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Relaxin Family Peptide Receptor 1 (RXFP1) Activation Stimulates the Peroxisome Proliferator-Activated Receptor Gamma

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Abstract

Relaxin (Rlx) has antifibrotic effects in a number of tissues. Many of these effects are similar to those induced by the activators of peroxisome proliferator-activated receptor γ (PPAR γ), raising the possibility that a mechanism for Rlx's antifibrotic effects may involve activation of the PPAR γ pathway. This study investigates the effect of Rlx on PPARs and their mechanism of upregulation. It shows that Rlx stimulates ligand-independent PPAR activation in a dose-dependent manner. The combined effect of Rlx and PPAR γ agonists was superadditive, suggesting that both agents might be used together for an increased antifibrotic effect. Rlx caused increased expression of the PPAR γ target genes CD36 and LXR α . Rlx's effect was mimicked by forskolin, and partially blocked by pertussis toxin, suggesting that Rlx is working through a cAMP/PKA pathway to activate PPAR γ . A better understanding of this pathway might help in the amelioration of fibrotic diseases.

Introduction

Rlx is known to be a reproductive hormone by virtue of its action in softening of the cervix and vagina¹. Its action involves widespread remodeling of the extracellular matrix involving altered secretion and degradation of matrix components². The role of Rlx has been extended to nonreproductive tissues. Rlx has demonstrated antifibrotic actions on a number of cells, tissues and organs including lung, liver, heart and kidney^{2, 3}. Experimentally induced fibrosis in mouse lung and rat kidney was restored to a normal phenotype after Rlx treatment². Rlx-null mice developed pulmonary, cardiac and renal fibrosis, which could be reversed with Rlx treatment, suggesting a protective function for Rlx against fibrosis⁴. In the liver and liver fibroblasts, Rlx increased matrix metalloproteinase activity, decreased TIMP expression and reduced collagen production, suggesting a useful role in the treatment of hepatic fibrosis^{5, 6}. Many of these effects are similar to those seen with agonists of PPAR γ .

PPARs are nuclear transcription factors that heterodimerize with the 9-cis retinoic acid receptor (RXR) to induce transcription of target genes through their binding to PPAR response elements (PPRE)⁷. Three types of PPARs (PPAR α , β/δ and γ) have been identified, and all bind to similar PPRE sequences⁷. Recent studies have implicated PPARs

in the control of fibrosis^{8–10}. Treatment with PPAR ligands, or expression of PPARs, suppresses fibrosis in organs such as heart, liver, kidney, and lung⁸. PPAR γ agonists have been shown to prevent experimentally-induced fibrosis in some of these organs^{9, 11}.

Because Rlx and PPAR γ share a number of antifibrotic effects, it is possible that they possess common signaling pathways. Activation of PPAR γ may be one possible downstream mechanism for the antifibrotic effects of Rlx. Therefore, we examined the effect of Rlx on PPAR γ activity.

Material and methods

HEK-293T cells and THP1 monocytes were used for this study. To measure PPRE activity, 293T cells were transiently transfected with a RXFP1 expression plasmid¹² (kindly provided by Aaron Hsueh, Stanford University), along with a reporter plasmid containing three tandem copies of the acyl CoA oxidase PPRE upstream of the firefly luciferase gene¹³ (ACO-PPRE-luc) (kindly provided by Brian Seed, Harvard University), and a renilla luciferase construct as a transfection control. Fugene-6 (Roche) was used as transfection reagent. Cells were treated for 24 hours with porcine Rlx (kindly provided by O.D. Sherwood, University of Illinois at Urbana-Champaign) or human InsL3 or Rlx-3 (Phoenix Pharmaceuticals). In some experiments, agonists of PPAR α (WY14643, Calbiochem), PPAR β/δ (GW0742), PPAR γ rosiglitazone (rosi), 15-deoxy δ 12,14-prostaglandinJ2 (15dPGJ2), and the PPAR γ ligand blocker (GW9662) (Cayman) were used. To elucidate signaling pathways, cells were treated with Forskolin, isobutylmethyl xanthine (IBMX), L-NAME (Sigma), Pertussis toxin, PD98059, LY294002 (Calbiochem), or PD169316 (Cayman). Firefly and renilla luciferase activities were measured using Dual-Glo luciferase assay kit (Promega). The gene expression levels of CD36 and LXR α were determined using Taqman real-time RT-PCR (Applied Biosystems), using 18S ribosome of RNA as an endogenous control, as shown previously¹⁴. Western blots for CD36 were performed using a rabbit polyclonal antibody (Abcam), as shown previously⁵.

Results

Rlx increased PPRE activity

A PPRE reporter assay was performed to investigate the effects of Rlx, Rlx-3 and InsL3 on PPARs. Rlx increased PPRE activation in a concentration-dependent manner, maximally at 1nM, with an EC₅₀ of 24 pM. In contrast, InsL3, which has low affinity for RXFP1 did not activate PPRE even at a concentration of 100nM, and Rlx-3, which has lower affinity for RXFP1 as compared to Rlx¹⁵, activated PPRE only at the highest concentration tested (100 nM) (data not shown). HEK293T cells which were not transfected with RXFP1 did not show any change in PPRE activity in response to Rlx (data not shown). These results suggest that Rlx increases the activity of one or more PPARs, and that the effect of Rlx is consistent with activation of RXFP1.

Rlx increased the activity of PPAR γ

To further study the involvement of individual PPARs, specific PPAR agonists were used in the PPRE reporter assay. Rlx alone, in the absence of exogenous PPAR agonists, increased the activity by more than 70% as compared to control. PPAR α and PPAR β/δ agonists (WY14643 and GW0742 respectively) had little or no effect on activity of PPRE compared to control (data not shown). The combination of PPAR α or PPAR β/δ agonists with Rlx produced an activity which was similar to that of Rlx alone (data not shown). In contrast, the PPAR γ agonist rosi increased PPRE activity, and the combined effect of Rlx with rosi was more than additive (Rlx 169.2 $\pm$ 9.1%, rosi 158.7 $\pm$ 15.0%, Rlx + rosi 270 $\pm$ 25.9%). Similar

effects were also seen with 15dPGJ2, another PPAR γ agonist (15dPGJ2 $135.5 \pm 22.1\%$, Rlx + 15dPGJ2 $248 \pm 41.6\%$). Because PPAR γ agonists in combination with Rlx had a superadditive increase in PPRE activity, we chose to focus on the effect of Rlx on PPAR γ .

Rlx stimulated ligand-independent PPAR γ activation

The previous data showed Rlx activation of PPAR γ in the absence of exogenous ligand. PPAR γ is activated by binding of its ligand, but can also be regulated in a ligand independent manner by coactivators, corepressors and post translational modifications such as phosphorylation^{16–18}. To determine whether the increase in PPRE activation by Rlx is dependent on ligand binding, we studied Rlx-mediated activation of PPAR γ using a PPAR γ ligand binding blocker (GW9662). In the presence of GW9662, PPRE activation in response to rosi was reduced, but there was no effect on the Rlx-induced response. GW9662 partially blocked the response of Rlx and rosi combined, reducing the response to the level of Rlx alone. This suggests that Rlx stimulates ligand-independent PPAR γ activation.

Rlx increased PPAR γ target genes

An increase in PPAR γ activity by Rlx should result in an increase in the expression level of PPAR γ target genes such as CD36 and LXR α . To determine the effects of Rlx on target genes, HEK293T cells transiently transfected to express RXFP1 were treated with Rlx, and the expression levels of CD36 and LXR α were determined by real-time RT-PCR. Rlx increased the gene expression of PPAR γ target genes as compared to control. These results were confirmed in THP1 cells, which naturally express RXFP-1. Furthermore, Rlx combined with rosi caused a synergistic increase in the gene expression level of CD36. The increase in CD36 by Rlx was further validated by western blotting using THP-1 cells. These data suggest the activation of PPAR γ in response to Rlx.

Rlx increased cAMP to induce PPAR γ activation

Rlx has been reported to activate a number of signaling pathways¹. To investigate possible signaling pathways triggered by Rlx to stimulate PPAR γ activity, we exposed 293T cells expressing RXFP1 to various signaling inhibitors. Inhibitors of MAPK (PD98059, 20uM), p38-MAPK (PD169316, 1uM), PI-3K (LY294002, 50uM), or nitric oxide (L-NAME, 3mM) had no effect on Rlx-stimulated PPAR γ activity. In contrast, the adenylate cyclase stimulator forskolin (2uM) mimicked the Rlx effects. There was no effect of the PDE inhibitor IBMX (200uM), but the G $_i$ pathway inhibitor pertussis toxin (100ng/ml) partially blocked Rlx's effects. Taken together, these data suggest that Rlx acts through a cAMP-mediated mechanism that may involve G $_s$ and G $_i$ pathways.

Discussion

Rlx has diverse functions. It has been shown to activate many pathways making it a pleiotropic hormone¹. We have shown for the first time evidence of Rlx activation of the nuclear receptor PPAR γ . All the three PPARs known so far can recognize similar PPRE region in genes and activate them. Rlx enhanced the PPRE activity in a dose- dependent manner suggesting that Rlx increased the activity of one or more PPARs. Rlx-3 only activated PPRE at a high concentration (100nM), approximately 1000 times less potent than Rlx. This was surprising, because Rlx-3 is only 30–50 fold less potent than Rlx at stimulating cAMP production through RXFP1¹⁵. One possible explanation could be because of Rlx-3 triggering a signaling pathway different from that of Rlx. Rlx did not stimulate PPRE in untransfected cells, which do not express RXFP1. Taken together, this suggests that Rlx activation of PPRE activity is mediated through RXFP1.

PPRE activity was increased by PPAR γ agonists, and when combined with Rlx caused a superadditive effect. In contrast, PPAR α and PPAR β/δ agonists had little or no effect on PPRE activity with or without Rlx. This suggests that PPAR γ is the likely target of Rlx, therefore, we focused on PPAR γ . However, a possible role for the remaining two PPARs can not be ruled out at this stage of investigation. Rlx increased the gene expression of the PPAR γ target genes CD36 and LXR α , in cells overexpressing RXFP1. Rlx and the PPAR γ agonist rosi caused a synergistic increase in the expression of CD36 in THP1 cells. This suggests a relationship between Rlx and PPAR γ .

PPARs are transcription factors consisting of a ligand binding domain and a DNA binding domain. One of the possible ways Rlx could activate PPAR γ is through ligand binding. On ligand binding, PPAR γ heterodimerizes with RXR to bind and stimulate PPRE activation⁷. The presence of coactivators, corepressors or phosphorylation can also affect PPAR γ activity¹⁶⁻¹⁷. The ligand blocker GW9662 modifies the ligand binding site of PPAR γ and blocks the binding of ligand to PPAR γ . GW9662 had no inhibitory effect on Rlx activation of PPRE, suggesting that Rlx works independent of a ligand binding mechanism. Ligand-independent activation of PPAR γ by Rlx might involve other regulators of PPAR γ activity, such as PGC1 α , p300, CBP, RXR, its ligand retinoic acid and many others, yet to be studied¹⁹⁻²⁰.

Rlx has been shown to activate cAMP/PKA, nitric oxide, MAPK and other pathways making it very diverse in action¹. Rlx activation of PPAR γ seems to involve a cAMP-mediated mechanism that may involve the Gs and Gi pathways, because forskolin mimicked Rlx effects and pertussis toxin partially blocked Rlx's effects. However, as stated earlier, Rlx-3-induced cAMP production through RXFP1 is only 30–50 fold less potent than relaxin¹⁵, yet in our study Rlx-3 only detectably stimulated PPRE activation at a very high concentration (100nM), suggesting that cAMP production alone may not be responsible for relaxin's stimulation of PPAR γ . However, in our study, Rlx does not seem to act on PPAR γ activity through the MAPK, P38-MAPK, PI3K, or Nitric oxide pathways, as inhibitors of these pathways did not reduce the PPRE activity.

In summary, we have shown that Rlx increases the activity of PPAR γ through a ligand-independent mechanism. This activation of PPAR γ seems to involve cAMP/PKA pathway. The combination of Rlx with PPAR γ agonists produced a synergistic effect on expression of PPAR γ target genes. PPAR γ has diverse biological implications in diseases ranging from obesity, diabetes, immunological disorders, asthma, atherosclerosis, cancer, fibrosis and Alzheimer's disease²¹⁻²⁴. The synergistic effects of Rlx with PPAR γ agonist and better understanding of the signaling pathway will help to develop new therapies in diseases involving PPAR γ .

Acknowledgments

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Key words

(PPAR)	Peroxisome Proliferator Activated Receptor
(Rlx-3)	Relaxin-3
(Insl3)	Insulin-like Peptides
(PPRE)	Peroxisome Proliferator Response Element

(RXFP-1) Relaxin Family Peptide Receptor1**References**

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