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# Relaxin-induced increased coronary flow through stimulation of nitric oxide production

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1 Relaxin (RLX) is a multifunctional hormone which, besides its role in pregnancy and parturition, has also been shown to influence the cardiovascular system. In this study, we investigated the effect of RLX on coronary flow of rat and guinea-pig hearts, isolated and perfused in a Langendorff apparatus. RLX was either added to the perfusion fluid at a concentration of $5 \times 10^{-9}$ M for a 20-min perfusion, or given as a bolus into the aortic cannula at concentrations of $10^{-9}$ M, $5 \times 10^{-9}$ M and $10^{-8}$ M dissolved in 1 ml of perfusion fluid.

2 RLX, given either for a 20-min perfusion or as a bolus in the aortic cannula to guinea-pig and rat isolated hearts, increased the coronary flow and the amount of nitrite, a stable end-product of nitric oxide (NO) metabolism, that appeared in the perfusates in a concentration-dependent fashion.

3 The increase in coronary flow and in nitrite in the perfusates induced by RLX was significantly reduced by pretreatment with the nitric oxide synthase (NOS) inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA, $10^{-4}$ M).

4 The effects of RLX on coronary flow and nitrite amounts in the perfusates were compared with those induced by the endothelium-dependent vasodilator agent, acetylcholine (ACh, $10^{-8}-10^{-7}$ M), and by the endothelium-independent vasodilator agent, sodium nitroprusside (SNP, $10^{-7}-10^{-6}$ M). The results obtained show that RLX is more effective than ACh and SNP in increasing coronary flow.

5 The results of this study show that RLX increases coronary flow through stimulation of NO production; hence this hormone should be regarded as a novel agent capable of improving myocardial perfusion.

**Keywords:** Relaxin; isolated heart; coronary flow; nitric oxide

## Introduction

Relaxin (RLX) is a peptide hormone known for its role in pregnancy and parturition (Bryant-Greenwood, 1982; Sherwood, 1994). Recently, several lines of evidence suggest that RLX has additional functions on several organs and systems, including blood vessels and heart. It has been shown that RLX has a powerful vasodilator action in various organs, such as rat uterus and mesocaecum, mouse mammary gland, and pigeon crop sac (Bani & Bigazzi, 1984; Bigazzi *et al.*, 1986; 1988; Vasilenko *et al.*, 1986; Bani *et al.*, 1988). Moreover, RLX has been reported to decrease blood pressure and to blunt the response to vasoconstrictors in mesenteric vasculature in spontaneously hypertensive rats (St-Louis & Massicotte, 1985; Massicotte *et al.*, 1989). RLX has also been found to have positive chronotropic and inotropic effects on the rat heart (Parry *et al.*, 1990; Kakouris *et al.*, 1992; Ward *et al.*, 1992), most likely by binding to high affinity specific receptors in the heart atria (Osheroff *et al.*, 1992; Osheroff & Ho, 1993). More recently, cardiocytes derived from the atria of rats have been shown to secrete detectable amounts of RLX (Taylor & Clark, 1994), which it has been suggested may act via autocrine and/or paracrine routes to regulate cardiovascular structure and function, thus strengthening the idea that the heart is a physiological target organ for RLX. Based on these cardiac effects of RLX, some authors (Kakouris *et al.*, 1992) have suggested that the hormone may be responsible for the elevation of cardiac output during human pregnancy (Durr, 1989), when serum concentrations of RLX also become elevated (Bell *et al.*, 1987). In this study we aimed to clarify whether RLX influences the vascular tone of the coronary system by determining the coronary flow in isolated, perfused hearts of the guinea-pig and rat. In recent studies carried out in our laboratory on mast cells (Masini *et al.*, 1994), platelets and arterial smooth muscle cells in culture, RLX has been found to stimulate the production of nitric oxide (NO), which is known to be a powerful vasodilator agent (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Therefore, we were prompted to search for a relationship between possible changes in coronary flow and NO production by the heart upon RLX administration, and to compare the effects of RLX with those exerted by endothelium-dependent and -independent vasodilator agents.

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## Methods

### *Isolation and perfusion of guinea-pig and rat hearts*

Male guinea-pigs, weighing 300-400 g, and male Wistar albino rats, weighing 200-300 g, were used. The schedule of the experiments was designed in compliance with the guidelines for animal care and use of the University of Florence (Italy). The animals were anaesthetized with ethyl ether, killed by decapitation, exanguinated, and the hearts removed. The hearts were placed in a Langendorff apparatus and perfused through a cannula inserted into the aorta with modified Tyrode solution at a constant pressure of 40 cm water. In this way, the aortic semilunar valve remains persistently closed and the perfusion fluid enters the coronary arteries directly. The perfusion solution was composed of (mM): $Na^+$ 149.3, $K^+$ 2.7, $Ca^{2+}$ 1.8, $Mg^{2+}$ 1.05, $Cl^-$ 145.4, $HCO_3^-$ 11.9, $HPO_4^-$ 0.3 and (+)-glucose 5.6. It was maintained at 37°C and gassed with a mixture of 95% $O_2$ and 5% $CO_2$, giving a final pH of 7.48 (Dieterich & Löffelholz, 1977). Once the heart was perfused, the coronary effluents were collected and used for determinations of coronary flow and for biochemical assays. The electrocardiogram was monitored by a bipolar surface electrode

for the overall experimental period. A strain-gauge transducer was connected to a clip hooked to the heart apex and coupled with a thermic writing oscillograph. In this way, heart rate and force of contraction were recorded.

### *Treatments*

After 30 min of stabilization, the hearts were treated with RLX. In some experiments on guinea-pig hearts, RLX was injected as a bolus into the aortic cannula at concentrations of $10^{-9}$, $5 \times 10^{-9}$, and $10^{-8}$ M dissolved in 1 ml of perfusion fluid. The perfusates were collected in the 2 min following RLX administration in graduated tubes and the coronary flow measured. In other experiments on guinea-pig and rat hearts, RLX was added to the perfusion fluid at a concentration of $5 \times 10^{-9}$ M (30 ng $ml^{-1}$) for a 20-min perfusion. This concentration of the peptide was chosen because it allowed a clearcut response of the heart to be obtained in pivotal experiments. To evaluate coronary flow, the perfusates were collected over 5-min intervals in graduated tubes for the entire duration of the experiments. Two ml of each sample was used for lactate dehydrogenase (LDH) determination. The remainder of the samples was used to evaluate NO production, as described below.

Coronary flow and NO production were also determined in experiments with acetylcholine (ACh, $10^{-8}$–$10^{-7}$ M), an endothelium-dependent vasodilator agent, administered in the presence of physostigmine ($10^{-7}$ M) to inhibit tissue acetylcholinesterase, or with sodium nitroprusside (SNP, $10^{-7}$–$10^{-6}$ M), an NO-donor which causes endothelium-independent vasodilatation. These drugs were given for a 20-min perfusion.

### *Evaluation of NO production*

To investigate whether RLX influences coronary flow through a NO-mediated mechanism, perfusates from guinea-pig hearts were collected following injection of RLX as described above, and then lyophilized and resuspended in a fixed volume of water for determination of nitrite ($NO_2^-$), which is the stable end-product of NO metabolism. The amounts of $NO_2^-$ in the perfusates were measured spectrophotometrically by the Griess reaction. The Griess reagent (aqueous solution of 1% sulphanilic acid and 0.1% N-1-naphthylethylendiamine dihydrochloride in 2.5% $H_3PO_4$) forms a stable chromophore with $NO_2^-$, absorbing at 546 nm $\lambda$. The values were determined by comparison with standard concentrations of sodium nitrite and expressed as net amounts of $NO_2^-$ $ml^{-1}$ (Salvemini *et al.*, 1992). A possible involvement of the L-arginine-NO pathway in the response of the coronary vessels to RLX was also investigated in guinea-pig isolated hearts by studying the effect of $N^G$-monomethyl-L-arginine (L-NMMA), which is a powerful inhibitor of nitric oxide-synthase (NOS). The drug was added to the perfusion fluid at a concentration of $10^{-4}$ M and maintained for 1 h before giving RLX, as a bolus or as a 20-min perfusion, at the same concentrations as above. The perfusates were collected and the coronary flow and $NO_2^-$ amounts were evaluated.

In some experiments, guinea-pigs were pretreated for 24 h with i.p. injections of *Escherichia coli* lipololysaccharide (LPS, 1 mg $kg^{-1}$, dissolved in 0.5 ml saline). LPS is known to increase the endogenous generation of NO (Marletta *et al.*, 1988). After the animals were killed the hearts were isolated and perfused as described above. RLX was added as a bolus at the noted concentrations. The perfusates were collected and the coronary flow and $NO_2^-$ amounts were evaluated.

In some of the experiments with ACh and SNP in the guinea-pig hearts, the drugs were added to the perfusion fluid after a 1-h pretreatment with L-NMMA ($10^{-4}$ M).

### *LDH determination*

LDH was determined in the perfusates by measuring spectrophotometrically the catalysed reaction of pyruvate to lactate in the presence of $NADH^+$, according to Bergmeyer & Bernt (1974). The values were expressed as mu $min^{-1}$ $g^{-1}$ of tissue (wet wt.).

### *Chemicals*

Pure porcine RLX, prepared according to Sherwood & O'Byrne (1974), was a generous gift of Dr O.D. Sherwood. RLX was dissolved in modified Tyrode solution used for heart perfusion. In order to avoid adhesion of RLX to glass and plastic ware of the perfusion apparatus, they were coated with silicone before use. Kits for LDH determination were purchased from Boehringer Mannheim (Mannheim, Germany). SNP, sulphanilic acid, N-1-naphthylethylenediamine dihydrochloride, sodium nitrite, physostigmine sulphate, ACh chloride (batch no. 2340078), and LPS (serotype 0127 : B8) were from Sigma Chemical Co. (St Louis, MO, USA).

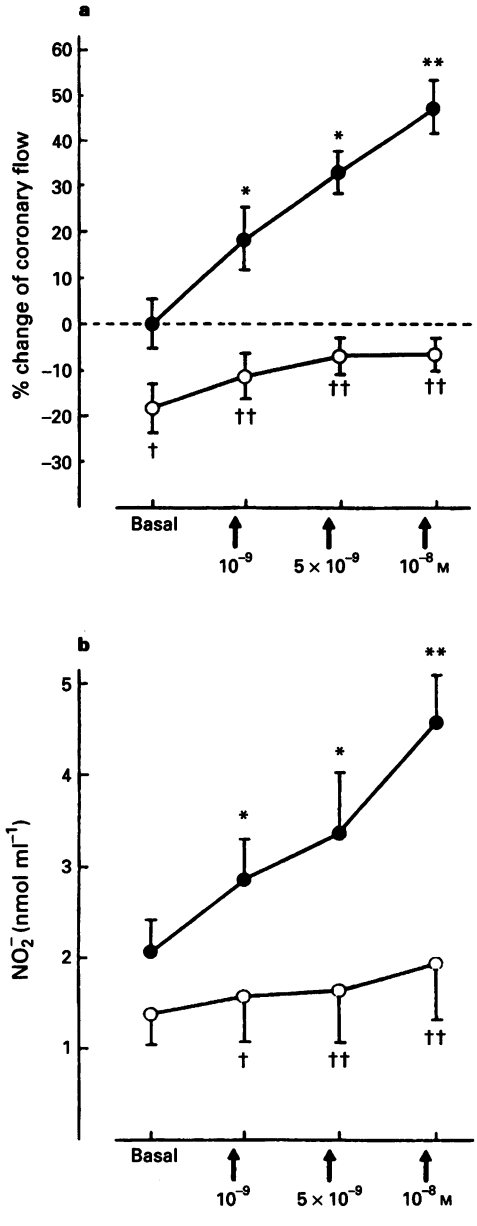


**Figure 1** Effect of relaxin (●) on coronary flow (a) and nitrite ($NO_2^-$) amounts in the perfusates (b) in guinea-pig isolated hearts. Relaxin (RLX) was injected as a bolus into the aortic cannula at different concentrations. Pretreatment with L-NMMA (○) totally inhibits both effects of relaxin. The values are expressed as means (±s.e. mean) of 5 separate experiments. *$P<0.01$; **$P<0.001$ versus the basal value. †$P<0.01$; ††$P<0.001$ versus RLX alone.

L-NMMA was obtained from Ultrafine Chemicals Ltd. (Manchester, U.K.). All the chemicals were of suprapure quality.

### *Statistical analysis*

For each parameter assayed, data are expressed as the mean (±s.e. mean) for the number of experiments, as indicated. The significance of differences was evaluated by Student's *t* test for unpaired values and, for multiple comparisons, data were analysed with ANOVA followed by Student's *t* test. $P<0.05$ was considered significant.

## Results

### *Effects of RLX on coronary flow and NO production*

Treatment of guinea-pig isolated hearts with RLX given as a bolus in the aortic cannula increased coronary flow significantly and in a concentration-dependent fashion (Figure 1a), starting from a pre-drug value of $3.25\pm0.78$ ml min$^{-1}$. No latency was observed between the RLX administration and the onset of flow increase. The RLX-induced increase in coronary flow was shown to be paralleled by a significant elevation in the amounts of $NO_2^-$ in the perfusates (Figure 1b), indicating a marked enhancement of NO production. One-hour pretreatment of the hearts with L-NMMA not only decreased significantly the basal coronary flow but also totally inhibited the effects of RLX on both coronary flow and $NO_2^-$ amounts in the perfusates (Figure 1a, b). Treatment of the hearts with RLX for a 20-min perfusion resulted in a prompt and significant increase in coronary flow, by a mean of $38.7\pm4.1\%$ over the basal value ($3.84\pm0.63$ ml min$^{-1}$) within 10 min of perfusion (Figure 2a). Coincidentally, the amounts of $NO_2^-$ in the perfusates increased significantly (Figure 2b). Similarly to the experiments with RLX given as a bolus, a 1-h perfusion of the hearts with L-NMMA before administration of RLX reduced significantly the effect of the peptide on cor-

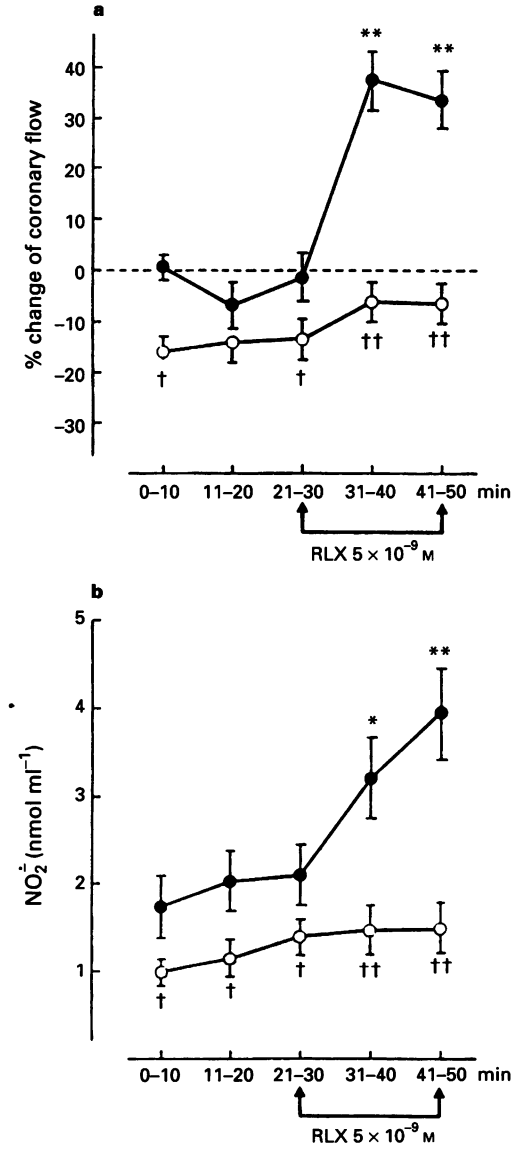


**Figure 2** Effect of relaxin (RLX) (●) on coronary flow (a) and nitrite ($NO_2^-$) amounts in the perfusates (b) in guinea-pig isolated hearts. Relaxin was given for a 20-min perfusion. Pretreatment with L-NMMA (○) abrogates the effects of relaxin. The values are expressed as means (±s.e. mean) of 5 separate experiments. *$P<0.05$; **$P<0.01$ versus the basal value. †$P<0.01$; ††$P<0.001$ versus RLX alone.

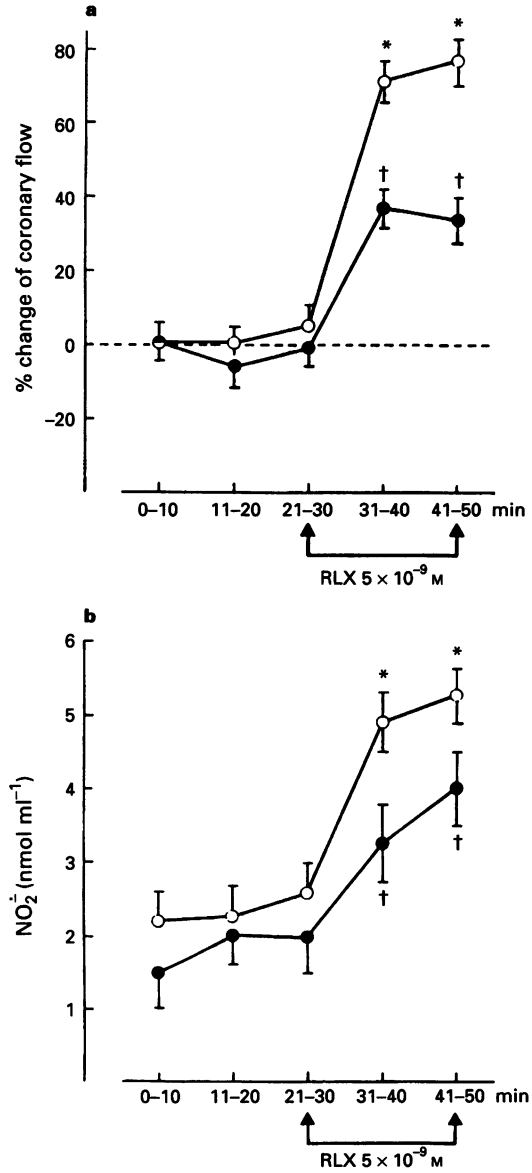


**Figure 3** Effect of relaxin on coronary flow (a) and nitrite ($NO_2^-$) amounts in the perfusates (b) in isolated hearts from control (●) and lipopolysaccharide (LPS)-treated (○) guinea-pigs. Relaxin was given for a 20-min perfusion. The values are expressed as means (±s.e. mean) of 4 separate experiments. *$P<0.001$ versus the basal value. †$P<0.01$ versus RLX alone.

onary flow and $NO_2^-$ amounts in the perfusates (Figure 2a, b). An involvement of the L-arginine-NO pathway in the response of the heart to RLX is further strengthened by the results obtained with LPS, showing that pretreatment of the animals with this drug enhanced significantly the RLX-induced increase in both coronary flow and $NO_2^-$ amounts in the perfusates (Figure 3a, b). These effects of RLX were significantly reversed by pretreatment of the hearts with L-NMMA (data not shown).

Similar results on coronary flow were obtained in rat isolated hearts following a 20-min perfusion with RLX at the same concentration as for the guinea-pig hearts (Figure 4). In fact, upon RLX treatment, a significant increase in coronary flow was obtained as compared with the pre-drug value ($2.96 \pm 0.21$ ml $min^{-1}$).

### *Other cardiac effects of RLX*

In the hearts of both guinea-pigs and rats, RLX also caused an increase in the rate of contraction. The mean increase in heart rate was 15.6% and 10.4% over the basal values, respectively, with no latency between the RLX administration and the onset of the positive chronotropic effect. No inotropic effect was ever observed at the RLX concentrations assayed.

### *LDH release*

Determination of LDH in the perfusates did not show any differences before and after treatment with RLX, even at the higher concentrations tested ($12.3 \pm 2.7$ versus $13.2 \pm 1.8$, respectively, $n = 4$).

### *Effects of ACh and SNP on coronary flow and NO production*

In guinea-pig isolated hearts, the effects of RLX on coronary flow and $NO_2^-$ formation were compared with those induced by a 20 min perfusion with ACh. As expected, ACh perfusion caused a significant, concentration-related increase in both the parameters assayed (Figure 5a, b). The effect of ACh ($10^{-7}$ M) was abated by pretreatment of the hearts with L-NMMA (Figure 5a,b), which has been shown not to have any anti-muscarinic action (Buxton *et al.*, 1993). Twenty-minute perfusion with SNP also caused a significant, concentration-related increase in the coronary flow. The effect of SNP ($10^{-6}$ M) was not abrogated by pretreatment of the hearts with L-NMMA (Figure 6). Comparing the effects of RLX with those of ACh and SNP, we found that RLX caused an increase of about 40% in the coronary flow over the basal value at a

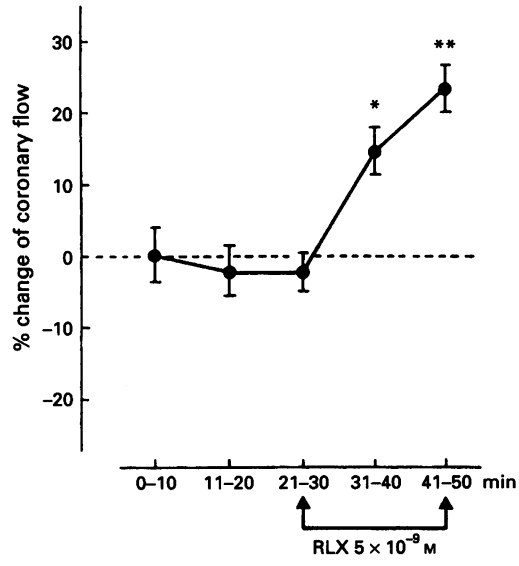


**Figure 4** Effect of relaxin (RLX) on coronary flow in rat isolated, perfused hearts. Relaxin was given for a 20-min perfusion. The values are expressed as means (±s.e. mean) of 4 separate experiments. $*P<0.05$; $**P<0.01$ versus the basal value.

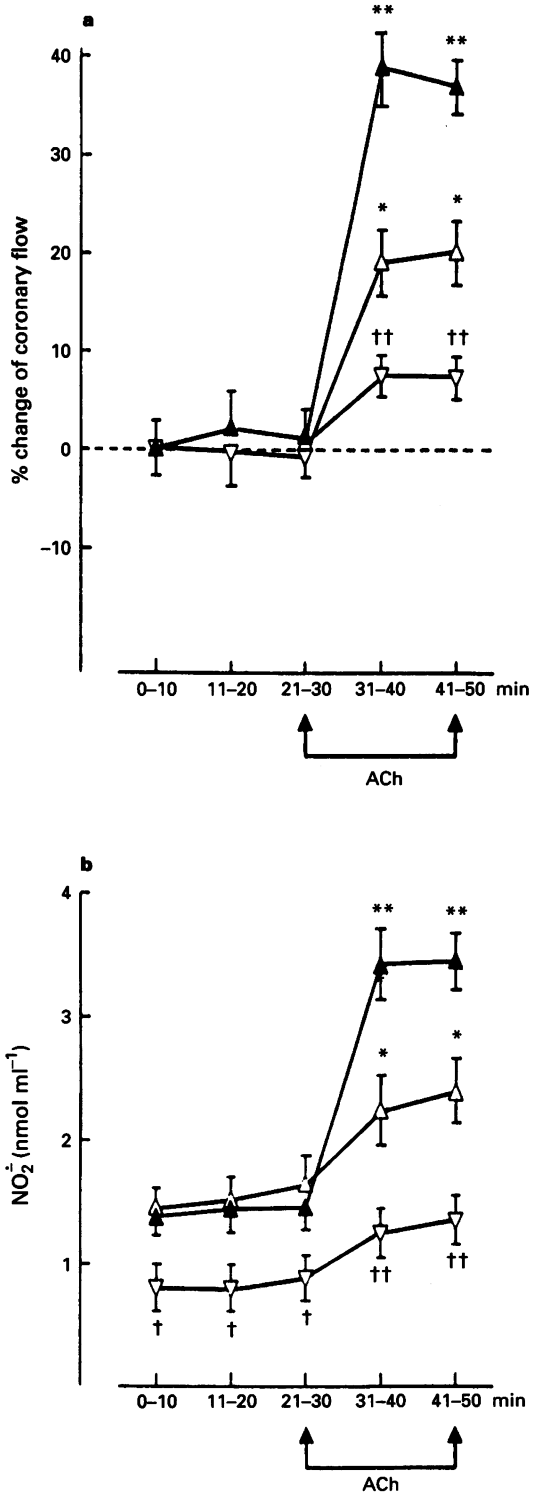


**Figure 5** Effect of acetylcholine (ACh, △: $10^{-8}$ M; ▲: $10^{-7}$ M) on coronary flow (a) and nitrite ($NO_2^-$) amounts in the perfusates (b) in guinea-pig isolated hearts. ACh was given for a 20-min perfusion. Pretreatment with $N^G$-monomethyl-L-arginine (▽) abrogates the effects of ACh. The values are expressed as means (±s.e. mean) of 5 separate experiments. $*P<0.05$; $**P<0.01$ versus the basal value. $\dagger P<0.01$; $\dagger\dagger P<0.001$ versus ACh alone ($10^{-7}$ M).

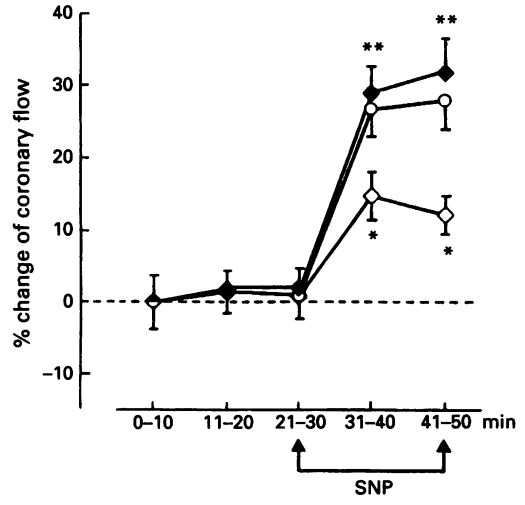


**Figure 6** Effect of sodium nitroprusside (SNP, ◇: $10^{-7}$ M; ◆: $10^{-6}$ M) on coronary flow in guinea-pig isolated hearts. SNP was given for a 20-min perfusion. Pretreatment with $N^G$-monomethyl-L-arginine (○) does not modify the effect of SNP. The values are expressed as means (±s.e. mean) of 4 separate experiments. $^*P<0.05$; $^{**}P<0.01$ versus the basal value.

concentration of $5 \times 10^{-9}$ M. A similar effect could be obtained with ACh, but at a higher concentration ($10^{-7}$ M), whereas SNP gave a lower increase of about 30% over the basal value, even at the higher concentration assayed ($10^{-6}$ M).

## Discussion

The results of this study show that RLX causes a significant, concentration-dependent increase in the coronary flow in guinea-pig and rat isolated, perfused hearts. This effect of RLX is in keeping with the vasodilator property of the peptide reported by our group and other authors in various organs (Bani & Bigazzi, 1984; Vasilenko *et al.*, 1986; Bigazzi *et al.*, 1986; 1988; Bani *et al.*, 1988). In the guinea-pig heart, the RLX-induced increase in coronary flow is paralleled by an elevation of endogenous production of NO, which is known to be a powerful vasodilator agent (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Suppression of the effects of RLX on coronary flow and NO generation by the NOS inhibitor, L-NMMA, and enhancement of these effects of RLX by the NOS inducer LPS, provide additional evidence for an involvement of the L-arginine-NO pathway in the response of the coronary vasculature to RLX. In the heart, NO may be generated by various components of the coronary vessels, such as endothelium and smooth muscle cells (Palmer *et al.*, 1988; Mollace *et al.*, 1991), as well as by perivascular mast cells (Masini *et al.*, 1991). In this context, it should be mentioned that mast cells and vascular smooth muscle cells have been shown by us to increase NO production upon RLX treatment (Masini *et al.*, 1994; Bigazzi *et al.*, 1995).

In the isolated heart perfused in the Langendorff apparatus, the perfusion fluid does not enter the left ventricle, hence the coronary flow is substantially independent of cardiac activity. Therefore, an influence of the RLX-induced increase in heart rate on the elevation of coronary flow can be ruled out.

RLX exerts its vasodilator effect on the coronary system at very low concentrations, in the range of nanomoles, like those measured in rat plasma under physiological conditions (Sherwood *et al.*, 1980). It should be noted that comparison of the effects of RLX with those of ACh and SNP revealed that 50 and 500 fold higher concentrations of these vasodilators are needed to obtain similar or even lower responses.

In recent years, attention has been paid to the possibility that the heart can release substances which have the ability to improve myocardial tissue perfusion, and which are potentially protective against ischaemic injuries occurring during cardiovascular diseases such as infarction and unstable angina (reviewed by Parratt, 1993). Among these substances, NO has been recognised as having pronounced beneficial effects, owing to its powerful vasodilator action (Rubanyi *et al.*, 1991; Vegh *et al.*, 1992). The newly recognised property of RLX to stimulate NO production in perfused hearts suggests that this hormone may play an important role in the regulation of myocardial perfusion. In this context, RLX may be a protective factor against ischaemic cardiac diseases in women, given that the incidence of coronary heart disease is very low among women during fertile life (Kannel & Abbott, 1987) when RLX is actively produced and released into blood by the corpora lutea, and increases markedly after the menopause (Manson *et al.*, 1992), coincidentally with cessation of ovarian cycles.

Taking into account the results of this study and the previously observed antiaggregatory properties of RLX (Bigazzi *et al.*, 1995), the possibility arises for the future use of RLX or RLX-derived drugs for the prevention and treatment of ischaemic heart disease, especially when the widespread therapeutic use of nitrovasodilators appears to be on the clinical horizon (Salvemini & Mollace, 1994).

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