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# Relaxin up-regulates inducible nitric oxide synthase expression and nitric oxide generation in rat coronary endothelial cells<sup>1</sup>

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## SPECIFIC AIMS

Nitric oxide (NO) -mediated vasodilatation appears to be a primary effect of relaxin (RLX), a peptide hormone of the insulin/IGF family produced mainly by the ovaries and best known for its actions on the female reproductive system that has recently been shown to markedly dilate heart blood vessels. We used rat coronary endothelial (RCE) cells in primary culture as a model to address the hypothesis that RLX can have a direct action on the coronary endothelium by inducing changes in its ability to generate NO and, if so, to clarify the possible mechanisms of action and influence on cell function.

## PRINCIPAL FINDINGS

### 1. RLX increases the expression of inducible NO synthase (NOS II) protein and mRNA in RCE cells but has no effect on the constitutive NO synthase (NOS III)

Examination of RCE cell cultures immunostained with antibodies specific for NOS II and NOS III isoforms revealed that the control cells were almost negative for NOS II and showed a clear-cut cytoplasmic immunoreactivity for NOS III. A 24 h incubation with RLX (60 ng/ml, prepared from a highly purified porcine RLX preparation, 2500 to 3000 U/mg, provided by O. D. Sherwood) caused a marked increase in the immunoreactivity for NOS II, leaving NOS III immunoreactivity unchanged. Western blot analysis confirmed that control RCE cells did not express NOS II but did express detectable amounts of NOS III. A 24 h incubation with RLX (60 ng/ml) caused the expression of NOS II and had a negligible effect on NOS III. Addition of dexamethasone (8  $\mu$ mol/l), a well-known inhibitor of the NOS transcription factor NF- $\kappa$ B, together with RLX completely abolished the RLX-induced expression of NOS II but had no significant effect on NOS III. Replacement of the authentic hormone with an equivalent concentration of RLX inactivated with 1,2-ciclo-

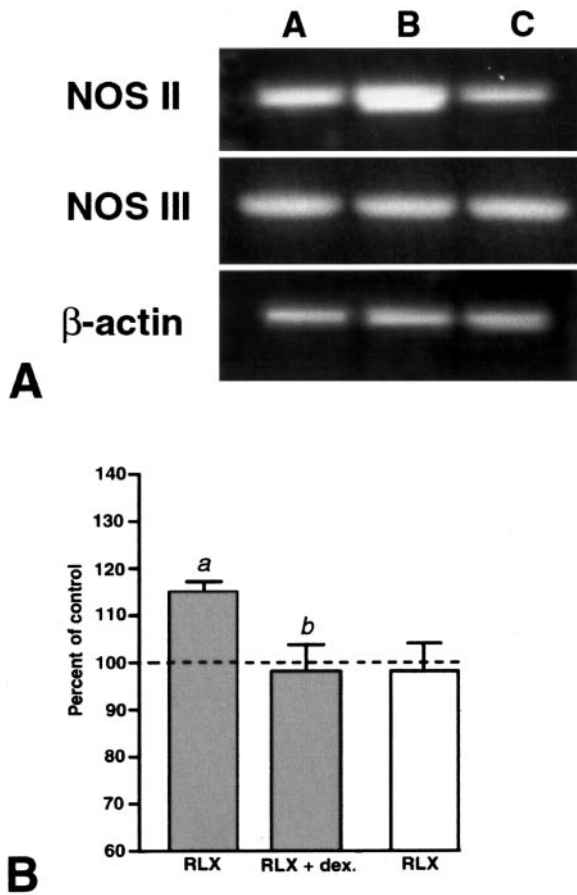
hexanedione, which binds to the arginines of the receptor binding domain of RLX and prevents the ligand-receptor interaction, failed to increase NOS II expression, thus confirming the specificity of the RLX effect. Evaluation of NOS mRNA by RT-PCR (Fig. 1) showed that, under basal conditions, RCE cells expressed both NOS II and NOS III mRNAs. A 24 h incubation with RLX at the noted concentration caused a clear-cut, significant elevation of the amount of NOS II mRNA and had no effect on NOS III mRNA. As expected, addition of dexamethasone (8  $\mu$ mol/l) together with RLX caused a marked, significant reduction of NOS II mRNA, which dropped to a level similar to that of the control cultures, and a negligible reduction of NOS III mRNA.

### 2. RLX increases NO release and intracellular NO generation by RCE cells

Determination of nitrite, the stable end product of NO metabolism, in RCE cell supernatants with the Griess reaction (expressed as nmol of nitrite/ml of culture medium) showed that, in a 24 h incubation in medium with no RLX added, RCE cells produced detectable amounts of NO (8.8 $\pm$ 0.1). Addition of RLX (60 ng/ml) to the RCE cell cultures caused a significant increase in nitrite in the medium (17.9 $\pm$ 1.2;  $P$ <0.001 vs. control). This effect of RLX was abrogated by the addition of the NOS inhibitor L-NMMA (10  $\mu$ mol/l) during the last 20 min of RLX incubation (9.7 $\pm$ 0.4;  $P$ <0.001 vs. RLX alone), as well as by a 24 h incubation with dexamethasone (8  $\mu$ mol/l) together with RLX (3.8 $\pm$ 0.1;  $P$ <0.001 vs. RLX alone). In one experiment, inactivated RLX given in the place of authentic RLX was ineffective in increasing nitrite amount (8.9). An RLX-induced increase in endogenous generation of

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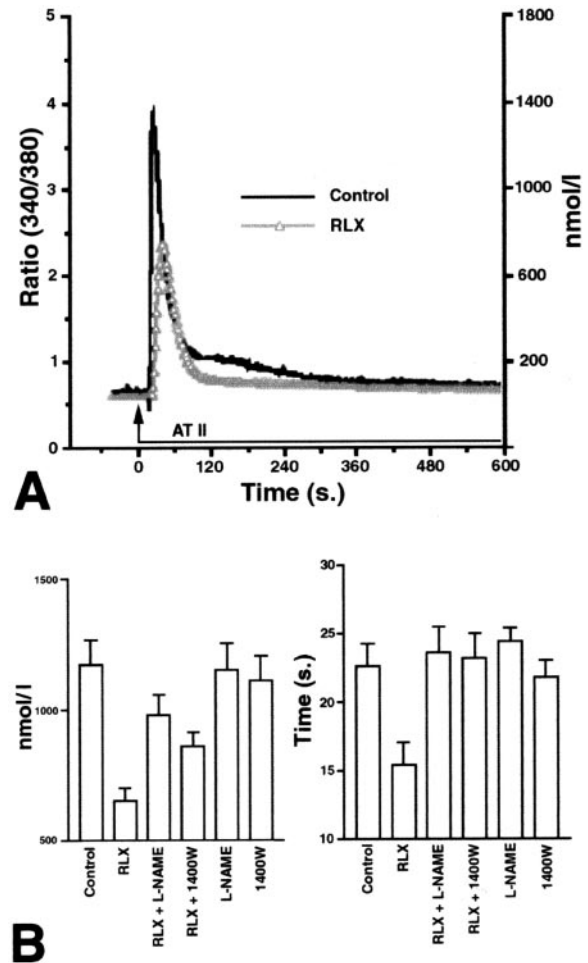
**Figure 1.** Analysis of NOS mRNA expression by RT-PCR. *A*) Representative gel electrophoresis of amplification products for NOS II, NOS III, and the housekeeping control gene  $\beta$ -actin from RCE cell cultures. Lanes, *A*: untreated controls; *B*: cultures treated with RLX (60 ng/ml for 24 h); *C*: cultures treated with RLX and dexamethasone (8  $\mu$ mol/l for 24 h). *B*) Densitometric analysis of the bands from 3 separate experiments expressed as % changes of the values of the controls (one-way ANOVA). Gray columns: NOS II. *a*)  $P < 0.05$  vs. control; *b*)  $P < 0.05$  vs. RLX alone. Open column: NOS III. RLX vs. control: not significant.

NO ( $[\text{NO}]_i$ ) by RCE cells is also shown by image analysis studies with the NO-sensitive fluorescent probe DAF-2/DA. A 24 h incubation with RLX at the noted concentration increased  $[\text{NO}]_i$  in DAF-2/DA-loaded RCE cell cultures challenged with the vasoconstrictor agonist angiotensin II (AT II). This effect of RLX was prevented by the NOS inhibitor L-NAME (10  $\mu$ mol/l) added 20 min before challenging the cells with AT II.

### 3. RLX depresses the agonist-induced intracellular $\text{Ca}^{2+}$ ( $[\text{Ca}^{2+}]_i$ ) transient in RCE cells

In basal conditions,  $[\text{Ca}^{2+}]_i$  in RCE cells evaluated by loading the cells with the  $\text{Ca}^{2+}$ -sensitive fluorophore Fura 2-AM was similar in control and RLX-treated cells ( $110 \pm 7$  vs.  $126 \pm 7$  nmol/l, respectively). Stimulation of control RCE cells with the vasoconstrictor agonists AT II or  $\alpha$ -thrombin caused a prompt increase in  $[\text{Ca}^{2+}]_i$ , attaining a peak 60 s after addition of the agonists and

with a decay time of  $21.6 \pm 1.7$  s (Fig. 2A). A 24 h incubation of RCE cells with RLX dramatically decreased both peak and decay time of the agonist-induced  $[\text{Ca}^{2+}]_i$  rise (Fig. 2A). A 20 min preincubation with either the nonspecific NOS inhibitor L-NAME (10  $\mu$ mol/l) or the selective NOS II inhibitor 1400W (1  $\mu$ mol/l) modified the agonist-induced  $[\text{Ca}^{2+}]_i$  transient in control and RLX-treated RCE cells in different ways (Fig. 2B). Although in the control cells, only



**Figure 2.** Evaluation of  $[\text{Ca}^{2+}]_i$ . *A*) Representative tracings of  $[\text{Ca}^{2+}]_i$ -associated fluorescence in Fura 2-AM-loaded RCE cells from control and RLX-treated cultures on challenge with angiotensin II (AT II, 1  $\mu$ mol/l). RLX (60 ng/ml for 24 h) markedly reduces both the peak and decay time of the  $[\text{Ca}^{2+}]_i$  transient as compared with the control. AT II (1  $\mu$ mol/l) was added at time 0 (arrow) and maintained throughout the experiment as indicated. *B*) Percent  $[\text{Ca}^{2+}]_i$  increase over the basal (left chart) and  $[\text{Ca}^{2+}]_i$  transient decay time (right chart) in 3 separate RCE cell culture experiments. Compared with controls, RLX (60 ng/ml for 24 h) caused a significant decrease of both the parameters assayed. Coincubation with the nonspecific NOS inhibitor L-NMMA (10  $\mu$ mol/l for 20 min) or the selective NOS II inhibitor 1400W (1  $\mu$ mol/l for 20 min) blunted the effect of RLX. Significance of differences (one-way ANOVA): RLX vs. control:  $P < 0.001$  (increase),  $P < 0.01$  (decay time); RLX vs. RLX + L-NAME:  $P < 0.01$  (both); RLX vs. RLX + 1400W:  $P < 0.05$  (increase),  $P < 0.01$  (decay time); RLX + L-NAME vs. RLX + 1400W: not significant (both).

L-NAME caused a moderate increase in the decay time of  $[Ca^{2+}]_i$  transient, in the RLX-treated cells both NOS inhibitors were able to significantly raise both peak and decay time. 1400W was only slightly less effective than L-NAME in blunting the effect of RLX. These findings suggest a predominant role of NOS II in mediating the action of RLX on  $[Ca^{2+}]_i$  in RCE cells.

## CONCLUSIONS AND SIGNIFICANCE

There is general agreement that under physiological conditions, the vasodilatory action of NO is primarily an endothelium-dependent process. In fact, endothelial cells contain the constitutive  $Ca^{2+}$ /calmodulin-dependent NOS III isoform that continuously produces small amounts of NO, which cause relaxation of the vascular wall by an autocrine/paracrine mechanism devoted to a moment-to-moment regulation of the vascular tone. Endothelial cells can also express inducible NOS II, which synthesizes higher amounts of NO than NOS III and can be up-regulated by different stimuli, especially inflammatory cytokines and mediators. The current study provides evidence that the vasorelaxant hormone RLX has a direct effect on endothelial cells from rat coronary vessels. This effect consists mainly of the up-regulation of NOS II mRNA and protein, apparently mediated by activation of a dexamethasone-sensitive transcription factor. This factor could be NF- $\kappa$ B, because it has been shown to bind to the NOS II gene promoter and to be involved in the induction of NOS II caused by inflammatory cytokines and lipopolysaccharides. NF- $\kappa$ B was also found to mediate RLX-induced NOS II expression by arterial smooth muscle cells *in vitro*.

In RCE cells, the up-regulation of NOS II ostensibly leads to increased NO generation, which is concurrent with a diminished responsiveness of the cells to vasoconstrictors as judged by the clear-cut decrease in the agonist-induced  $[Ca^{2+}]_i$  transient observed in the RLX-treated cells vs. the untreated controls. These effects of RLX on coronary endothelial cells may account for the overall vasodilatory properties of this hormone in heart blood vessels, as reported in our previous studies where RLX was found to increase coronary flow by acting through the NO biosynthetic pathway.

At variance with estrogens or insulin, which up-regulate the constitutive, endothelial-type NOS III in human and bovine endothelial cells *in vitro*, RLX does not seem to influence NOS III isoform in RCE cells. In fact, NOS III expression in terms of detectable protein and mRNA is not significantly modified by a 24 h incubation with RLX at the concentration tested. This

finding suggests that NOS III is not substantially involved in the response of RCE cells to RLX. This view is also indirectly supported by the results of experiments on the depression by RLX of agonist-induced  $[Ca^{2+}]_i$  transient. In these experiments, the reduction of the effect of RLX afforded by the selective NOS II inhibitor 1400W was nearly the same as that afforded by the nonspecific NOS inhibitor L-NAME, suggesting that NOS II contributes the major amount of biologically active NO in response to RLX. Evidence shows that NOS II may function as a 'constitutive' enzyme in some cell types under physiological conditions. Once synthesized on the action of different inducers, NOS II is active for hours to days and generates large amounts of NO, which can have beneficial effects such as antiatherogenic and antiapoptotic. At variance with inflammatory cytokines, which lead to massive NOS II induction and consequent generation of harmful NO levels, RLX could up-regulate NOS II in RCE cells within a physiological range. This property of RLX may therefore underlie a variety of cardiovascular protective functions of endothelial-derived NO beyond vasodilatation. These include the reduced expression of chemokines and adhesion molecules as well as the inhibition of smooth muscle cell proliferation in the coronary vascular wall. RLX may afford significant protection against cardiovascular ischemic disease, in agreement with the results of our previous studies in rat and guinea pig hearts subjected to ischemia and reperfusion. In the above studies, RLX was also found to significantly decrease neutrophil extravasation into the damaged myocardium, an effect that can be ascribed to reduced neutrophil adhesion to the coronary endothelium possibly mediated by increased generation of NO by the endothelial cells.

In conclusion, the effects of RLX on coronary endothelial cells reported here could account for at least part of the cardiovascular protective action of this hormone we demonstrated in animal models. To our knowledge, RLX emerges as the only hormone capable to up-regulate NOS II in endothelial cells. Together with estrogens, which can modulate endothelial NO generation acting *via* the constitutive NOS III isoform, RLX can be regarded as a physiological regulator of heart microcirculation and hence of myocardial blood perfusion. Further support for this view comes from previous findings that the heart possesses specific receptors for RLX. On these grounds, the presence of circulating RLX in women during fertile life may help explain the low incidence of coronary heart disease compared with age-matched men and postmenopausal women. **FJ**