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Relaxin Activates the MAP Kinase Pathway in Human Endometrial Stromal Cells

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Abstract The reproductive hormone, relaxin, is structurally similar to insulin and insulin-like growth factor (IGF). Although a number of cellular responses to relaxin have been described, intracellular signaling mechanisms that link relaxin receptor engagement to alterations in gene expression remain uncharacterized. In the present study, relaxin treatment of a well-characterized target, human endometrial stromal cells, resulted in rapid activation of p42/44 mitogen-activated protein (MAP) kinase, as well as of MAPK (or ERK) kinase (MEK). Using a selective chemical inhibitor of MEK, it was further demonstrated that MEK phosphorylation is critical for relaxin-induced MAP kinase activation. Relaxin treatment also induced MAP kinase activation in THP-1 monocytic cells and in human smooth muscle cells, indicating that it may be a major signaling transducer utilized by the relaxin receptor. In contrast to insulin or IGF-1, relaxin did not trigger the PI 3-kinase/Akt pathway, perhaps accounting in part for relaxin's unique biological profile. Relaxin was also found to cause activation of the transcription factor CREB, a substrate of the MAP kinase pathway. Finally, activation of the MAP kinase pathway was shown to be essential for optimal stimulation of expression of the gene for vascular endothelial growth factor J. Cell. Biochem. 85: 536–544, 2002.

Key words: relaxin; signaling; MAP kinase; endometrial cells; THP-1 cells; smooth muscle cells; VEGF

Relaxin is a naturally occurring peptide hormone belonging to the insulin family [Sherwood, 1994], which is secreted in both males and females. In women, the corpus luteum, a glandular structure in the ovary, is the primary source of circulating relaxin, which reaches a peak concentration of approximately 1 ng/ml during pregnancy. We and others have previously shown that relaxin can regulate the expression of a variety of genes, including collagens and MMP-1 [Unemori and Amento, 1990; Samuel et al., 1996; Qin et al., 1997], insulin-like growth factor binding protein-1 [Tseng et al., 1992], vascular endothelial growth factor (VEGF) [Unemori et al., 1999, 2000], basic fibroblast growth factor [Lewis et al., 2002], and cyclooxygenase-2 (COX-2) [Unemori et al., 2002]. These gene products are believed to act as effectors, which contribute to relaxin's broad range of functions. However, the specific signal transduction mechanisms by which relaxin regulates any of these genes remain unclear. It has previously been shown that at the cellular level, relaxin treatment induces tyrosine phosphorylation of certain proteins [Osheroff and King, 1995; Palejwala et al., 1998], of which a protein with the molecular weight of 200 kD was postulated to be the candidate relaxin receptor. In addition, relaxin engagement of its receptor increases intracellular cyclic AMP levels in several cell types [Fei et al., 1990; Parsell et al., 1996]. While these results have helped us understand some of relaxin's intracellular signaling events, specifics of the transduction pathway utilized by the relaxin receptor have not been established.

On the basis of relaxin biology, and in particular, its structural similarity to insulin and IGF-1, we reasoned that relaxin may utilize major signaling pathways shared by other growth factors of the insulin family. In the present study, we show that relaxin employs discrete signaling pathways in normal human endometrial (NHE) cells, preferentially activating the MAP kinase pathway. In addition, MAP kinase activation occurs in two other cellular targets of relaxin, THP-1 monocytic leukemia cells and smooth muscle cells (SMCs). We also show that activation of the MAP kinase

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pathway is required for full relaxin-mediated transcriptional activation of the VEGF gene.

MATERIALS AND METHODS

Reagents

Recombinant human relaxin (rhRlx) was manufactured by Connetics Corporation (Lot 63601, 1.5 mg/ml in 20 mM acetate, pH 5.0). Kits containing antibodies, which recognize MAPK and pMAPK (cat# 9100), MEK and pMEK (cat# 9120), Akt and pAkt (cat# 9250), Jun N-terminal kinase (JNK) and pJNK (cat# 9250), and CREB and pCREB (cat# 9190) were purchased from New England Biolabs, Inc. A specific inhibitor of MEK (MEKI) (U0126, cat# 662005) was purchased from Calbiochem, Inc. and stock solutions prepared in DMSO. Recombinant human platelet-derived growth factor-BB (PDGF-BB) (cat# 220-BB-010) was purchased from R&D Systems.

Cell Culture

NHE stromal cells, described previously [Fei et al., 1990; Unemori et al., 1999], were seeded in 60-mm^2 tissue culture dishes (0.5×10^6 cells) and grown to confluence in DMEM:F12, supplemented with 10% newborn calf serum and 2 mM L-glutamine. Cells were washed with PBS 24 h later, and treated with vehicle (acetate buffer), rhRlx (10 ng/ml, or ~2 nM), or PDGF-BB (2 nM)in serum-free DMEM:F12 medium containing 0.2% BSA or lactalbumin hydrolysate (LH) for 5, 10, 30, and 60 min. For inhibitor experiments, NHE cells were pre-incubated with MEK inhibitor (10 μM) for 2 h, and then treated with acetate plus MEKI or with rhRlx plus MEKI for 5, 10, and 60 min. Control NHE cultures were subjected to 2 h pretreatment with an equivalent concentration of DMSO vehicle, and then treated for 5, 10, and 60 min with acetate plus DMSO or with rhRlx plus DMSO.

THP-1 cells were purchased from the American Type Culture Collection (ATCC # TIB202) (Rockville, MD) and grown in HyQ IMDM medium (HyClone), supplemented with 10% fetal calf serum. For experiments, 10×10^6 cells were placed in 100-mm^2 wells in serum-free IMDM containing 0.2% BSA or LH, and treated 24 h later with rhRlx (10 ng/nl) or vehicle for 5, 10, 30, and 60 min.

Primary cultures of human coronary artery SMCs (CASMC) and pulmonary artery SMCs (PASMC) were purchased from Clonetics and cultured as prescribed by the manufacturer in SmGM medium containing 5% FBS. Cells were seeded at 5×10^5 cells per 60 mm^2 wells, and washed 24 h later with PBS. Cells were then treated in serum-free SmBM containing 0.2% BSA or LH with rhRlx (10 ng/ml) or vehicle for 5, 10, 30, and 60 min.

Preparation of Cell Extracts

After treatment, as described above, cells were rinsed in ice-cold phosphate-buffered saline and solubilized in lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin). Lysates were clarified by centrifugation at 15,000*g* for 10 min. Protein concentration in the lysates was determined using a Bio-Rad protein assay reagent (Bio-Rad).

SDS–PAGE and Western Blotting

Cell lysates (30 μg of protein per lane) from NHE cells, THP-1 cells, and SMCs were electrophoresed on a 12% SDS–polyacrylamide gel and transferred to nitrocellulose membranes, according to standard procedures. Following a 1 h incubation in Tris-buffered saline containing 0.1% Tween and 3% dry milk, blots were incubated for 1 h at room temperature with primary antibodies diluted (1:1,000) in hybridization buffer. Following extensive washing with hybridization buffer, blots were incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (1:5,000) (Amersham Pharmacia Biotech) diluted in hybridization buffer. Secondary antibody was detected using the ECL system (Amersham Pharmacia Biotech).

Real-Time Quantitative RT-PCR

Real time quantitative RT-PCR was used to measure VEGF transcripts in NHE cells following rhRlx treatment. NHE cells were treated in serum-free DMEM:F12 medium with vehicle or rhRlx (10 ng/ml) in the presence or absence of MEKI (10 μM). At 2 h, the cells were lysed in Trizol reagent (GIBCO-BRL) and RNA extracted. The resulting cDNA was subjected to real-time PCR quantification using the TaqMan Sequence Detection system on the ABI Prism 7700 Instrument (PE Applied Biosystems, Inc.). The TaqMan Sequence Detection system uses

templates and fluorogenic probes ('FAM' and 'JOE') to measure, in real time, fluorescence emission, which increases in direct proportion to the amount of specific amplified product, thus allowing for quantitative analysis of gene expression. Oligonucleotide primer/probe sets (Table I) were designed for human VEGF mRNA transcripts (detecting all four isoforms), as well as for the internal control, 18S RNA, using Primer Express version 1.0 software (PE Applied Biosystems, Inc., Foster City, CA). RT-PCR was performed on 250 ng total RNA for the analysis of VEGF mRNA expression and 5 ng of total RNA to amplify transcripts of 18S RNA. All reactions utilized a 57°C annealing temperature with a total of 40 amplification cycles. The VEGF-specific signal was then normalized for expression of 18S RNA. Data were analyzed using Sequence Detector v1.6.3 software (Applied Biosystems/PE).

Statistical Analysis

Analysis of variance and Student–Newman–Keuls test for multiple group comparisons (Glantz, 1992) were used to assess for significant differences in relative VEGF (VEGF/18S) transcript expression.

RESULTS

rhRlx Stimulation Induces MAP Kinase Activation in NHE Cells

NHE cells have previously been shown to express high affinity binding sites for relaxin and to respond to relaxin stimulation with an intracellular burst of cAMP [Fei et al., 1990; Osheroff and King, 1995; Parsell et al., 1996]. As a first approach to characterizing the relaxin signal transduction cascade in these well-characterized target cells, we tested for rhRlx-induced activation of MAP kinase. NHE cells were treated with rhRlx (10 ng/ml) or PDGF-BB (2 nM) for 5, 10, 30, and 60 min, and cell lysates prepared. The PDGF-BB-stimulated cell lysates were used as a positive control for the ability to detect activated MAP kinase (Fig. 1A). rhRlx exposure rapidly triggered phosphorylation of MAP kinase, detectable as a characteristic doublet representing the 42 and 44 kDa isoforms [Marshall, 1995; Garrington and Johnson, 1999; Hunter, 2000]. Phosphorylation peaked within 5–10 min of rhRlx addition, similar in pattern to that seen following addition of PDGF-BB. Phosphorylation was undetectable by 30 min following addition of rhRlx and by 60 min following the addition of PDGF-BB, a profile typical of growth factor-stimulated MAP kinase activation. While rhRlx addition resulted in phosphorylation of MAP kinase, it did not alter the expression of MAP kinase protein itself (Fig. 1B).

TABLE I. Primers and Probes Used in Quantitative RT-PCR Analysis of VEGF and 18S mRNA

VEGF-sense	AGTTCATGGATGTCTACAGCGC
VEGF-anti-sense	TGGCTTGAAGATGTACTCGATCTC
VEGF-probe	'FAM'ACTGCCATCCAATCGA-GACCCTGG
18S RNA-sense	CAACTTTCGATGGTAGTCGC
18S RNA-anti-sense	GACTCATTCCAATTACAGGGCC
18S RNA-probe	'FAM'AAGGCAGCAGGCGCG-CAAATTA

rhRlx Treatment of NHE Cells Activates MEK

Typically, growth factor-regulated signaling activates MAP kinase (or ERK) through phosphorylation of the upstream activator, MEK [Marshall, 1995; Garrington and Johnson, 1999]. We, therefore, examined MEK phosphorylation in rhRlx-treated NHE cells. A rapid activation of MEK was detected by Western blot

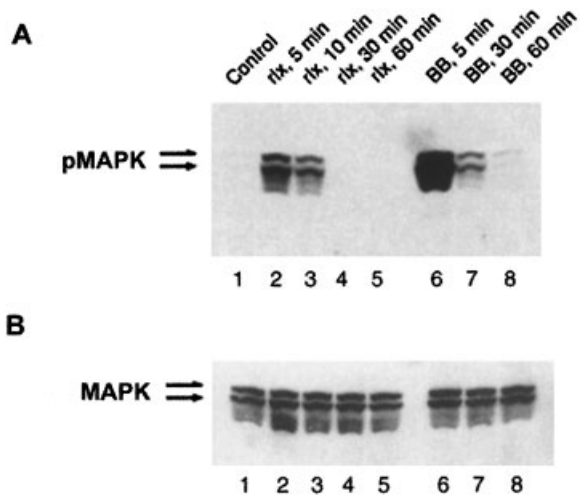

Fig. 1. rhRlx treatment induces p42/44 MAP kinase activation in NHE cells. **A**: Western blot analysis of cell extracts from NHE cells treated with acetate (Control) for 5 min or rhRlx (rlx) (10 ng/ml) for 5, 10, 30, and 60 min, as indicated above each lane, using phosphospecific anti-pMAPK antibodies. The positions of p42/44 phosphorylated MAPK protein isoforms are indicated by arrows. Cell lysates from PDGF-BB-(2 nM) (BB) treated cells (5, 30, and 60 min) were used as a positive control for the ability to detect activated MAP kinase. **B**: Western blot analysis of MAPK protein, using the same lysates as in (A). The positions of the two isoforms of MAP, p42 and p44, are indicated by arrows.

using antibodies specific for the phosphorylated form of MEK (Fig. 2A). The kinetics of MEK activation by rhRlx were similar to that of MAP kinase (Fig. 1A). MEK protein expression was not altered by rhRlx treatment (Fig. 2B). As expected, PDGF also activated MEK, albeit for slightly longer duration than that observed with MAPK phsphorylation, perhaps reflecting differential phosphatase activity [Bokemeyer et al., 1998].

Specific Inhibitor of MEK Blunts rhRlx-Induced MAPK Activation

In order to examine the contribution of MEK to MAPK activation, MAPK phosphorylation was assessed following inhibition of MEK activity using a specific inhibitor. NHE cells were pre-incubated with a specific chemical inhibitor of MEK (MEKI) for 2 h, and subsequently treated with DMSO (Control), rhRlx (10 ng/ml) (plus DMSO), or rhRlx plus MEKI (10 μM) for 5, 10, and 60 min. The addition of MEKI during the rhRlx treatment period completely inhibited rhRlx-induced MAP kinase activation (Fig. 3A), while leaving intact total MAPK protein expression (Fig. 3B).Therefore, in NHE cells, MEK activity is essential to the rhRlx-induced activation of MAP kinase. Notably, a basal level of MAPK activation was detectable in the experiment shown, and it was also inhibited by MEKI (Fig. 3A, lanes 1 and 2).

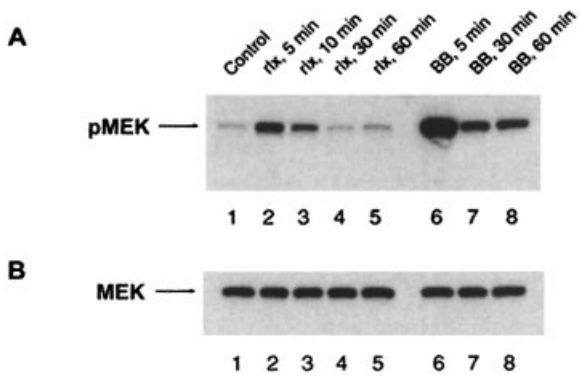

Fig. 2. rhRlx treatment of NHE cells activates MEK. **A**: Western blot analysis of cell extracts from NHE cells treated with acetate (Control) for 5 min or rhRlx (rlx) (10 ng/ml) for the times indicated above each lane. The blot was probed with phosphospecific anti-pMEK antibodies, after which phosphorylated MEK is apparent (arrow). PDGF-BB (BB) stimulated phosphorylation of MEK, as expected, and was used as a positive control, as in Figure 1. **B**: Western blot analysis of MEK protein (arrow), using the same lysates as in (A).

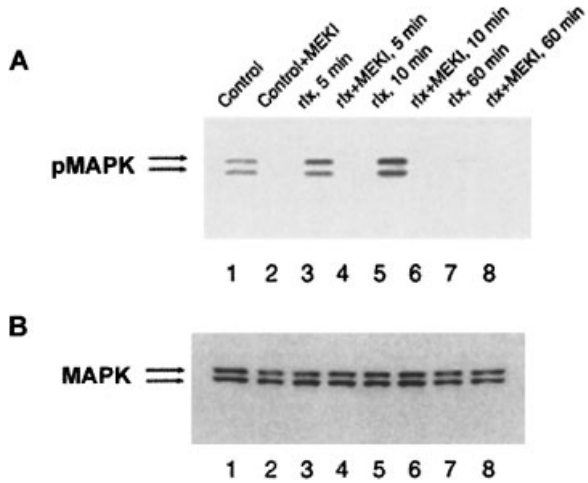

Fig. 3. Specific inhibitor of MEK blocks rhRlx-induced MAPK activation. NHE cells were pretreated for 2 h with MEK inhibitor (10 μM) (**lanes 2**, **4**, **6**, and **8**) or with an equivalent concentration of DMSO vehicle (**lanes 1**, **3**, **5**, and **7**). Cells were then treated with acetate plus DMSO for 5 min (Control, lane 1) or with rhRlx (rlx) plus DMSO (lanes 3, 5, and 7) or rhRlx plus MEKI (lanes 4, 6, and 8) for 5, 10, and 60 min, as indicated. Western blotting was performed using phosphospecific anti-MAPK antibodies (A) or using antibodies to total MAPK protein (B). Arrows indicate the positions of the phosphorylated p42/44 pMAPK doublet (A) or total p42/44 MAPK protein (B).

Activation of MAP Kinase in THP-1 and in Primary SMCs

We have previously demonstrated that THP-1 cells express high affinity rhRlx receptors and that rhRlx treatment stimulates cellular accumulation of cAMP in these cells [Parsell et al., 1996]. Scatchard analysis of radiolabeled rhRlx binding to THP-1 cells showed that these cells express much fewer receptors than NHE cells [Parsell et al., 1996]. Human SMCs from the pulmonary and coronary artery also bind rhRlx and respond by accumulating cAMP [unpublished communications]. In order to assess whether MAPK activation following rhRlx exposure is a response common to these cells, cell lysates from these cells following rhRlx treatment were subjected to Western blot analysis. rhRlx induced a modest and transient activation of MAP kinase in THP-1 cells (Fig. 4A,B), which tended to have a higher basal level of MAP kinase activity than NHE cells. Phosphorylation reached a maximum within 10 min of rhRlx addition and decreased to baseline by 30 min. In coronary artery and PASMC, rhRlx also induced rapid MAP kinase activation, demonstrating kinetics similar to that observed in NHE and THP-1 cells (Fig. 5A,B).

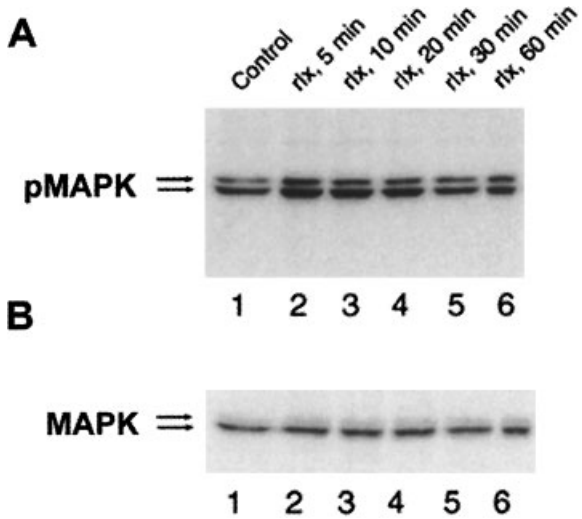

Fig. 4. Activation of MAP kinase by rhRlx in the monocytic cell line, THP-1. THP-1 cells were placed in serum-free medium for 24 h and then treated with acetate (Control) for 5 min or with rhRlx for the times indicated above each lane. Cell lysates were then analyzed for pMAPK (**A**) or MAPK (**B**) expression, as described in the legend for Figure 1.

Akt Kinase and JNK Are Not Activated in rhRlx-Treated Cells

Both insulin and IGF-1 activate the PI 3-kinase/Akt signaling pathway, which regulates cell proliferation, metabolism, and apoptosis [Franke et al., 1997]. For this reason, we assessed whether rhRlx activated this signaling pathway. While PDGF-BB, the positive control, activated Akt kinase rapidly and robustly (Fig. 6A,B), rhRlx treatment of NHE cells failed to induce Akt phosphorylation.

Another member of the MAPK family is JNK, which can be activated by specific MKKs [Minden et al., 1994; Garrington and Johnson, 1999]. As shown in Figure 6C, JNK was activated by PDGF-BB in NHE cells, but no detectable activation of JNK upon rhRlx treatment of the NHE cells was found by Western blotting using a phosphorylation-specific anti-JNK antibody.

Activation of Downstream CREB Transcription Factor by rhRlx Treatment

In an effort to search for the downstream effectors of the rhRlx-activated MAPK pathway, we tested for rhRlx-induced activation

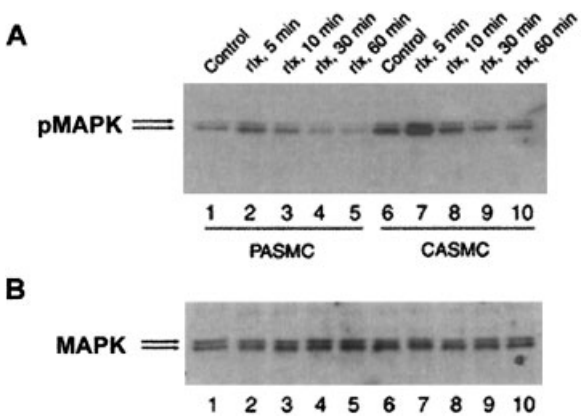

Fig. 5. Activation of MAPK by rhRlx in primary human SMCs. Cultures of PASMC or CASMC starved for 24 h in serum-free culture medium were exposed to acetate (Control) or rhRlx (10 ng/ml) for the times indicated above each lane. Cell lysates were analyzed for pMAPK (**A**) and total MAPK (**B**), as described in the legend to Figure 1.

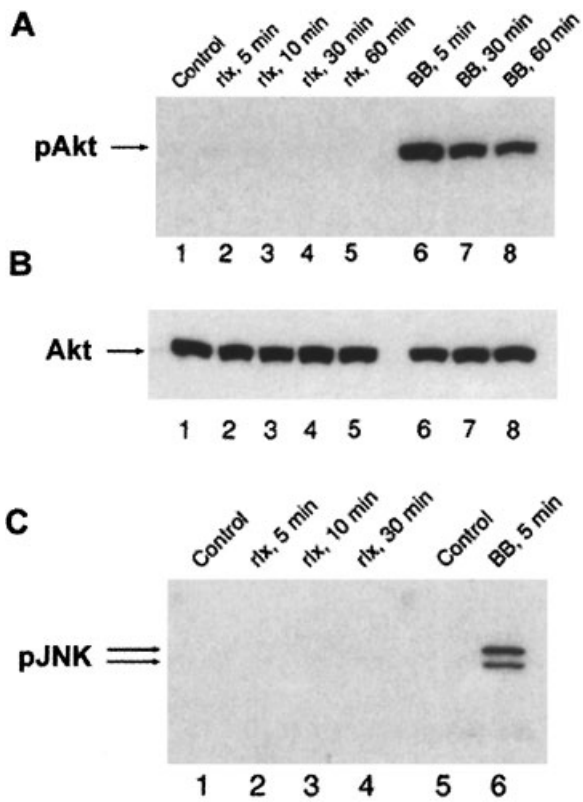

Fig. 6. Akt kinase and JNK are not activated in rhRlx treated cells. NHE cells were treated with acetate (Control) for 5 min or with rhRlx (10 ng/ml) for 5, 10, 30, and 60 min. Cell lysates were analyzed by Western blotting using phosphospecific anti-pAkt antibodies (**A**) and phosphospecific anti-pJNK antibodies (**C**). Anti-Akt Western blotting was performed to monitor the total amount of Akt protein per lane (**B**). PDGF-BB-stimulated cell lysates (BB) were used as a positive control for the ability to detect activated Akt kinase and JNK. Arrows indicate the position of activated pAkt (A), total Akt protein (B), and activated pJNK (C).

of CREB, a transcription factor, which could potentially regulate the expression of rhRlx target genes. In NHE cells treated with rhRlx, the activated form of CREB was detected using a specific antibody [Gonzalez and Montminy, 1989; Sheng et al., 1991], which recognizes CREB phosphorylated on Ser 133 (Fig. 7A,B).

MAP Kinase Pathway Activation Is Required for Optimal rhRlx-Induced VEGF Expression

It has been previously shown that the MAP kinase pathway can regulate VEGF expression in fibroblasts [Milanini et al., 1998]. Because rhRlx stimulates VEGF expression at the transcriptional level in NHE cells [Unemori et al., 2002], the potential role of MAPK in VEGF gene activation was examined using a specific inhibitor of MEK. NHE cells were treated with rhRlx for 2 h in the presence or absence of MEK inhibitor. Total RNA was then extracted for real-time, quantitative PCR analysis of VEGF mRNA and 18S RNA expression. Expression of 18S RNA was not different among treatment groups (data not shown). As shown in Figure 8, rhRlx addition induced an approximately five-fold upregulation of VEGF mRNA at 2 h. Treatment with the MEK inhibitor reduced the rhRlx induction by approximately 40%, indicating that the MAP kinase pathway is required for optimal rhRlx-stimulated VEGF expression.

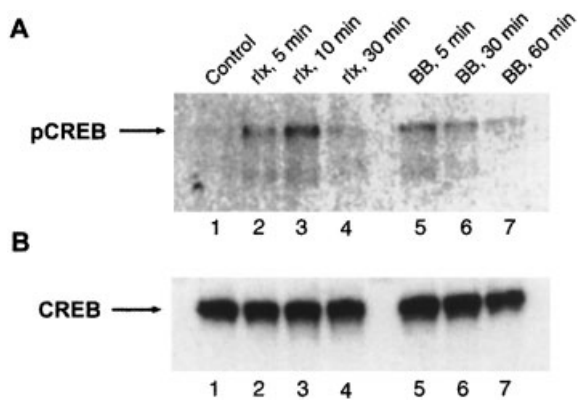

Fig. 7. Activation of CREB transcription factor by rhRlx. NHE cells were treated with acetate (Control) for 5 min or with rhRlx (rlx) (10 ng/ml) for 5–60 min, as indicated above each lane. Western blot analysis using phosphospecific anti-pCREB antibodies and anti-CREB revealed activation of CREB (arrow) (**A**) and total cellular CREB protein (arrow) (**B**). PDGF-BB (BB) was used as a positive control.

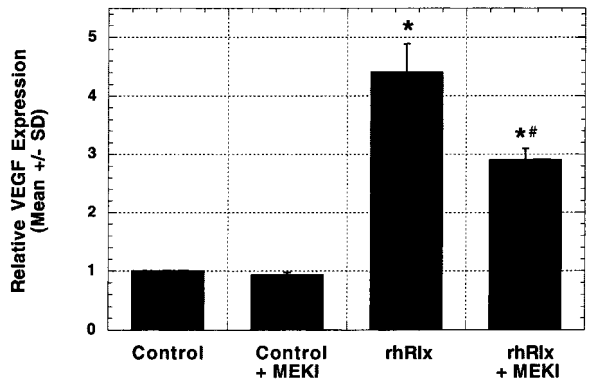

Fig. 8. MEKI partially inhibits rhRlx-induced VEGF expression. NHE cells were pretreated with MEKI or DMSO, as described in experimental procedures, and then treated with acetate (Control) or with rhRlx (10 ng/ml) for 2 h in the presence of DMSO or MEK inhibitor (μM). Transcripts were quantified by real time, quantitative RT-PCR, and VEGF mRNA expression was normalized for 18S expression. Relative VEGF expression in control cells is denoted as 1.0, and expression in MEKI-treated, rhRlx (plus DMSO)-treated, and rhRlx plus MEKI-treated cells is expressed relative to this control value. Bars = mean ± SD, n = 3 tissue culture experiments; $*P < 0.05$ vs. control cultures; $\#P < 0.05$ vs. rhRlx-treated by Student–Newman–Keuls test for multiple group comparisons.

DISCUSSION

Relaxin is believed to induce gene activation in target cells via engagement of a specific high affinity cellular receptor [Osheroff and King, 1995; Parsell et al., 1996]. Binding triggers rapid alterations in cellular metabolism [Parsell et al., 1996; Erikson et al., 2002], intracellular cAMP accumulation [Fei et al., 1990; Parsell et al., 1996; Dodge et al., 1999], and tyrosine phosphorylation of several proteins, including the putative relaxin receptor itself [Osheroff and King, 1995; Parsell et al., 1996; Palejwala et al., 1998]. The utilization of two signaling pathways by relaxin [Kuznetsova et al., 1999; Bartsch et al., 2001] is suggested by the observed increases in cAMP and by tyrosine phosphorylation of cellular proteins, which are believed to be downstream effects of engagement of a G-protein-coupled receptor and a tyrosine kinase receptor, respectively. Recently, cross-talk between these pathways has been described. Endothelin binding to its G-protein coupled receptor reportedly results in tyrosine phosphorylation of the tyrosine kinase receptor for EGF [Daub et al., 1996, 1997; Zwick et al., 1999]. In addition, Ma et al. [2000] have demonstrated that a G protein can activate the tyrosine kinase Src through physical interaction.

Results of the present study indicate that relaxin binding to its cognate receptor does cause activation of a specific tyrosine phosphorylation-related signaling cascade in a number of target cell types, including those in which cAMP responses to relaxin occur.

A screening strategy utilizing well-characterized anti-phosphospecific antibodies, which recognize activated forms of signaling molecules, was used to identify factors in relaxin signaling. Insulin and IGF-I are closely related to relaxin in overall structure, and therefore, these gene family members are hypothesized to share certain common signal transduction pathways [Bedarkar et al., 1982]. However, mapping studies have demonstrated that the receptor binding region of relaxin is distinct from those of insulin and IGF-I [Bullesbach and Schwabe, 1994], and therefore, it was also anticipated that there would be differences, as well.

Similar to observations with insulin and IGF-I signaling in target cells, rhRlx treatment of three cell types resulted in activation of MAPK. The degree of activation of MAPK in NHE cells by rhRlx appeared to be roughly a third of that induced by PDGF-BB, which has been extensively characterized as a potent activator of this pathway [Fantl et al., 1993]. This difference may lie in the fact that there are far fewer relaxin receptors on these cells (~1,000 per cell) [Osheroff and King, 1995; Unemori et al., 1999] than the number of PDGF receptors typical on mesenchymal cells (roughly 300,000 per cell) [Heldin et al., 1981]. Alternatively, the peaks in activation kinetics may have been missed in the time course studied here. The NHE stromal cells, THP-1 monocytic cells, and the two smooth muscle cell types studied here demonstrate similar kinetics in that the peak phosphorylation of MAPK occurred within 10 min of relaxin exposure. The findings in NHE cells suggest that relaxin-induced MAPK activation occurs through MEK. The upstream regulator of MEK/MAPK remains to be determined, although there is evidence suggestive of c-Raf involvement [Palejwala et al., 2001]. There is also evidence, however, that B-Raf, rather than c-Raf, may function as a positive link between MAP kinase and cAMP pathways [Wu et al., 1993; Vossler et al., 1997].

In contrast to insulin and PDGF, rhRlx did not activate Akt kinase or JNK in NHE cells. Both MAP kinase and PI 3-kinase/Akt pathways are known to be required for growth factor-induced cellular proliferation [Fantl et al., 1992; Hu et al., 1995; Franke et al., 1997; Rodriguez-Viciana et al., 1997]. The unique signaling pattern of rhRlx may help explain why rhRlx does not promote cellular proliferation in these cellular targets [unpublished communications].

The transcription factor CREB is one of the major nuclear factors, which transduces cAMP activation of gene transcription [Haus-Seuffert and Meisterernst, 2000]. In addition, CREB phosphorylation can also occur via activation of the MAP kinase pathway, via activation of p90rsk [Frodin and Gammeltoft, 1999]. In this study, we show that rhRlx treatment of NHE cells results in CREB activation. Its phosphorylation in these cells may be the downstream result of a MEK/MAPK/p90rsk/CREB activation cascade, or activation of the cAMP/PKA pathway [Telgmann et al., 1997], or both. Further work is required to show how these pathways, including p90rsk, may interact to cause activation of CREB.

Activation of the MAP kinase pathway is required for optimal expression of VEGF in NHE cells. Indeed, profound inhibition of MEK activity using a chemical inhibitor resulted in a 40% reduction in expression of VEGF transcripts, indicating that MEK contributes, in part, to VEGF activation. CREB may be at least one of the potential transcriptional regulators of VEGF in these cells, since both inhibition of cAMP [Unemori et al., 1999] and inhibition of MEK lead to decreased levels of VEGF expression. Although the VEGF promoter does not have a consensus cAMP response element, it does have an AP-2 site, which has been shown to transduce cAMP signaling [Imagawa et al., 1987; Gao et al., 1997]. Since upregulated VEGF expression occurs in response to relaxin treatment in NHE stromal cells [Unemori et al., 1999], THP-1 cells [Unemori et al., 2000], wound macrophages [Unemori et al., 2000], cardiac fibroblasts [Lewis et al., 2002], and SMCs [Liu et al, unpublished communications], the MAP kinase pathway may be a critical regulator of relaxin phenotype in a number of cell types.

In summary, rhRlx exposure of a number of cell types results in MAPK activation, which implicates a signaling cascade involving MEK/MAPK/CREB. Importantly, signaling molecules Akt and JNK are not activated following rhRlx exposure. This specific cascade may be of

fundamental importance in determining the phenotypic response of target cells to relaxin, and may, at least in part, delineate the relaxin response from the response to related family members, insulin and IGF-I.

NOTE ADDED IN PROOF

Two G-protein-coupled relaxin receptors were identified subsequent to acceptance of this manuscript [Hsu et al., Science 295:671–674, 2002].

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