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IGFBP-3, Hypoxia and TNF- α Inhibit Adiponectin Transcription

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Abstract

The thiazolidinedione rosiglitazone, an agonist ligand for the nuclear receptor PPAR- γ , improves insulin sensitivity in part by stimulating transcription of the insulin-sensitizing adipokine adiponectin. It activates PPAR- γ -RXR- α heterodimers bound to PPAR- γ response elements in the adiponectin promoter. Rosiglitazone-stimulated adiponectin protein synthesis in 3T3-L1 mouse adipocytes has been shown to be inhibited by IGFBP-3, which can be induced by hypoxia and the proinflammatory cytokine, TNF- α , two inhibitors of adiponectin transcription. The present study demonstrates that IGFBP-3, the hypoxia mimetic agent cobalt chloride, and TNF- α inhibit rosiglitazone-induced adiponectin transcription in mouse embryo fibroblasts that stably express PPAR- γ 2. Native IGFBP-3 can bind RXR- α and inhibited rosiglitazone-stimulated promoter activity, whereas an IGFBP-3 mutant that does not bind RXR- α did not. These results suggest that IGFBP-3 may mediate the inhibition of adiponectin transcription by hypoxia and TNF- α , and that IGFBP-3 binding to RXR- α may be required for the observed inhibition.

Keywords

Insulin-like Growth Factor Binding Protein-3 (IGFBP-3); Adiponectin; Retinoid X Receptor- α (RXR- α); Hypoxia; Tumor Necrosis Factor- α (TNF- α)

Adiponectin, a protein synthesized and secreted by white adipose tissue, circulates in plasma at high concentrations, promotes insulin-sensitivity and has anti-atherosclerotic, anti-thrombotic and anti-inflammatory activity [1;2;3]. Low circulating levels of adiponectin in obese or overweight patients are thought to contribute to the pathogenesis of the metabolic syndrome, a cluster of risk factors for type 2 diabetes mellitus and cardiovascular disease with abdominal obesity and insulin resistance playing a central role [3;4;5]. Adiponectin binds to adiponectin receptors in liver and skeletal muscle, activating AMP-activated protein kinase to stimulate fatty acid oxidation and decrease triglyceride content [6].

Drugs that increase circulating adiponectin levels provide a potential therapeutic strategy to overcome the contributions of low adiponectin levels to the pathogenesis of the metabolic syndrome [7]. Thiazolidinediones, high affinity agonist ligands for the nuclear receptor

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peroxisome proliferator-activated receptor- γ (PPAR- γ), induce adiponectin gene expression and increase insulin sensitivity [8;9;10]. They activate PPAR- γ -retinoid X receptor- α (RXR- α) heterodimers that are constitutively bound to PPAR- γ response elements (PPREs) in the promoter of adiponectin [11] and other target genes [12] to stimulate transcription [9;11].

IGF binding protein-3 (IGFBP-3), the most abundant IGF-binding protein in plasma, inhibits the increase in adiponectin protein in 3T3-L1 mouse adipocytes induced by the thiazolidinedione rosiglitazone [13]. IGFBP-3 binds to RXR- α [14;15] and disrupts retinoic acid receptor (RAR)-RXR- α nuclear receptor heterodimers resulting in transcription inhibition [14;15]. In the present study, we examine whether IGFBP-3 also inhibits thiazolidinedione-induced adiponectin transcription triggered by activated PPAR- γ -RXR- α heterodimers and, if so, whether binding to RXR- α is required. We previously showed that an IGFBP-3 mutant which does not bind RXR- α is able to induce apoptosis in human prostate cancer cells [16].

In obesity, adipose tissue exists in a chronic inflammatory state that induces the synthesis of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) [17]. Hypoxia also occurs in fat depots as their mass increases [18;19;20], and the hypoxic microenvironment is thought to contribute to the development of the inflammatory state [20;21]. Both the hypoxic microenvironment [19;22] and increased TNF- α [10;23] in adipose tissue in obesity inhibit adiponectin gene expression. IGFBP-3 expression is induced by both hypoxia [24;25] and TNF- α [13;26;27;28], raising the possibility that IGFBP-3 might mediate their inhibitory effects on adiponectin transcription.

In the present study, we determined whether IGFBP-3, hypoxia and TNF- α inhibit rosiglitazone-stimulated, PPAR- γ -mediated adiponectin promoter activity. Mouse embryonic fibroblasts that stably express PPAR- γ 2 [29;30] were transfected with a luciferase reporter plasmid containing 3 copies of the PPRE sequence of the human adiponectin promoter [11]. Native IGFBP-3, but not the IGFBP-3 mutant that does not bind RXR- α , decreased PPRE-dependent adiponectin promoter activity. The hypoxia mimetic agent, cobalt chloride [21], and TNF- α also decreased rosiglitazone-stimulated adiponectin promoter activity, suggesting that inhibition of adiponectin transcription may be a common mechanism by which they contribute to the pathogenesis of insulin resistance in obesity.

Material and Methods

Materials

Non-glycosylated recombinant human IGFBP-3 was obtained from Novozymes Gro-Pep (Adelaide, Australia). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). Cobalt chloride and TNF- α were obtained from Sigma.

Cell Cultures

Mouse embryonic fibroblasts (MEF) stably expressing PPAR- γ 2 (MEF-PPAR- γ 2) were a kind gift of Dr. Kai Ge (NIDDK) [29;30]. The cells were cultivated in DMEM (Gibco-Invitrogen Corporation; Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) in a humidified atmosphere containing 5% CO₂ at 37 °C, and were split at subconfluent densities to prevent them from differentiating into adipocytes.

Adiponectin PPRE-luciferase reporter and IGFBP-3 expression plasmids

A luciferase reporter plasmid, pGL3-Adipo-PPRE-SV40-Luc, was constructed containing 3 copies of the PPRE element of the human adiponectin promoter (TGACTT T TGCCCC) [11] inserted upstream from the SV40 promoter-luciferase transcriptional unit. Sense and antisense oligonucleotides (5'-

ATGCATGCTAGCGTGACTTTTGCCCCAAGTGACTTTTGCCCCAAGTGACTTTTGC
 CCCAAAGATCTATGCAT-3' and 5'-
 ATGCATAGATCTTTGGGGCAAAGTCACCTGGGGCAAAGTCACCTGGGGCA
 AAAGTCACGCTAGCATGCAT-3'), respectively, were inserted into the NheI and BglII sites
 of pGL3-Promoter Vector (Promega; Madison, WI). The sequences were confirmed by DNA
 sequencing. Mutation of the adiponectin PPRE abolished thiazolidinedione induction of
 adiponectin transcription [11].

Plasmids expressing a fusion protein of yellow fluorescent protein (YFP) with wild-type
 IGFBP-3, YFP-IGFBP-3 [31], or with an IGFBP-3 mutant in which the heparin binding domain
 (HBD) was mutated so that it does not bind RXR- α , YFP-HBD-11m-IGFBP-3 [16], were
 previously described.

Adiponectin promoter assay

MEF-PPAR- γ 2 fibroblasts (8×10^3 cells / well) were plated in 24-well plates (Costar, Corning
 Life Sciences, Corning, NY) and transfected with 25 ng of pGL3-Adipo-PPRE-SV40-Luc or
 pGL3-Promoter vector using Lipofectamine Plus (Invitrogen) according to the manufacturer's
 instructions. In some experiments, the cells were cotransfected with 100 ng of YFP-WT-
 IGFBP-3, YFP-HBD-11m-IGFBP-3 or YFP plasmid DNA [16]. After 24 h, the cells were
 washed with PBS and treated with rosiglitazone (0.4 mM final concentration) or dimethyl
 sulfoxide vehicle, and with IGFBP-3, cobalt chloride or TNF- α as indicated in medium
 containing 2.5% charcoal-treated fetal bovine serum. After 24 h, luciferase activity was
 measured in cell lysates using a dual luciferase reporter assay system (Promega, Madison, WI)
 and a Centro XS3 Microplate Luminometer LB 960 (Berthold Technologies, Oak Ridge, TN).

Results

IGFBP-3 inhibits rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity in MEF-PPAR- γ fibroblasts

To examine the effect of IGFBP-3 on rosiglitazone-stimulated adiponectin promoter activity,
 MEF-PPAR- γ fibroblasts cells were transiently transfected with a luciferase reporter plasmid
 containing a basal SV40 promoter with or without 3 copies of the human adiponectin promoter
 PPRE, and a plasmid expressing a wild-type IGFBP-3-YFP fusion protein or YFP alone, and
 treated with rosiglitazone or vehicle (Fig. 1A). Rosiglitazone stimulated luciferase activity
 approximately 4-fold in the pGL3 reporter plasmid containing the human adiponectin PPRE
 (lane 10 vs lane 4). No significant promoter activity was seen in the presence or absence of
 rosiglitazone using the pGL3 control plasmid that contains only the basal SV40 promoter (lanes
 1-3 and 7-9).

Coexpression of YFP-wild-type IGFBP-3 inhibited rosiglitazone-stimulated adiponectin
 promoter activity approximately 35% (lane 11 vs lane 10), but had no effect on adiponectin
 promoter activity in the absence of rosiglitazone (lane 5 vs lane 4). These results indicate that
 expression of wild-type IGFBP-3 specifically inhibited rosiglitazone-stimulated adiponectin
 PPRE-dependent promoter activity. Recombinant human IGFBP-3 protein inhibited
 rosiglitazone-stimulated adiponectin promoter activity to the same extent as expressed
 IGFBP-3 (Fig. 1B).

IGFBP-3 can bind to RXR- α , the heterodimer partner of PPAR- γ , RAR and other nuclear
 receptors, and has been shown to inhibit transcription mediated by RXR-RAR heterodimers
 in human prostate cancer cells [14]. To determine whether IGFBP-3 needed to bind to RXR- α
 to inhibit rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity, we
 transfected the HBD-11m-IGFBP-3 mutant that does not bind RXR- α . In contrast to wild-type

IGFBP-3 (Fig. 1A, lane 11), HBD-11m-IGFBP-3 did not inhibit rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity (lane 12), consistent with the hypothesis that direct interaction of IGFBP-3 with RXR- α is required for IGFBP-3 inhibition of PPRE-dependent adiponectin promoter activity.

Rosiglitazone-induced adiponectin promoter activity is inhibited by hypoxic conditions and by the cytokine TNF- α

In obesity, increased synthesis of proinflammatory cytokines such as TNF- α [17] and hypoxic conditions in adipose tissue contribute to the development of a chronic inflammatory state [21]. Both of these conditions reduce adiponectin protein and mRNA in 3T3-L1 cells under basal conditions [10;19;20;22;23], and induce IGFBP-3 [13;24;25;26;27;28]. We examined whether the inhibition of adiponectin expression induced by these factors occurred at the transcriptional level as a result of the inhibition of PPRE-dependent adiponectin promoter activity.

Cobalt chloride, an inducer of the transcription factor Hypoxia Inducible Factor- α (HIF- α) [21;32], a major regulator of genes involved in the response to hypoxia [33], was used to mimic hypoxia. MEF-PPAR- γ 2 fibroblasts were transfected with pGL3-Adipo-PPRE-SV40-Luc and, after 24 h, were incubated with rosiglitazone (lanes 5-8) or vehicle (lanes 1-4) and increasing concentrations of CoCl₂ (Fig. 2). In the absence of CoCl₂, rosiglitazone stimulated luciferase activity approximately 4-fold (lane 5 vs lane 1). In rosiglitazone-stimulated cells, CoCl₂ inhibited luciferase activity in a concentration-dependent manner. Inhibition was ~50% at 10 μ M CoCl₂ (lane 6) and ~85% at 100 μ M (lane 8). By contrast, in control cells inhibition was observed only at the highest CoCl₂ concentration (100 μ M; lane 4). It previously was reported that hypoxia (1% O₂) inhibited adiponectin promoter activity in 3T3-L1 mouse adipocytes under basal [19] or thiazolidinedione-stimulated [20] conditions using 3.6 kb [19] or 1.3 kb [20] promoter fragments. Our results extend these findings by showing that the adiponectin PPRE alone is sufficient to confer dose-dependent inhibition of promoter activity by a hypoxia-mimetic agent when the promoter is activated by rosiglitazone.

TNF- α can promote insulin resistance associated with obesity by several mechanisms including inhibition of adiponectin expression [10;17;23]. TNF- α reduced adiponectin mRNA and protein levels in 3T3-L1 cells under basal conditions [10;23], but TNF- α treatment did not decrease adiponectin mRNA in thiazolidinedione-stimulated cells, even though the cytokine inhibited adiponectin promoter activity using a 2.0 kb promoter fragment in both thiazolidinedione-stimulated and basal cells [10]. We examined the effect of TNF- α on rosiglitazone-stimulated adiponectin promoter activity in MEF-PPAR- γ 2 fibroblasts. Cells were transfected with pGL3-Adipo-PPRE-SV40-Luc, and stimulated with rosiglitazone in the presence or absence of 50 ng/ml of recombinant TNF- α (Fig. 3). TNF- α decreased rosiglitazone-stimulated PPRE-dependent adiponectin promoter activity by ~55%. Since hypoxia and TNF- α induce IGFBP-3 expression transcription [13;24;25;26;27;28], and all three treatments inhibit PPRE-dependent adiponectin promoter activity, these results raise the possibility that IGFBP-3 may, at least in part, mediate the inhibition of adiponectin transcription by hypoxia and TNF- α .

Discussion

Circulating levels of the insulin-sensitizing adipokine, adiponectin, are reduced in obesity, contributing to insulin resistance and the metabolic syndrome, and greatly increasing the risk of type 2 diabetes and cardiovascular disease [3;4;5] (Fig. 4). Increasing adiponectin synthesis pharmacologically might help disrupt this pathogenetic process. Thiazolidinediones are insulin-sensitizing agonistic ligands for the nuclear receptor PPAR- γ that can activate PPAR- γ -RXR- α heterodimers bound to a PPRE in the adiponectin promoter to stimulate adiponectin

transcription [8;9;10;11]. The present study shows that IGFBP-3, hypoxia and TNF- α , three agents that promote insulin resistance *in vivo*, inhibit human adiponectin PPRE-dependent promoter activity stimulated by the thiazolidinedione rosiglitazone in mouse fibroblasts.

Expression of IGFBP-3 in transgenic mice induced hyperglycemia, glucose intolerance and insulin resistance [34], and IGFBP-3 inhibited insulin-stimulated glucose uptake in 3T3-L1 mouse adipocytes [13;35]. Treatment of 3T3-L1 cells with IGFBP-3 also decreased rosiglitazone-stimulated adiponectin protein expression [13]. IGFBP-3 can bind to the PPAR- γ heterodimer partner, RXR- α , and inhibited transcription stimulated by RAR-RXR- α by disrupting the heterodimer complex [14;15]. This led us to postulate that IGFBP-3 also might inhibit rosiglitazone-stimulated adiponectin transcription mediated by PPAR- γ -RXR- α heterodimers. Using immortalized mouse embryo fibroblasts that stably expressed PPAR- γ 2 [29;30], we confirmed that rosiglitazone stimulated adiponectin-PPRE-dependent promoter activity. Cotransfection with YFP-IGFBP-3 fusion proteins inhibited rosiglitazone-stimulated promoter activity (Fig. 4). Inhibition was a direct effect of IGFBP-3 since similar inhibition was observed using recombinant human IGFBP-3 protein.

To determine whether IGFBP-3 inhibition of adiponectin promoter activity required its binding to RXR- α , we determined whether rosiglitazone-induced adiponectin transcription would be inhibited by an IGFBP-3 mutant that does not bind RXR- α [14;16]. We previously reported that the RXR- α -non-binding IGFBP-3 mutant, YFP-HBD-11m-IGFBP-3, retained the ability of wild-type IGFBP-3 to induce apoptosis in human prostate cancer cells, indicating that direct binding of IGFBP-3 to RXR- α was not required for its pro-apoptotic activity [16]. When mouse embryo fibroblasts were co-transfected with YFP-HBD-11m-IGFBP-3 and a luciferase reporter plasmid containing 3 copies of the human adiponectin PPRE, no inhibition of rosiglitazone-stimulated promoter activity was observed, in contrast to the inhibition observed when wild-type YFP-IGFBP-3 was transfected. The inability of the RXR- α -non-binding IGFBP-3 mutant to inhibit thiazolidinedione-stimulated adiponectin PPRE-dependent promoter activity is consistent with the possibility that direct binding of IGFBP-3 to RXR- α in the RXR- α -PPAR- γ heterodimer might be required for the inhibition of transcription, as described for RAR-RXR- α heterodimers [14;15]. Further studies are necessary to determine whether the lack of inhibition is due to the inability of the IGFBP-3 mutant to bind RXR- α , however, since the COOH-terminal region that is mutated in HBD-11m-IGFBP-3 is highly basic and contains a functional nuclear localization signal as well as binding sites for other proteins besides RXR- α [31;36;37]. (During the final stages of preparation of this manuscript, it was reported that wild-type IGFBP-3 (but not an IGFBP-3 mutant analogous to our RXR- α nonbinding mutant) also can bind to PPAR- γ , providing an alternative mechanism by which IGFBP-3 can inhibit adiponectin transcription [38].)

Two pathophysiologic conditions that develop in obese adipose tissue, hypoxia and chronic inflammation, contribute to the pathogenesis of insulin resistance and the metabolic syndrome [17;18;19;21] (Fig. 4). Both hypoxia [19;22] and the proinflammatory cytokine TNF- α [10; 23] inhibited adiponectin expression in 3T3-L1 adipocytes and human adipose tissue fragments. It is intriguing that hypoxia [24;25] and TNF- α [13;26;27;28] also induce IGFBP-3 transcription (Fig. 4). This led us to examine whether a hypoxia-mimetic agent, cobalt chloride [21;32], and TNF- α could inhibit thiazolidinedione-stimulated adiponectin PPRE-dependent promoter activity in mouse embryo fibroblasts as did IGFBP-3. It previously was shown that hypoxia inhibited adiponectin transcription under basal [19] and thiazolidinedione-stimulated [20] conditions, and that TNF- α inhibited transcription under both basal and thiazolidinedione-stimulated conditions [10]. In these studies, 1.3-3.6 kb adiponectin promoter fragments were used, so that the observed inhibition of thiazolidinedione-stimulated promoter activity could not be specifically attributed to the PPRE in the adiponectin promoter. By contrast, our studies show that both hypoxia and TNF- α inhibited rosiglitazone-stimulated adiponectin promoter

activity using a reporter plasmid containing only 3 copies of the PPRE from the human adiponectin promoter, establishing that the PPRE itself is sufficient for the observed inhibition.

In summary, we have shown that IGFBP-3, hypoxia, and the pro-inflammatory cytokine TNF- α inhibit thiazolidinedione-stimulated adiponectin transcription, and may contribute to the pathogenesis of insulin resistance and the metabolic syndrome in patients with abdominal obesity (Fig. 4). Our results suggest that binding of IGFBP-3 to RXR- α in RXR- α -PPAR- γ heterodimers may be required for inhibition, and raise the possibility that the induction of IGFBP-3 by hypoxia and TNF- α may contribute to their ability to inhibit adiponectin transcription and promote insulin resistance.

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Fig. 1.

IGFBP-3 inhibits rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity in MEF-PPAR- γ 2 fibroblasts. A) Transient transfection of IGFBP-3. MEF-PPAR- γ 2 fibroblasts were transiently transfected with pGL3-Adipo-PPRE-SV40-Luc (lanes 4-6 and 10-12) or pGL3 promoter vector (lanes 1-3 and 7-9), and cotransfected with wild-type YFP-IGFBP-3 (lanes 2, 5, 8 and 11), mutant YFP-HBD-11m-IGFBP-3 (lanes 3, 6, 9 and 12) or YFP (lanes 1, 4, 7 and 10). After 24 h, the cells were treated with rosiglitazone (lanes 7-12) or dimethylsulfoxide vehicle (lanes 1-6). Luciferase activity was measured after 24 h incubation. The Mean $\pm$ SD of two experiments is plotted. B) Recombinant IGFBP-3 protein. MEF-PPAR- γ 2 fibroblasts were transfected with the pGL3-Adipo-PPRE-SV40-Luc reporter plasmid. After 24 h, rosiglitazone together with recombinant human IGFBP-3 (gray bar) or vehicle (black bar) were added and the incubation continued for another 24 h before luciferase activity was measured. Relative luciferase activity (Mean $\pm$ SD, n=2) is plotted. Control (vehicle) is plotted as 1.0.

Fig. 2.

Effect of the hypoxia-mimetic agent CoCl_2 on rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity. MEF-PPAR- γ 2 fibroblasts were transfected with pGL3-Adipo-PPRE-SV40-Luc. After 24 h, the cells were incubated with rosiglitazone (lanes 5-8) or vehicle (lanes 1-4) in the presence of increasing concentrations of CoCl_2 : 0 (lanes 1 and 5), 10 (lanes 2 and 6), 40 (lanes 3 and 7) and 100 (lanes 4 and 8) μM . Luciferase activity was measured 24 h later. Relative Luciferase Activity (Mean $\pm$ SD, $n=2$) is plotted.

Fig. 3. Effect of TNF- α on rosiglitazone-stimulated adiponectin PPRE-dependent promoter activity. MEF-PPAR- γ 2 fibroblasts were transfected with pGL3-Adipo-PPRE-SV40-Luc. After 24 h, the cells were stimulated with rosiglitazone and coincubated with TNF- α (50 ng/ml) or vehicle. Luciferase activity was measured 24 h later. Relative Luciferase Activity (Mean $\pm$ SD in two experiments) is plotted for Control (*black bar*) and TNF- α -treated (*gray bar*).

Fig. 4.

Schematic diagram illustrating a possible pathway by which hypoxia, chronic inflammation, and IGFBP-3 inhibit adiponectin transcription. Reduced adiponectin levels contribute to the development of insulin resistance and the metabolic syndrome, increasing the risk of type 2 diabetes and cardiovascular disease. In obesity, adipose tissue exists in a hypoxic state of chronic inflammation. Both hypoxia and the pro-inflammatory cytokine TNF- α inhibit adiponectin expression.

Rosiglitazone activates RXR- α -PPAR- γ heterodimers bound to a PPRE in the adiponectin promoter to stimulate adiponectin transcription. We show that an hypoxia-mimetic agent (cobalt chloride), TNF- α , and IGFBP-3 inhibit rosiglitazone-stimulated adiponectin transcription. Although the inhibitory effects of hypoxia and TNF- α may be direct, we hypothesize that IGFBP-3, whose transcription is induced by hypoxia and TNF- α [13;24;25; 26;27;28], may mediate their inhibition of adiponectin transcription by binding to RXR- α [14], disrupting RXR- α -PPAR- γ complexes so that they cannot activate the PPRE in the adiponectin promoter.