Relaxin Activates Peroxisome Proliferator-activated Receptor \( \gamma \) (PPAR\( \gamma \)) through a Pathway Involving PPAR\( \gamma \) Coactivator 1\( \alpha \) (PGC1\( \alpha \))

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Relaxin Activates Peroxisome Proliferator-activated Receptor γ (PPARγ) through a Pathway Involving PPARγ Coactivator 1α (PGC1α)*

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Background: Relaxin activates peroxisome proliferator-activated receptor γ (PPARγ) by an unknown PPARγ ligand-independent mechanism.

Results: Expression of PPARγ coactivator 1α (PGC1α) was increased by relaxin through distinct signaling pathways.

Conclusion: The mechanism for relaxin regulation of PPARγ is through increased PGC1α levels.

Significance: Discovery of new targets of relaxin signaling may provide further understanding of its pleiotropic effects.

Relaxin is a polypeptide hormone that belongs to the insulin-like family of peptides (1, 2). The earliest functions attributed to relaxin involved pregnancy, due to detection of relaxin in the circulation during the first trimester in humans, and its involvement in implantation, and additional functions which vary among species. However, now a number of the relaxin non-reproductive functions have been elucidated, including regulation of vascular tone, vasodilation, fibrosis prevention, and reversal, and action as a neuropeptide (1). The first relaxin receptors identified were the leucine-rich G protein-coupled receptors 7 and 8 (LGR7 and LGR8), later renamed relaxin family peptide receptor (RXFP1) 1 and 2, respectively (3, 4). Relaxin has higher affinity for RXFP1 as compared with RXFP2 in vitro, but has only been shown to activate RXFP1 in vivo, and therefore RXFP1 is considered the cognate relaxin receptor (5). Upon activation, RXFP1 couples to multiple G-proteins including Gαi, Gαo, and Gq, to modulate cAMP levels through a complex mechanism involving phosphatidylinositol 3-kinase (PI3K) and protein kinase Cζ (5–8). In addition to increased cAMP production and activation of cAMP-dependent protein kinase (PKA), relaxin activation of RXFP1 has been tied to other signaling pathways, including tyrosine kinase, the mitogen-activated protein kinase ERK1/2, nitric oxide, and glucocorticoid activation (2, 9–11). Thus, relaxin has shown diverse and complex signaling. We recently identified another signaling pathway activated by relaxin, involving peroxisome proliferator-activated receptor gamma (PPARγ) (12).

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. To date, three PPAR isoforms have been identified, including PPARα, PPARβ/δ, and PPARγ (13). The PPARs function as transcription factors with their heterodimerization partners the retinoid X receptors. Upon ligand binding, the DNA binding domains of PPARγ require activation, resulting in increased PGC1α levels. This provides a mechanism for the ligand-independent activation of PPARγ in response to relaxin.

Conclusion: The mechanism for relaxin regulation of PPARγ is through increased PGC1α levels.

Significance: Discovery of new targets of relaxin signaling may provide further understanding of its pleiotropic effects.

Relaxin activation of its receptor RXFP1 triggers multiple signaling pathways. Previously, we have shown that relaxin activates PPARγ transcriptional activity in a ligand-independent manner, but the mechanism for this effect was unknown. In this study, we examined the signaling pathways of downstream of RXFP1 leading to PPARγ activation. Using cells stably expressing RXFP1, we found that relaxin regulation of PPARγ activity requires accumulation of cAMP and subsequent activation of cAMP-dependent protein kinase (PKA). The activated PKA subsequently phosphorylated cAMP response element-binding protein (CREB) at Ser-133 to activate it directly, as well as indirectly through mitogen activated protein kinase p38 MAPK. Activated CREB was required for relaxin stimulation of PPARγ activity, while there was no evidence for a role of the nitric oxide or ERK MAPK pathways. Relaxin increased the mRNA and protein levels of the coactivator protein PGC1α, and this effect was dependent on PKA, and was completely abrogated by a dominant-negative form of CREB. This mechanism was confirmed in a hepatic stellate cell line stably that endogenously expresses RXFP1. Reduction of PGC1α levels using siRNA diminished the regulation of PPARγ by relaxin. These results suggest that relaxin activates the cAMP/PKA and p38 MAPK pathways to phosphorylate CREB, resulting in increased PGC1α levels. This provides a mechanism for the ligand-independent activation of PPARγ in response to relaxin.

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3 The abbreviations used are: LGR, leucine-rich G protein-coupled receptor; PPARγ, peroxisome proliferator-activated receptor γ; PGC1α, PPARγ coactivator 1α; RXFP1, relaxin family peptide receptor 1; PKA, cAMP-dependent protein kinase; MAPK, mitogen-activated protein kinase; CREB, cAMP response element-binding protein; PI3K, phosphoinositide 3′-kinase; PKCζ, calcium-dependent protein kinase Cζ; PPRE, peroxisome proliferator response element; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; GSNO, S-nitroso glutathione.
Cells were treated with purified porcine relaxin (kindly provided by Dr. O. David Sherwood, University of Illinois at Urbana-Champaign) as indicated in the figure legends. Relaxin was used at a concentration of 1 nM, which is in the range sufficient for activation of canonical cAMP-related pathways through RXFP1 (2). Forskolin, the p38 MAPK inhibitor PD-169316, the PKA inhibitor H89, the PI3K inhibitor LY294002, the ERK1/2 inhibitor PD98059, the nitric-oxide synthase inhibitor L-NAME, and pertussis toxin were purchased from EMD Biochemicals. The cyclic nucleotides Sp-6-Bnz-cAMPS and Sp-8-pCPT-2’′O-Me-cAMPS were from Biolog. The nitric oxide donors SNAP (N-nitroso-N-acetyl-d,l-penicillamine) and GSNO (N-nitrosothioglutathione) were from Sigma.

**Reporter Assays**—Cells were cotransfected with firefly and Renilla luciferase reporter plasmids as described above. After 24 h, cells were treated as described for the individual figures, then assays were performed 48 h after transfection. Activation of reporter constructs was monitored using the Dual-Glo Luciferase assay (Promega) and measured in a Spectramax M5 plate reader. The data are expressed as the firefly luciferase activity normalized to the Renilla luciferase in the same sample.

**Quantitative Real-time PCR and Conventional RT-PCR**—Total cellular RNA was extracted using the Purelink RNA Mini kit (Invitrogen), and treated with RNase-free DNase to remove genomic DNA. The RNA concentration was measured using the Ribogreen assay (Invitrogen). A total of 2 μg of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) in a total volume of 20 μl. The cDNA was then subjected to TaqMan real-time PCR. The reaction mixtures were comprised of 2 μl of cDNA (diluted 1:15), 10 μl of Taqman universal PCR master mix, 2.5 μl Taqman primer/probe mix in a final volume of 20 μl per reaction. The human gene expression assays (Applied Biosystems) used were PGC1α (Hs00173304_m1) and TATA binding protein (TBP) (4333769F). Gene expression was normalized to the level of TBP within each sample using the relative ΔΔCT method. Gene expression is shown as relative expression to control. The data shown are representative of three independent experiments. For conventional RT-PCR analysis, total RNA was extracted and cDNA prepared as above from HEK-RXFP1, THP1, HepG2, and LX2 cells. Expression of RXFP1 was determined using intron-spanning primers for human RXFP1 (sense 5′-TCTTGGTTAATTTG GCCGC-3′, antisense 5′-CATTAACTCAGGT GCCATCTCC-3′, or GAPDH (sense 5′- ACCACACTGTCATCCACGT-3′, antisense 5′-TCCACCACCTGTGTGCTGA-3′) as a loading control.

**cAMP and PKA Assays**—The production of cAMP production was measured by treating the cells with the indicated concentrations of relaxin in the presence of the phosphodiesterase inhibitors IBMX (0.5 mM) and Ro20–1724 (0.1 mM) for 30 min at room temperature. The level of cAMP was measured using the cAMP-Glo assay (Promega). The level of PKA activation was determined using the Pep-Tag assay (Promega) according to the manufacturer’s instructions.

**Western Blots**—Cells were treated as described for the individual experiments, using mammalian protein extraction reagent (M-PER, Pierce, Thermo Scientific, Rockford, IL) contain-
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**RESULTS**

Relaxin Activates PKA to Activate PPAR Activity—One major signaling pathway triggered by relaxin is via induction of cAMP production and activation of cAMP-activated protein kinase (PKA). To examine the role of cAMP/PKA in activation of PPARγ, HEK-293 cells stably expressing RXFP1 (HEK-RXFP1 cells) were used. We previously showed that relaxin increased cAMP production and activation of a PPAR response element (PPRE) via activation of PPARγ in these cells, and that these responses were dependent on RXFP1 (12). Relaxin also increased PKA activity in these cells, and that these responses were dependent on RXFP1 (12). Relaxin also increased PKA activity in these cells, which was blocked by treatment with the PKA inhibitor H89 (Fig. 1A). Using a PKA reporter, treatment of HEK-RXFP1 cells with either forskolin, a direct adenylyl cyclase activator, or relaxin, increased transcriptional activity through a PPAR response element (PPRE) via activation of PPARγ in these cells, and that these responses were dependent on RXFP1 (12). Relaxin also increased PKA activity in these cells, which was blocked by treatment with the PKA inhibitor H89 (Fig. 1A).

Because relaxin activates multiple signaling pathways in cells expressing RXFP1, a panel of activators and inhibitors of various pathways was used to screen for potential mechanisms for activation of PPARα. One major signaling pathway triggered by relaxin is via induction of cAMP production and activation of cAMP-activated protein kinase (PKA).

**FIGURE 1.** The cAMP/PKA pathway activates PPAR transcription through a PPRE reporter. A, HEK-RXFP1 cells were pretreated for 30 min in the presence of indicated concentration of the PKA inhibitor H89, then treated with 1 nM relaxin, and PKA activity was measured by phosphorylation of a PKA substrate. B, HEK-RXFP1 cells transfected with PPRE and Renilla luciferase reporters were pretreated for 30 min in the absence and presence of H89 (20 μM), then treated with 10 μM forskolin or 1 nM relaxin for 24 h prior to dual luciferase assay. Data are presented as PPRE luciferase assay relative to control, mean ± S.E., *p < .05; **, p < .01; ***, p < .001, n = 3. C, Western blot showing knockdown of PKA or p38 MAPK after treatment with siRNA. D, HEK-RXFP1 cells were cotransfected with plasmids encoding PKI or an inactive PKI mutant (PKImut) and PPRE and Renilla luciferase reporters, then treated with relaxin as described above, then subject to dual luciferase assay. ***, p < .001; n = 3. E, Western blot showing knockdown of PKA or p38 MAPK after treatment with siRNA. F, HEK-RXFP1 cells were cotransfected with PKA siRNA or nontargeting control siRNA and PPRE and Renilla luciferase reporters, then treated with relaxin as described above, then subject to dual luciferase assay. ***, p < .001, n = 3.
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**FIGURE 2.** p38 MAPK is a downstream mediator of PKA-induced PPAR activity. **A**, HEK-RXFP1 cells transfected with PPRE and Renilla luciferase reporters were pretreated 30 min with the p38 MAPK inhibitor PD169316 (20 μM) or vehicle, then treated for 24 h with or without 1 nM relaxin then subject to PPRE luciferase assay. The data are expressed as the PPRE luciferase activity relative to untreated cells, mean ± S.E., n = 3, *p < 0.05, **p < 0.01. **B**, HEK-RXFP1 cells were treated with 1 nM relaxin for the indicated times, and lysates were analyzed by Western blot for total and phosphorylated p38 MAPK. **C**, cells were pretreated 30 min with 20 μM H89 or vehicle, then treated with 1 nM relaxin for 15 min, and lysates were analyzed by Western blot for total and phosphorylated p38 MAPK. **D**, HEK-RXFP1 cells were cotransfected with p38 MAPK siRNA or nontargeting control siRNA and PPRE and Renilla luciferase reporters, then treated with relaxin as described above, then subject to dual luciferase assay. *, p < 0.05, ***, p < 0.001, n = 3, E, cells were cotransfected with PKA siRNA or nontargeting control siRNA, then treated with relaxin for 15 min before Western blot analysis for phosphorylated and total p38 MAPK.

relaxin-induced PPARγ activation (Fig. 1C). Both forskolin and the PKA-specific activator Sp-6-Bnz-cAMPS caused increased PPRE transcriptional activity, but the exchange protein activated by cAMP (Epac) activator Sp-8-CPT-cAMPS had no effect. The relaxin-stimulated PPRE activation was completely inhibited by H89, and partially inhibited by pertussis toxin and the PI3K inhibitor LY294002. The ERK1/2 inhibitor PD98059 inhibited by H89, and partially inhibited by pertussis toxin and the p38 MAPK inhibitor. The partial reduction by PD169316 partially blocked the effect. The nitric oxide donor SNAP and GSNO did not stimulate transcriptional activity, and the nitric-oxide synthase inhibitor L-NAME did not inhibit the ability of relaxin to activate PPARγ, suggesting that the nitric oxide is not a mediator of the relaxin effects.

*p*38 MAPK Is a Downstream Mediator of PKA-induced PPAR Activity*—Because the p38 MAPK inhibitor reduced the relaxin effect (Fig. 1C), this pathway was studied in more detail. The p38 MAPK inhibitor caused a partial decrease in the activation of the PPRE reporter by relaxin (Fig. 2A), but had no significant effect on the baseline levels. To determine whether relaxin causes phosphorylation and activation of p38 MAPK, HEK-RXFP1 were treated with relaxin for various periods of time, and phosphorylation of p38 MAPK was monitored (Fig. 2B). Relaxin caused a rapid and transient increase in p38MAPK phosphorylation that was maximal between 5 and 15 min, with a return to baseline levels with increased time. Furthermore, the relaxin-induced p38 MAPK activation was completely blocked by treatment with H89 (Fig. 2C), suggesting that p38 MAPK is activated downstream of PKA.

**CREB Mediates Relaxin-induced PPAR Activity**—A common downstream target of both PKA and p38MAPK activation is cAMP response element-binding protein (CREB). Relaxin treatment of HEK-RXFP1 cells stimulated CREB transcriptional activity of HEK-RXFP1 cells transfected with a CREB luciferase reporter (Fig. 3A). Furthermore, overexpression of the dominant-negative CREB (KCREB) resulted in a complete loss of relaxin-induced PPRE activation (Fig. 3B). Treatment with relaxin resulted in phosphorylation of CREB at Ser-133 (Fig. 3C). The phosphorylation of CREB in response to relaxin was completely blocked by a PKA inhibitor, and partially blocked by the p38 MAPK inhibitor. The partial reduction by the p38 MAPK inhibitor of CREB phosphorylation (36.0 ± 2.1%) compares favorably with the decrease in relaxin-stimulated PPRE activity (47.4 ± 2.7%, Fig. 2A). Together, these data suggest that relaxin causes activation of CREB downstream of PKA and p38 MAPK.

PGC1α Is the Mechanism for Activation of PPARγ Activity by Relaxin—We have shown earlier that relaxin activation of PPARγ in HEK-RXFP1 cells occurs by a ligand-independent mechanism (12). The PPARγ coactivator PGC1α is capable of activating PPARγ in the absence of PPARγ ligand, and furthermore is regulated by both cAMP/PKA and p38 MAPK in a number of cells (29, 30). Therefore, the effect of relaxin on the expression level of PGC1α was tested. Relaxin increased the RNA expression level of PGC1α in HEK-RXFP1 cells (Fig. 4A). The increased RNA expression was evident by 2 h of treatment, and started decreasing by 4 h. This increase in expression level...
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FIGURE 3. CREB mediates relaxin-induced PPAR activity. A, HEK-RXFP1 cells were transfected with CRE and Renilla luciferase reporters. The cells were treated for 24 h with or without 1 nM relaxin and subjected to dual luciferase assay. B, HEK-RXFP1 cells were transfected with the dominant-negative CREB construct (pCMV-KCREB), or empty vector (pCMV), and the PPRE and Renilla luciferase reporters. The cells were treated for 24 h with or without 1 nM relaxin and subjected to dual luciferase assay. The data are expressed as the luciferase activity relative to untreated cells, mean ± S.E., n = 3. *** p < 0.001 compared with untreated control. C, HEK-RXFP1 cells were pretreated for 30 min with 20 μM H89, 20 μM PD169316 (PD16), or vehicle, then treated 30 min in the presence or absence of 1 nM relaxin. Lnysates were analyzed by Western blotting for total and phosphorylated CREB. D, HEK-RXFP1 cells were transfected with PKA, p38 MAPK, or nontargeting siRNA, then were treated 30 min with or without 1 nM relaxin. The activation of CREB phosphorylation with by the p38 MAPK inhibitor (Fig. 5E). Furthermore, relaxin stimulated phosphorylation of CREB was reduced by both H89 and the p38 MAPK inhibitor (Fig. 5F). The degree of reduction of relaxin-stimulated CREB phosphorylation by with the p38 MAPK inhibitor (38.4 ± 4.8%) compares favorably to the response seen in HEK-RXFP1 cells. Finally, the expression level of PGC1α was increased in response to relaxin (Fig. 5G). Taken together, these results confirm that the signaling pathways leading to PGC1α expression and PPARγ activation are intact in LX2 cells endogenously expressing RXFP1.

DISCUSSION

We have previously shown that relaxin treatment of HEK-RXFP1 cells caused increased transcriptional activity of PPARγ (12). This activation did not require addition of an exogenous PPARγ ligand, nor was it inhibited by a PPARγ ligand-binding inhibitor, suggesting a ligand-independent mechanism of activation. Recently, another study showed relaxin activation of PPARγ in parenchymal brain arterioles, where it regulated hypertrophic outward remodeling (22). In contrast to activation of PPARγ in HEK-RXFP1 cells, the effect in brain arterioles appears to be ligand-dependent, suggesting that the mechanisms of relaxin regulation of PPARγ are tissue-specific. We sought to elucidate the mechanisms for the ligand-independent activation of PPARγ in HEK-RXFP1 cells, and in a cell line naturally expressing RXFP1, the LX2 hepatic stellate cells.

Relaxin binding to RXFP1 activates a number of signal transduction pathways, the most completely studied of which is through cAMP and PKA. Depending on the cell type, RXFP1 couples to multiple G-proteins to activate these pathways. In a number of cells, including the stably transfected HEK-RXFP1 cells, and some cells endogenously expressing RXFP1 such as THP-1 and HeLa cells, RXFP1 alters cAMP levels in a biphasic manner, initially coupling to Go to decrease cAMP, followed by a delayed activation of adenylyl cyclase by Gi. The involvement of PGC1α in the ability of relaxin to stimulate PPARγ activation was examined using siRNA-mediated knockdown of PGC1α (Fig. 4D). Furthermore, coexpression of the dominant-negative KCREB construct decreased the relaxin-stimulated increase in PGC1α protein levels (Fig. 4E). The involvement of PGC1α in the ability of relaxin to stimulate PPARγ activation was examined using siRNA-mediated knockdown of PGC1α (Fig. 4, F and G). The PGC1α-siRNA reduced the stimulation of PPARγ activity by relaxin, while control siRNA had no effect, suggesting that PGC1α mediates the effect of relaxin on PPARγ. Overall, these results suggest that relaxin increases PGC1α expression through PKA and CREB activation.

Relaxin Regulates PGC1α in Cells Endogenously Expressing RXFP1—The signaling pathways activated by relaxin in HEK-RXFP1 cells were tested in cells naturally expressing RXFP1. In the liver, the myofibroblastic hepatic stellate cells are the predominant RXFP1-expressing cells (31, 32). A human hepatic stellate cell line (LX2) that recapitulates many of the properties of activated hepatic stellate cells (27) was used. Relaxin induced an increase in cAMP levels (Fig. 5A) and activated PKA (Fig. 5B), confirming that LX2 cells are responsive to relaxin. Expression of RXFP1 was detected in LX2 cells at a modest level compared with HEK-RXFP1 cells or THP1 monocytes previously shown to express high levels of RXFP1 (Fig. 5C). Relaxin treatment resulted in an increase in transcription through the PPRL reporter, consistent with activation of PPARγ (Fig. 5D). Similar to the results using HEK-RXFP1 cells, relaxin stimulated phosphorylation of p38 MAPK (Fig. 5E). Furthermore, relaxin stimulated phosphorylation of CREB was reduced by both H89 and the p38 MAPK inhibitor (Fig. 5F). The degree of reduction of relaxin-stimulated CREB phosphorylation with the p38 MAPK inhibitor (38.4 ± 4.8%) compares favorably to the response seen in HEK-RXFP1 cells. Finally, the expression level of PGC1α was increased in response to relaxin (Fig. 5G). Taken together, these results confirm that the signaling pathways leading to PGC1α expression and PPARγ activation are intact in LX2 cells endogenously expressing RXFP1.
Depending on the cell type, other pathways are triggered by RXFP1, including nitric oxide, ERK1/2 and p38 MAPK (1, 2). Importantly, all of these pathways have been implicated in the regulation of PPARγ/H9253 (33–35). In a number of cell types, activation results in activation of various nitric-oxide synthases to mediate physiological responses (2, 10, 36). However, in the present study, activation of PPARγ/H9253 was not stimulated by the addition of nitric oxide donors, nor was the effect of relaxin reduced with a nitric-oxide synthase inhibitor, suggesting that the nitric oxide pathway was not involved in regulation of PPARγ/H9253 activation. Similarly, ERK1/2 inhibitor had no effect on relaxin-stimulated PPARγ/H9253 activity, suggesting that this pathway is not involved in this effect. There have been few studies of the role of p38 MAPK in mediating relaxin effects. In human periodontal ligament cells, relaxin regulation of matrix metalloproteinase expression was reduced by a p38 MAPK inhibitor (37), and relaxin potentiated BNP2-mediated p38 MAPK activation in multipotent mesenchymal cells (38). Relaxin induced activation of p38 MAPK in vascular smooth muscle cells, but not HeLa or vascular endothelial cells (39) or renal fibroblasts (40). In the present study, a p38 MAPK inhibitor partially blocked relaxin’s effects, and relaxin treatment rapidly and transiently induced phosphorylation of p38 MAPK, which returned to baseline by 30 min. Therefore, it is possible that the earlier studies, which employed a much longer period of treatment (45–60 min), may have missed the peak of p38 MAPK phosphorylation in those cells. The phosphorylation of p38 MAPK in HEK-RXFP1 was downstream of PKA, as it was completely blocked by H89.

A major effector of gene transcription downstream of cAMP/PKA signaling is CREB. Relaxin treatment of human endometrial stromal cells resulted in rapid and transient phosphorylation of p38 MAPK, which returned to baseline by 30 min. Therefore, it is possible that the earlier studies, which employed a much longer period of treatment (45–60 min), may have missed the peak of p38 MAPK phosphorylation in those cells. The phosphorylation of p38 MAPK in HEK-RXFP1 was downstream of PKA, as it was completely blocked by H89.

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**FIGURE 4.** PGC1α is the mechanism for activation of PPARγ activity by relaxin. A, HEK-RXFP1 cells were treated at the indicated times with 1 nM relaxin, and the mRNA for PGC1α was quantified by real time RT-PCR relative to that of TBP. Data are expressed as fold expression of PGC1α compared with untreated cells. B, HEK-RXFP1 cells were transfected with PGC1α-promoter and Renilla luciferase reporters, treated 24 h with 1 nM relaxin, then subject to dual-luciferase assay. C, HEK-RXFP1 cells were treated with 1 nM relaxin for the indicated times, and lysates were analyzed by Western blotting PGC1α and GAPDH as indicated. D, HEK-RXFP1 cells were pretreated 20 μM H89 or vehicle, then incubated for 2 h in the presence or absence of 1 nM relaxin. The mRNA for PGC1α was quantified by real time RT-PCR relative to that of TBP. Data are expressed as fold expression of PGC1α compared with untreated cells. E, HEK-RXFP1 cells were transfected with KCREB or control plasmid, then cells were treated with 1 nM relaxin for 4.5 h. Lysates were analyzed by Western blotting for PGC1α and GAPDH as indicated. F and G, HEK-RXFP1 cells were cotransfected with control or PGC1α siRNA, PPRE and Renilla reporter vectors. After 24 h, the levels of PGC1α were reduced as determined by Western blot (F). Cells were then treated with or without 1 nM relaxin for 24 h and subjected to dual luciferase assay (G). The data are expressed as the luciferase activity relative to untreated cells, mean ± S.E., n = 3. *, p < 0.05; **, p < 0.01.
tor, suggesting a bifurcation in signaling at PKA, through p38 MAPK and converging at CREB. This concept was further supported by the use of the dominant negative form of CREB, which completely inhibited PPRE activation in response to relaxin.

Previously, we reported that relaxin activation of PPARγ was ligand-independent, but the mechanism was unknown. The transcriptional activity of PPAR can be regulated by phosphorylation. We found no evidence of altered PPARγ phosphorylation in response to relaxin (data not shown). Another mechanism is by association with coactivator and corepressor proteins. Of particular interest was PGC1α, which was discovered as a coactivator of PPARγ in brown adipose tissue, where it acts to regulate proteins involved in thermogenic programming, such as uncoupling protein-1 (43). PGC1α is capable of ligand-independent binding and activation of PPARγ (44). Furthermore, PKA, p38 MAPK and CREB all participate in activation of PGC1α. In muscle and liver, increased PGC1α expression was triggered by cAMP/PKA or p38 MAPK activation, and in many cases, CREB was found to be a downstream target of p38 MAPK (25, 45–49). The expression of PGC1α is regulated by CREB through a consensus CRE sequence in the PGC1α promoter (25, 45). Therefore, PGC1α was possible mechanism for ligand-independent activation of PPARγ by relaxin.

As discussed earlier, cells expressing RXFP1 differ in their signaling properties in response to relaxin, and it is important to compare results in cells with induced expression (such as HEK-RXFP1 cells) with cells endogenously expressing RXFP1 (50, 51). We previously showed that primary rat and mouse hepatic cells expressed RXFP1, and responded to relaxin.
In this study, we used LX2 cells, a human hepatic stellate cell line that recapitulates many of the properties of primary cells (27). Relaxin treatment caused an elevation of cAMP and activation of PKA, and RXFP1 mRNA was detected in LX2 cells, confirming that RXFP1 is expressed and active in these cells. Relaxin also activated transcription through the PPRE reporter vector. Both p38-MAPK and CREB were phosphorylated in response to relaxin, and CREB phosphorylation was reduced completely by a PKA inhibitor and partially by a p38-MAPK inhibitor. Finally, PGC1α expression was increased by relaxin treatment of LX2 cells. These data suggest that the RXFP1 signaling pathways leading to PGC1α expression are similar in both HEK-RXFP1 and LX2 cells.

In summary, we have provided the first evidence of ligand-independent activation of PPARγ by relaxin. The overall scheme is shown in Fig. 6. Relaxin activates RXFP1 which stimulates cAMP through PI3K-dependent and independent pathways to generation to activate PKA. This leads to phosphorylation of CREB, directly via PKA and indirectly by p38-MAPK. Activation of CREB leads to expression of PGC1α, which associates with PPARγ to increase its transcriptional activity. It is important to note that PGC1α is also phosphorylated by a number of kinases, including PKA and p38-MAPK, to regulate transcriptional activity and protein stability (29, 30). Therefore, although we found that PGC1α gene expression was up-regulated in response to relaxin, we cannot rule out a role for PGC1α phosphorylation in mediating relaxin’s effects. Nevertheless, these studies provide the first evidence of PGC1α as a target of relaxin signaling, and provide the mechanism for the ligand-independent activation of PPARγ by relaxin.

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REFERENCES

1075–1084
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1492–1504
18793–18799


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