforms appear unchanged upon RLX treatment. Nonetheless, the present study offers first evidence for the physiological expression of NOS I by HUVEC. We can rule out the possibility that this finding might be an artefact because: (i) the primers used to identify NOS I mRNA by RT–PCR were designed over the 3′-untranslated region (UTR) of the gene, whose sequence differs from those of the other NOS isoforms; (ii) preadsorption of anti-NOS I antibodies with the specific epitope abolished the immunostaining. NOS I, also referred to as neuronal-type NOS (nNOS), has been localized not only in central and peripheral neurons and in other cells of neuroectodermal origin, but also in skeletal and cardiac muscle and in visceral epithelial cells and smooth muscle (reviewed in Förstermann *et al*., 1995; Wolf, 1997; Vannucchi *et al*., 2002). At present, endothelial cells have been shown to be able to express functional NOS I only in non-physiological, experimental conditions, i.e. upon transfection of exogenous NOS I gene through an adenoviral vector (Channon *et al*., 1996) or upon deletion of the NOS III gene in knockout mice (Huang *et al*., 2002). This latter finding, however, suggests that endothelial NOS I gene can be actually expressed. The current finding of a constitutive expression of NOS I mRNA and protein by HUVEC raises the question of its biological meaning. It could be that this additional Ca^{2+}/calmodulin-dependent NOS represents a redundant mechanism involved in the moment-to-moment regulation of the vascular tone in the umbilical vein, in synergy with NOS III.

Acknowledgements

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