Relaxin improves TNF-α-induced endothelial dysfunction: the role of glucocorticoid receptor and phosphatidylinositol 3-kinase signalling

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Aims Human relaxin-2 influences renal and cardiovascular functions. We investigated its effects on experimental endothelial dysfunction.

Methods and results Acetylcholine-mediated vasodilation of rat aortic rings, impaired by 48 h tumour necrosis factor-α (TNF-α) treatment, was dose-dependently improved by relaxin co-incubation, an effect sensitive to phosphatidylinositol 3-kinase (PI3K) inhibition and the glucocorticoid receptor (GR) antagonist RU-486. TNF increased endothelial nitric oxide synthase (eNOS) phosphorylation at Thr495 and decreased total eNOS expression and both basal and stimulated eNOS activity. Relaxin co-incubation did not affect eNOS expression but improved its activity via PI3K-dependent Thr495 dephosphorylation and Ser1177 phosphorylation, and additional Ser633 phosphorylation. Via GR, relaxin attenuated the TNF-related stimulation of endothelin-1 expression, superoxide and nitrotyrosine formation, and arginase II expression. Relaxin restored, via GR-c/EBP-β-mediated promoter stimulation, the compromised expression of superoxide dismutase-1 (SOD1). In rat aortic endothelial cells, relaxin activated protein kinase B (Akt) and repressed TNF-induced nuclear factor-κB and activator protein-1.

Finally, the relevance of the different findings to the model used was proved by pharmacological interventions.

Conclusion Relaxin improved endothelial dysfunction by promoting eNOS activity, suppressing endothelin-1 and arginase-II expression, and up-regulating SOD1 via GR, GR-c/EBP-β, and PI3K-Akt pathways. This corroborates the notion that it functions as an endogenous and potentially therapeutic vasoprotector.

Keywords Relaxin • Endothelial dysfunction • eNOS • Glucocorticoid receptor • PI3-kinase

1. Introduction

The peptide relaxin belongs to the insulin superfamily; the receptor for H2 relaxin, the major circulating form in humans, was discovered in 2002 and named relaxin family peptide receptor 1. It is well accepted that binding to this receptor stimulates cAMP and also the NO pathway. Additionally, involvement of ETB receptors and agonistic effects on the human glucocorticoid receptor (GR) have been described for relaxin signalling. Numerous studies identified relaxin as a vasodilatory and anti-fibrotic factor and contributed to our understanding of its highly complex and cell type-sensitive signal transduction.

Endothelial dysfunction is characterized by impaired vascular reactivity owing to inflammatory processes, reduced NO availability, and increased oxidative stress. The cytokine tumour necrosis factor-α (TNF-α) represents one key molecule therein which initiates the chronic overproduction of ROS, mainly by up-regulating NADPH oxidase. TNF-α also impairs the bioavailability of NO, e.g. by down-regulating the expression of endothelial nitric oxide synthase (eNOS) and by ROS-mediated scavenging of NO. Furthermore, TNF-α

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contributes to an increased expression of ET-1, a potent pro-inflammatory vasoconstrictor.\textsuperscript{9}

Vasodilatory effects and increased NO signalling after relaxin incubation in the rat coronary endothelium were first described by Bani-Sacchi et al.\textsuperscript{10} We previously identified relaxin as a functional ET-1 antagonist by showing a relaxin-induced up-regulation of the vasoprotective endothelial ETB receptor.\textsuperscript{3} We and others furthermore demonstrated relaxin’s potential of inhibiting early vascular inflammation.\textsuperscript{11,12} Altogether, these findings render relaxin a potential candidate drug for the treatment of endothelial dysfunction. Here, we demonstrate relaxin’s favourable effects on TNF-α-induced experimental endothelial dysfunction and investigate its signalling.

2. Methods

2.1 Animals and aortic rings

After intraperitoneal anaesthesia (thiopental sodium, 80 mg/kg body weight) of male Wistar rats (Charles River), weighing 350 ± 30 g, animals were exsanguinated after the cessation of corneal reflex (which indicated lethal anaesthesia) by cutting the carotid artery and the jugular vein. Thoracic and abdominal aortas were excised, cleaned of connective tissue, and cut into 2 mm rings. The rings were mounted on platinum hooks in modified four-channel vessel baths (Harvard Apparatus) with oxygenated Krebs–Henseleit buffer. The assessment of maximum contractility and endothelium-dependent vasodilation was previously described.\textsuperscript{13}

This investigation conforms with the European Commission Directive 2010/63/EU.

2.2 Experimental design

Aortic rings were treated for 48 h with recirculating medium (4 mL) (see Supplementary material online) containing vehicle, 250, 500, or 1000 pg/mL TNF-α with 0, 1, 5, or 10 nmol/L of H2 relaxin (Phoenix) \((n = 8\) each). Following the 48 h treatment, medium was exchanged and, after identical phenylephrine-induced pre-contraction, responses to Ach (or bradykinin) and the endothelium-independent NO donor SNP \((10 \text{ nmol/L to } 10 \text{ μmol/L each})\) were tested.

Then, experiments were run with 500 pg/mL TNF plus vehicle, the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin \((100 \text{ nmol/L})\), the GR antagonists RU-486 \((50 \text{ nmol/L})\) and D60 \((5 \text{ μmol/L})\), the ETB antagonist A-192621 \((500 \text{ nmol/L})\), 5 nmol/L relaxin, the PKC-zeta inhibitor speciosterol sulphate C \((1 \text{ μmol/L})\), or combinations \((n = 6 \text{ each})\). Four rings per aorta were perfused as described and then processed for citrulline assays, NOS western blot analysis, and determinations of nitrotyrosine formation, superoxide levels, and gene expression. In other experiments, Ach (or bradykinin) and SNP tests were also performed after 48 h prior to processing rings for western blotting and citrulline assays. For all drugs mentioned above, it had been ascertained that they did not affect the different readouts in combination with vehicle or TNF-α alone.

In another setting (Figure 6), rings were pre-contracted identically after the 48 h procedure and perfused for 2 h in new medium containing vehicle, relaxin \((5 \text{ nmol/L})\), TNF \((500 \text{ pg/mL})\), or TNF plus relaxin, and one of the following \((n = 8 \text{ each})\): vehicle, the ETA receptor-selective antagonist A-127722 \((100 \text{ nmol/L})\), plus the ETB receptor antagonist A-192621 \((500 \text{ nmol/L})\), the non-selective NO synthase inhibitor L-NAME \((100 \text{ μmol/L})\), the iNOS-selective inhibitor 1400W \((5 \text{ μmol/L})\), the nNOS inhibitor S-methyl-l-thiocitrulline \((L-SMTMC) (10 \text{ μmol/L})\), 1 mmol/L l-arginine over 1 h followed by the arginase inhibitors BEC \((200 \text{ μmol/L})\) or l-norvaline \((10 \text{ mmol/L})\), or the superoxide dismutase-1 (SOD1) inhibitor disulfiram \((0.5 \text{ μmol/L})\).\textsuperscript{24} Thereafter, pre-contraction was re-adjusted and Ach challenges were performed.

2.3 Superoxide generation

This was assessed in aortic rings by lucigenin chemiluminescence as described by others.\textsuperscript{13}

2.4 Nitrotyrosine levels

Nitrotyrosine levels were measured in homogenates of aortic rings by ELISA according to the manufacturer’s instructions (Upstate, Millipore) (see Supplementary material online).

2.5 Endothelin-1 in buffer

Endothelin-1 was determined using a radio-immunoassay kit (Bachem) as described previously.\textsuperscript{25}

2.6 Western blot analysis of NOS expression and phosphorylation in aortic rings

Protein expression of (phospho)-eNOS was detected by western blot analysis in tissue homogenates (see Supplementary material online). We used antibodies against eNOS, phospho-eNOS(Ser633), phospho-eNOS(Ser1177), phospho-eNOS(Thr495), nNOS, and iNOS.

2.7 Citrulline assay for eNOS activity

eNOS activity was measured in a standard citrulline assay in homogenates of aortic rings (see Supplementary material online).

2.8 Prepro-endothelin-1 mRNA

Total RNA extracts from aortic rings were transcribed into cDNA, and PCR was performed with 1 ng of cDNA template, 200 nmol/L prepro-ET-1 primers, and SYBR Green PCR master mix (Applied Biosystems) (see Supplementary material online).

2.9 Cells

Primary rat aortic endothelial cells (RAEC) were prepared from rat aortas by collagenase incubation (see Supplementary material online). Confluent RAEC were detached with trypsin/EDTA and propagated in DMEM supplemented with 15% horse serum, 5% FCS, 75 μg/mL ECGS, 20 mmol/L HEPES, 2 mmol/L glutamin, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Cells were identified as endothelial by their cobblestone morphology and by staining with factor VIII antigen. Cells from passages 3–6 were used in these studies.

2.10 In vitro kinase assay for activated protein kinase B

This kit obtained from Cell Signaling (#9840) was handled according to the supplier’s instructions. After immunoprecipitating activated protein kinase B (Akt) from cell extracts, an in vitro kinase assay was performed using GSK-3 fusion protein as a substrate. We also determined phospho-Akt(Thr308) using an antibody from Cell Signaling (#9275).

2.11 Western blot analysis of SOD1 in aortic rings and RAEC

For western blotting of lysed aortic rings, see above. From RAEC, 20 μL of whole-cell extracts were separated on a 17.5% SDS–PAGE gel. After blotting, membranes were incubated with an SOD1 antibody (see Supplementary material online).

2.12 PDK4 and NADPH oxidase mRNA, and NADPH oxidase activity in RAEC

mRNA was quantified with SYBR Green PCR (Applied Biosystems) (see Supplementary material online). NADPH oxidase activity in
3. Results

3.1 TNF-α-induced endothelial dysfunction is reversed by relaxin treatment through PI3K- and GR-related mechanisms

The TNF treatment enhanced the phenylephrine effect which was mitigated by relaxin co-treatment (Figure 1A); this required dose adjustments to ensure identical pre-contractions prior to Ach.

As shown in Figure 1B–D, the Ach response of aortic rings was blunted significantly by a 48 h treatment with 250, 500, and 1000 pg/mL TNF-α, diminishing maximum Ach effects by ~40, 50, and 80%, respectively. Co-application of relaxin, at 1, 5, and 10 nmol/L, significantly and dose-dependently mitigated this effect. Responses to the direct NO donor SNP were not affected by TNF or relaxin (Figure 1E). Almost identical results were obtained with regard to the bradykinin response (not shown).

Co-incubation with A-192621 did not interfere with relaxin’s potential to reverse endothelial dysfunction produced by 500 pg/mL TNF-α, whereas RU-486, D06, and wortmannin partly, and their combinations completely, reversed the relaxin effect (Figure 1F). The selective progesterone receptor antagonist ZK-230211, the ERK-1/2 inhibitor PD-98059, and the selective PKC-zeta inhibitor sphecosterol did not affect the relaxin effect (n = 4, not shown); for validation of the dosages used, see Supplementary material online. Comparable results were obtained using bradykinin instead of Ach (not shown).

3.2 Relaxin increased arginase activity via PI3K

Basal eNOS activity after the different 48 h treatments increased to ~200% compared with control after the relaxin treatment (Figure 2A); co-incubation with wortmannin prevented this effect. TNF-α drove eNOS activity down to <25% of control. This effect was significantly ameliorated by relaxin treatment (Figure 2A). The relaxin-related action towards TNF-α was prevented by co-incubation with wortmannin but not with the antagonists RU-486 and A-192621. PD-98059 and sphecosterol had no effect (not shown).

nNOS activity was determined at ~10% of basal calcium-dependent activity, without change in the different groups; we never measured relevant calcium-independent (iNOS) activity (not shown).

Regarding total eNOS protein expression (Figure 2C and E) and its different phosphorylations immediately after the different 48 h treatments, relaxin alone significantly increased eNOS phosphorylation at Ser1177 and Ser633. Co-incubation with wortmannin inhibited relaxin-induced Ser1177 phosphorylation. Total eNOS expression was reduced by TNF-α treatment, to ~50% compared with control, and remained unaffected by relaxin co-incubation (Figure 2C and E). TNF-α also induced a marked phosphorylation of the Thr495 residue. Co-incubation with relaxin caused a significant phosphorylation of Ser1177 and Ser633 and attenuated the TNF-α-stimulated increase of Thr495 phosphorylation, from >400 to ~200% of control values, this effect being sensitive exclusively to wortmannin.

A relevant difference compared with the values obtained under non-stimulated conditions was the markedly higher eNOS activity after Ach stimulation (Figure 2B, D, and F) (976 ± 103% of non-stimulated controls). Again, TNF-α significantly decreased eNOS activity, and this effect was mitigated by relaxin co-incubation. In this setting, wortmannin but also RU-486 reversed the relaxin effect on
TNF-α. Neither relevant iNOS nor nNOS activity was discernible after Ach stimulation (not shown).

It has to be noted that the Ach challenge markedly affected eNOS phosphorylation status compared with non-stimulated control conditions (Figure 2D and F, Ser1177, 634 ± 56%; Ser633, 410 ± 38%; Thr495, 33 ± 5%). Relaxin alone moderately but significantly elevated eNOS phosphorylation at Ser1177 and Ser633. Again, wortmannin antagonized the relaxin effect at Ser1177. TNF-α treatment decreased eNOS protein expression to ≈50% of controls, and led to a significant increase in Thr495 phosphorylation. Relaxin lowered TNF-induced phosphorylation at Thr495. Only wortmannin mitigated the described relaxin effect towards TNF-α.

nNOS protein could be detected at a constant level throughout the different groups; iNOS protein was not detectable except in control experiments upon cytomix stimulation (TNF-α, interleukin-1β, and interferon-γ, 10 ng/mL each) (data not shown).

3.3 Relaxin suppresses TNF-α-mediated ET-1 stimulation in aortic rings via GR

TNF-α raised circulating ET-1, which was counteracted by relaxin at the gene expression level, decreasing stimulated ET-1 peptide from ≈300 to 200% of control levels. RU-486, in contrast to A-192621 and wortmannin, was shown to prevent the suppressive relaxin effect (data not shown).

3.4 Relaxin blunts TNF-α-stimulated superoxide generation and protein nitration in a GR-dependent manner and counteracts suppression of SOD1 by TNF-α via a GR-c/EBP-β pathway

TNF-α treatment increased superoxide generation, to ≈230%, and nitrotyrosine levels, to ≈260% of control values, which was blunted.

Figure 1  TNF-α over 48 h compromises endothelium-dependent vasodilation–restoration by 1, 5, or 10 nmol/L relaxin (Rlx). (A) Responses to 1 μmol/L phenylephrine (in mN), resting force 5 mN. (B) 250 pg/mL TNF-α; (C) 500 pg/mL TNF-α; (D) 1000 pg/mL TNF-α. (E) SNP acts independently of TNF-α or relaxin. (F) RU-486 (RU) (500 nmol/L), D06 (5 μmol/L), and wortmannin (W) (100 nmol/L) significantly mitigated relaxin’s action. n = 8 each. P < 0.05; *, vs. control (vehicle); #, vs. TNF-α; §, vs. TNF plus relaxin.
significantly by relaxin (Figure 3A and B). Only the GR antagonists RU-486 and D06 blunted these inhibitory relaxin effects significantly. ZK-230211 had no effect on this relaxin action, nor had PD-98059 or spheciosterol (not shown). None of the drugs alone influenced SOD1 in the presence of TNF-α or vehicle (not shown).

Relaxin counteracted the TNF-α-mediated suppression of SOD1 protein in aortic rings (Figure 3C). In SOD1 promoter assays, relaxin alone clearly increased the SOD1 promoter activity at different time points (Figure 3D). The opposite action of TNF-α was (i) overridden by relaxin and (ii) completely abolished upon pre-treatment with curcumin, a down-regulator of AP-1, or with the JNK inhibitor SP600125, confirming reports by Afonso et al.,28 demonstrating critical involvement of the JNK-AP-1 pathway in the SOD1 down-regulation. The stimulatory relaxin effect, in turn, was entirely abrogated by RU-486.

Since analysis of the SOD1 promoter in humans and rats30 has indicated a strong stimulatory role of c/EBP proteins and since GR signaling may also involve GR-c/EBP complexes,31 we tested the functional role of both transcription factors in gene silencing experiments (Figure 3E). In RAEC, relaxin’s ability to stimulate SOD1 expression...
Figure 3  Superoxide generation (A) and nitrotyrosine levels (B) after treatment with TNF (500 pg/mL), relaxin (5 nmol/L), RU-486 (500 nmol/L), D06 (5 μmol/L), wortmannin (WM) (100 nmol/L), A-192621 (A) (500 nmol/L), and combinations. n = 6. P < 0.05; *, vs. control (vehicle); #, vs. both control and TNF-α. (C) Representative western blot of SOD1 in aortic rings (n = 4). (D) Luciferase assay of the full-length SOD1 promoter (p1499) in RAEC. n = 5. P < 0.05; *, vs. control (vehicle); #, vs. both control and TNF-α. Cells were treated with TNF, relaxin, RU-486, curcumin (Cur) (20 μmol/L), and the JNK inhibitor SP600125 (SP) (10 μmol/L). (D) Protein expression of SOD1 in RAEC after gene silencing of GR, C/EBP-α, or c/EBP-β (40 h treatment, n = 4).
remained unchanged after transfection with control or c/EBP-α siRNA. In contrast, the relaxin but not the TNF effect was totally lost after the silencing of GR or c/EBP-β. We then monitored the nuclear enrichment of GR and c/EBP-α/β following the relaxin treatment: relaxin but not TNF promoted rising levels of GR and c/EBP-β after 12 and 36 h of treatment (Figure 4A and B); c/EBP-α remained unchanged (data not shown). RU-486 alone was confirmed to induce nuclear translocation of GR but not of c/EBP-β\textsuperscript{15,31} (its antagonistic action, after binding to GR, resides in the prevention of transcriptional activity). Hence, RU did not prevent the nuclear GR rise.
but inhibited the relaxin-related increase in c/EBP-β. The complex bound to immobilized GR-specific oligos (Figure 4C) contained not only GR but also c/EBP-β, and the optical readings were consequently decreased upon interference with competing GR or c/EBP-β oligos. RU-486 alone, in contrast, merely translocated GR but not c/EBP-β. Corresponding findings were obtained when using immobilized c/EBP-specific oligos, except for RU alone, which did not result in c/EBP-β translocation (Figure 4D). The drugs and combinations thereof did not change the cytosolic protein levels of GR, c/EBP-β, or c/EBP-α as assessed by western blotting (data not shown, n = 4).

3.5 Relaxin attenuates stimulated arginase-II expression in RAEC but has no effect on increased NADPH oxidase activity

We could detect both arginase-I and -II under control conditions (Figure 5A). Whereas the protein expression of arginase-I remained unchanged, that of arginase-II was clearly elevated by 500 pg/mL of TNF over 48 h, to ~200% of control values (Figure 5B). Relaxin inhibited the stimulation of overall arginase activity in an RU-486-sensitive manner but did not affect the expression-normalized arginase activity, which was also increased by TNF (Figure 5C). RU, wortmannin, and A-192621 alone or in combination with either relaxin or TNF-α did not have any effect (data not shown).

Regarding NADPH oxidase in RAEC, TNF-α enhanced gene expression as well as enzyme activity significantly, without any effect of relaxin co-treatment (data not shown).

3.6 Relaxin-induced PI3K signalling inhibits TNF-related endothelial apoptosis

As shown in Figure 5D, TNF treatment increased apoptosis in RAEC over 48 h, with relaxin attenuating this effect in a PI3K-dependent manner. RU-486, PD-98059, or spheciosterol did not affect this readout (not shown).

3.7 Relaxin suppresses NF-κB- and AP-1-driven transcriptional activation in reporter gene assays, activates Akt, and up-regulates PDK4 in RAEC

TNF-α markedly activated transcriptional activation via NF-κB and AP-1, which was significantly inhibited by co-incubation with relaxin and dexamethasone. The relaxin and dexamethasone effects, in turn, were significantly and dose-dependently mitigated by RU-486. For more details, see Results and Figure S1 in Supplementary material online.

Relaxin (1 and 10 nmol/L) furthermore time- and dose-dependently activated PI3K-Akt signalling as shown in in vitro kinase assays. Levels of phospho-Akt (Thr108) and phospho-glycogen synthase kinase were markedly increased after relaxin incubation, and this effect was effectively blocked by wortmannin (100 nmol/L) (data not shown).

Relaxin also up-regulated the gene expression of PDK4 significantly; this was abolished by gene silencing of GR and by RU-486. Additionally, we found that relaxin activated ERK-1/2 and PKA, though this effect was insensitive to GR gene silencing or RU-486, whereas it did not affect p38, AMPK, or CaMKII at all (see Results and Table S1 in Supplementary material online).

3.8 Proof of pathophysiological relevance

In Figure 6A, blockade of both endothelin-1 receptors evoked a vasodilation in the control and relaxin groups but unmasked an endothelin-related vasoconstriction after TNF treatment which was significantly alleviated by relaxin. Non-selective NOS inhibition using L-NAME revealed the contribution of NO to basal vasotonus, which was markedly impaired after TNF treatment and clearly improved after relaxin co-treatment. The selective inhibition of iNOS or nNOS produced no relevant effects. Supplementation of L-arginine caused a slight vasodilation in the control and relaxin groups. The subsequent arginase inhibition significantly dilated rings pre-treated with TNF, this effect being mitigated by relaxin co-administration. Eventually, SOD1 inhibition by disulfiram resulted in a vasoconstriction in rings given TNF plus relaxin. Mere administration of vehicle showed no effect during this 2 h intervention (not shown).

Subsequent Ach challenge in the subgroups with arginase or SOD1 inhibition (Figure 6B), after re-adjusting identical pre-constriction, revealed the significant contribution of arginase to the deleterious TNF effects: arginase inhibition improved the Ach response in the TNF group. Moreover, the involvement of SOD1 in relaxin-related protection became evident since disulfiram worsened this Ach response. In the control and relaxin groups, arginase or SOD1 inhibition had no effect (data not shown).

In Figure 6C, the improved eNOS activity under TNF plus arginase inhibition confirmed the corresponding contraction data under basal (Figure 6A) and Ach-stimulated conditions (Figure 6B).

4. Discussion

Meanwhile, relaxin is an established player in reno-cardiovascular homeostasis. Here, relaxin restituted impaired vascular reactivity: it (i) re-established a more physiological eNOS activity; (ii) lowered the levels of stimulated pro-inflammatory and vasoconstrictive ET-1; and (iii) decreased the production of ROS and RNS, an effect attributable to elevated expression of SOD1 and mitigation of a key player in eNOS uncoupling, arginase-II. These effects involved signalling via GR and PI3K-Akt. For SOD1, the relaxin pathway specifically encompassed GR-c/EBP-β complexes. In additional cell-based experiments, relaxin promoted Akt activity and repressed the activation of the transcription factors NF-κB and AP-1. The PI3K-Akt-related anti-apoptotic action of the peptide (Figure 5D) is likely to contribute to its protective effect here because an increased rate of programmed cell death is known to diminish endothelial NOS activity. The relevance of the different findings to the model used was confirmed in our final data set (Figure 6).

TNF-α compromises endothelium-dependent vasodilation by reducing NO bioavailability via diminished eNOS synthesis and activity, ROS generation, and by inducing vasoconstrictive ET-1. We first showed in rat aortic rings (Figure 1) that the Ach response was impaired by TNF-α and then demonstrated dose-dependent restitution upon co-incubation with relaxin. This relaxin effect turned out to be susceptible to GR blockade and PI3K inhibition. In general, both the relaxin-GR and the relaxin-PI3K axes are established signalling pathways of the peptide, although their involvement in this particular context was until now unknown.

We then demonstrated that TNF-α induced a marked decrease of basal and stimulated eNOS activity (Figure 2A and B), an effect...
originating from the combination of reduced eNOS expression (Figure 2C–F), its oxidative uncoupling through elevated levels of ROS/RNS (Figure 3), and its abnormal phosphorylation pattern (Figure 2C–F). This reduced basal and stimulated eNOS activity were significantly restored by relaxin co-incubation (Figure 2A). Low eNOS protein expression after TNF-α treatment was not altered by relaxin but compensated for by augmentation of eNOS activity via enhanced phosphorylation at Ser1177 and Ser633 as well as dephosphorylation at Thr495.

Intriguingly, when relaxin was given alone, wortmannin merely inhibited stimulated Ser1177 phosphorylation, which fits well with the notion that the Ser633 residue is prevalently regulated via PKA.

Figure 5 Arginase-I and arginase-II expression (A and B) and activity (C) and TNF-related apoptosis (D) in RAEC. Treatment over 48 h with control (vehicle), TNF-α (500 pg/mL), relaxin (Rlx) (5 nmol/L), RU-486 (RU) (500 nmol/L) (not shown in D), wortmannin (WM) (100 nmol/L), A-192621 (A) (500 nmol/L) (not administered in D), and combinations. n = 6. P < 0.05; *, vs. control; #, vs. TNF-α.
another established relaxin pathway, and Thr495 is mainly a target of PKC. After TNF treatment, however, the wortmannin-related inhibition of relaxin’s action also included the inhibition of the marked dephosphorylation at Thr495. Dephosphorylation of Thr495, in contrast to Ser1177 phosphorylation, has not been reported so far as a particular effect of PI3K; this finding remains to be elucidated, and it might also be an indirect effect.

ROS/RNS up-regulated via TNF-α-mediated activation of NF-κB are central mediators of endothelial dysfunction. After relaxin co-treatment, superoxide production and nitrotyrosine levels (Figure 3) were markedly reduced and we revealed one key mechanism of this action: the up-regulation of SOD1 (Figures 3 and 4). This enzyme, a major defendant against ROS, is down-regulated by TNF-α. Relaxin increased its protein expression through GR-c/EBP-β-mediated promoter stimulation. We previously identified relaxin as a GR agonist and demonstrated activation, nuclear translocation, and binding of the relaxin–GR complex to specific glucocorticoid response elements of the DNA. Relaxin-related GR activation has also been confirmed by others. Our recent report on the stimulation of the human relaxin promoter by relaxin–GR signalling suggested a pathway not involving GR homodimers but heterogenic GR complexes—in this work, we demonstrate the involvement of c/EBP-β as a GR co-factor in another stimulatory relaxin effect. Besides, the assembly of GR-c/EBP-β complexes and its functional importance have already been shown for the classical glucocorticoid-dependent GR pathway. Our finding of relaxin/GR-mediated up-regulation of PDK4, a classical genomic target of GR, further supported the relevance of this pathway. In parallel, we excluded conceivable non-genomic GR-related mechanisms such as activation of PKA, AMPK, JNK, ERK-1/2, or CaMK.

Relaxin blunted the TNF-α-mediated up-regulation of arginase-II in RAEC (Figure 5). Arginase-II is abundantly expressed, activated, and up-regulated by TNF via the RhoA pathway (which also involves NF-κB), and it has been linked to endothelial dysfunction. We proved the pathophysiological relevance of arginase up-regulation (Figure 6); however, it was beyond our scope to further elucidate the ensuing cascade of events. The prevailing scientific opinion is that the enzyme starves eNOS from L-arginine, which then results in uncoupling, superoxide production, peroxynitrite rise, and oxidative damage to the catalytic centre of eNOS.

The mitigating relaxin effect on TNF-α-stimulated arginase-II expression was significantly attenuated by the GR antagonist RU-486. It is accepted that GR can bind to activated NF-κB or AP-1 and inhibit the stimulation of specific genes (transrepression), a fact that underlies the results we obtained here for relaxin–GR signalling in our NF-κB- and AP-1-luciferase assays.

Another putative pathway, the relaxin-induced up-regulation of endothelial ETB receptors, was obviously not critical to the actions of the peptide described herein. Although co-incubation with relaxin mitigated the ET-1-increasing effect of TNF-α, our results suggest that this effect is attributable to relaxin–GR-mediated

**Figure 6** Effects of ET-1 blockade and inhibition of NOS, arginase, or SOD1. Aortic rings were treated over 48 h with vehicle (control), relaxin (5 mmol/L), TNF (500 pg/mL), or relaxin plus TNF. On top of this treatment, rings were then perfused for another 2 h with the ETA antagonist A-127722 (100 nmol/L) plus the EET antagonist A-192621 (500 nmol/L), the non-selective NOS inhibitor L-NAME (100 μmol/L), the nNOS-selective inhibitor 1400W (5 μmol/L), the nNOS inhibitor L-SMTC (10 μM), 1 mmol/L L-arginine (Arg) over 1 h followed by the arginase inhibitors BEC (200 μmol/L) or L-norvaline (LNV) (10 mmol/L), or the SOD1 inhibitor disulfiram (DSF) (0.5 μmol/L). In (A), contraction changes over these 2 h are given in per cent of identical pre-contraction. P < 0.05; #, vs. control; *, vs. TNF-α. (B) In groups with arginase (Arg plus LNV) or SOD1 inhibition (DSF), pre-contraction was once more re-adjusted after the 2 h period and Ach challenges were performed. P < 0.05; *, vs. control; #, vs. TNF-α. (C) eNOS activity following arginine (Arg) plus BEC/LNV treatment, either immediately after the 2 h period (basal) or after additional ACh stimulation (stimulated). Values are per cent changes of the data obtained without arginine inhibition (which are summarized in Figure 2A and B) (example: basal eNOS activity following TNF and Arg plus BEC/LNV has changed by ~80%, i.e. from 21% under TNF alone in Figure 2A to 38% in this setting). P < 0.05; *, vs. control; #, vs. TNF-α.
suppression of stimulated ET-1 expression. The TNF-mediated stimulation of endothelin-1 depends on AP-1 signaling. We have shown here and also recently reported that relaxin inhibits TNF-induced AP-1 activation via GR.

In conclusion, this is the first comprehensive investigation into relaxin’s potential to protect against endothelial dysfunction. We are aware of the fact that the findings reported here in an in vitro model have to be confirmed in vivo. Relaxin remarkably ameliorated a TNF-a-induced vascular pathology via GR-mediated transrepression, GR-c/EBP-ß-mediated transactivation, and the PI3K-Akt-eNOS pathway. This may reinforce the concept that the peptide is greatly responsible for the well-known cardiovascular risk reduction of pre-menopausal women.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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