The Antidiabetic Agent LG100754 Sensitizes Cells to Low Concentrations of Peroxisome Proliferator-activated Receptor γ Ligands*

Received for publication, January 3, 2002, and in revised form, February 15, 2002

Published, JBC Papers in Press, February 27, 2002,
DOI 10.1074/jbc.C200004200

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Insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM) have reached epidemic status in industrialized societies (1, 2). Over 125 million people worldwide suffer from NIDDM, and these individuals face a dramatically increased risk for developing atherosclerotic heart disease, stroke, renal disease, blindness, and limb amputations. It is thus alarming that the number of NIDDM cases has increased 5-fold in the past decade, a trend that is predicted to continue. Equally worrisome is that NIDDM, initially defined as a disease of adult onset, is now appearing in adolescents.

Insulin responsiveness can be modulated by a number of processes including transcriptional cascades controlled by nuclear hormone receptors. Nuclear receptors comprise a superfamily of transcription factors that directly regulate gene expression in response to low molecular weight ligands. Upon binding these ligands, receptors undergo a conformational change that promotes an exchange of coregulatory proteins and ultimately a change in the rate of transcription of specific target genes (3). Compounds that bind to and activate the PPARγ subunit of the PPAR-RXR nuclear receptor heterodimer (4–6) alter transcription of genes involved in glucose and lipid homeostasis. Included among these target genes are lipid transporters (CD36, aquaporin), key metabolic enzymes (lipoprotein lipase, phosphoenolpyruvate carboxykinase, uncoupling protein-1), adipocyte-enriched signaling molecules (leptin, resistin, ACRP30, FIAF/PGAR), lipid-modulated nuclear receptors (LXRs), and an intermediate in the insulin signaling pathway (c-Cbl-associating protein) (2, 5, 7–15). A variety of cellular, molecular, and pharmacologic studies have shown that PPARγ activation results in increased adipogenesis, redistribution of fatty acids and triglycerides into fat, and ultimately improved insulin sensitivity (2, 6, 16). Indeed, PPARγ-specific ligands such as rosiglitazone are currently used for the clinical treatment of NIDDM (17).

PPARγ functions as part of a heterodimeric complex with a nuclear receptor known as RXR (4–6). RXR serves as a common heterodimeric partner for several nuclear receptors and is modulated by a class of ligands known as rexinoids. Since PPARγ functions as an obligate heterodimer with RXR, there has been an interest in developing RXR-specific rexinoids as potential treatments for NIDDM. A particularly interesting compound is LG754, which primarily activates PPAR-RXR heterodimers and retains potent antidiabetic properties (18). We now show that LG754 defines a novel rexinoid agonist that paradoxically has little intrinsic transcriptional activity. Instead, LG754 functions by enhancing the affinity of PPARγ for its ligands. LG754 thus defines a new class of receptor agonist that can be described as a ligand sensitizer. The fact that this PPARγ sensitizer relieves insulin resistance suggests that a relative deficiency in endogenous PPARγ ligands may play a primary role in the development of insulin resistance. This notion accounts for several critical paradoxes in our understanding of NIDDM.

MATERIALS AND METHODS

Plasmids and Reagents—The PPARγ luciferase reporter construct PPRE × 3 TK-Luc contains the herpesvirus thymidine kinase promoter (–105/+51) linked to three copies of the rat acyl-CoA oxidase PPRE (5′-AGGGGAGCAAGACACAGCAGTCGTCGGGAA-3′). The GAL4 reporter was as described previously (19). A cytomegalovirus expression vector with a T7 promoter was used to express the following proteins in cells and/or in vitro: PPARγ (mouse PPARγ1, GenBankTM accession number U10374), RXR (human RXRα, GenBankTM accession number X52773), Gal-PPB (human PBP, GenBankTM accession number AF238312, Val574–Ser649), VP-PPARγ (mouse PPARγ ligand binding domain, GenBankTM accession number U10374, Cys652–Tyr657). For two-hybrid studies, an RXR ligand binding domain expression vector was used that contains the SV40 Tag nuclear localization signal (APKKKKKKG) fused upstream of the RXR ligand binding domain (human RXRα, GenBankTM accession number X52773, Glu652–Thr662). Gal4 fusions contained the indicated fragments fused to the C-terminal end of the yeast Gal4 DNA binding domain; VP16 fusions contained the 78 amino acid herpesvirus VP16 transactivation domain. For bacterial expression, p160 coactivator proteins were expressed as fusion proteins containing GST upstream of the 3 receptor interaction domains of SRC-1 (human SRC-1, GenBankTM accession number U59302, Asp617–Asp629), ACTR (human ACTR, GenBankTM accession number AF036892, Gly617–Gln665) or GRIP1 (mouse GRIP1, GenBankTM accession number U39060, Arg617–Lys785). The PBP fusion contained the two

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The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, PPAR response element; LG268, LG10068; LG754, LG100754; PBP, PPARγ-binding protein; GST, glutathione S-transferase; CoA, coactivator.
The Antidiabetic Agent LG100754 Is a PPARγ Sensitizer

FIG. 1. LG754 possesses minimal intrinsic transcriptional activation activity. A, when compared with rosiglitazone, LG754 is a weak activator of PPARγ. CV-1 cells, which express endogenous RXR, were transiently transfected with a PPAR reporter (PPRE × 3 TK-Luc), a vector expressing PPARγ, and a β-galactosidase internal control. After transfection, cells were treated with control medium or medium containing 1 μM rosiglitazone or 1 μM LG754 as indicated. -Fold activation was determined relative to untreated cells. B, when compared with rosiglitazone in a mammalian two-hybrid assay, LG754 has only minimal ability to recruit PBP. CV-1 cells were transiently transfected with a Gal4 reporter and an expression vector for the Gal4 DNA binding domain linked to the receptor interaction domain of human PBP. Cells were also transfected with expression vectors for the VP16 transactivation domain linked to the ligand binding domain of PPARγ (VP-PPARγ) and a vector expressing the ligand binding domain of RXR. After transfection, cells were treated as in A.

FIG. 2. LG754 fails to effectively recruit coactivators to DNA-bound PPARγ-RXR heterodimers. Electrophoretic mobility shift complexes using a 32P-labeled PPAR response element (acyl-CoA oxidase) in vitro translated PPAR-RRX and affinity-purified GST (control) or GST-coactivator fusion proteins. LG268 (100 nM) and LG754 (1 μM) were added as indicated. The position of the PPARγ-RXR (>) and PPARγ-RXR-coactivator (>) complexes are shown.

a strong 10-fold increase in the recruitment of PPARγ to PBP, whereas LG754 had only a 2-3-fold effect on this interaction (Fig. 1B). The relative differences in coactivator recruitment by these two ligands closely paralleled the weak effect of LG754 on activation of the PPARγ-RXR heterodimer (Fig. 1A).

To further explore the effect of LG754 on coactivator recruitment, we utilized an electrophoretic mobility shift assay. Unlike the two-hybrid assay, this approach examines the effect of ligands on DNA-bound PPARγ-RXR heterodimers, i.e. native receptor complexes. Thus, PPARγ, RXR, and affinity-purified GST-coactivator fusion proteins were incubated with a 32P-labeled response element and separated by electrophoresis through a nondenaturing gel. In this experiment, we compared LG754 to LG268, another RXR-specific rexinoid with antidiabetic activity (22). LG268 effectively recruited the p160 family of coactivators (SRC1, ACTR, GRIP) but had no effect on the GST control (Fig. 2). A quantitatively smaller, but highly reproducible, shift was also seen with PBP (Fig. 2). This demonstrates that RXR ligands can recruit coactivators to the DNA-bound PPARγ-RXR heterodimer. In contrast, LG754 failed to recruit any of these proteins (Fig. 2), confirming that LG754 does not effectively recruit coactivators to the PPARγ-RXR complex. Thus, in contrast to previously described nuclear receptor agonists, LG754 is unique in that it does not possess strong transcriptional activating properties.

Since LG754 has only weak intrinsic transcriptional activity, its ability to mimic PPARγ ligands in vivo implies that this compound is functioning by an alternative mechanism. Previous studies have demonstrated that individual subunits of nuclear receptor heterodimers can have dramatic allosteric effects on its partner receptor. For example, the insect edysone receptor does not bind its ligand with high affinity, instead ligand binding requires association of the edysone receptor with its heterodimeric partner, ultraspiracle (USP) (23). The existence of such allosteric interactions among receptor heterodimers prompted us to ask whether LG754 can increase the affinity of the PPARγ-RXR complex for PPARγ ligands. Cell-based transfection assays were used to examine the effect of LG754 on rosiglitazone-mediated activation of PPARγ. CV-1 cells were transfected with PPARγ reporter and expression vectors and treated with suboptimal amounts of rosiglitazone (60 nM) in the presence of increasing amounts of LG754 (Fig. 3A). As expected, rosiglitazone (60 nM) activated PPARγ, but notably this activation was further enhanced by LG754. The optimal effect of LG754 was seen at a concentration of 1 μM (Fig. 3A), which is similar to the optimal doses required for adipogenesis in 3T3-L1 cells (18). We next examined the effect of LG754 on the PPARγ dose-response curve. As expected from Fig. 1A, LG754 had minimal activity by itself. However, it shifted the rosiglitazone dose response curve leading to a 3-fold increase in the apparent potency of rosiglitazone (Fig. 3B). LG754 had a similar effect on the...
FIG. 3. LG754, an antidiabetic RXR ligand, increases the apparent potency of PPARγ ligands. A, LG754 enhances the response to limiting doses of PPARγ ligands. CV-1 cells were transfected with a PPAR reporter construct along with expression vectors for PPARγ and a β-galactosidase internal control. Cells were treated with 60 nm rosiglitazone alone or in the presence of the indicated concentration of LG754. -Fold activation was plotted (n = 6); B, LG754 shifts the dose-response profile of rosiglitazone toward a higher potency. CV-1 cells were transfected with a PPAR reporter construct along with expression vectors for PPARγ and a β-galactosidase internal control. Cells were treated with the indicated concentrations of rosiglitazone in the absence (C) or presence (D) of 1 μM LG754, and normalized reporter activity was determined (means ± S.E., n = 4). C, LG754 shifts the dose-response profile of 15-deoxy-Δ12,14-prostaglandin J2 toward a higher potency. CV-1 cells were transfected as in B and treated with the indicated concentrations of 15-deoxy-Δ12,14-prostaglandin J2 in the absence (C) or presence (D) of 1 μM LG754. Normalized reporter activity was determined (means ± S.E., n = 6). D, LG754 increases binding of PPARγ to its ligand. Mobility shift experiments were performed as described in the legend to Fig. 2 but using unlabeled DNA and [125I]SB-236636 (21) as the PPARγ ligand. GST-PBP was included in the reaction and the DNA-bound PPARγ-RXR-PBP complex is shown. E, schematic diagram of the PPARγ ligand deficiency hypothesis. This model suggests that deficiencies in endogenous PPARγ ligands represent an early step in the development of insulin resistance in lipodystrophic diabetes, obesity-related diabetes, and in individuals with mutations in PPARγ.

To overcome this limitation, ligand binding was measured in a modified electrophoretic mobility shift assay where heterodimer-containing complexes can be separated and identified within the gel. Complexes were analyzed as in Fig. 2, but instead of using radiolabeled DNA, the complexes were visualized with a 125I-labeled PPARγ ligand (21). Note that LG754 significantly increased the amount of 125I-labeled PPARγ ligand in the complex (Fig. 3D) without affecting the total amount of complex formed (Fig. 2 and data not shown). These data suggest that LG754 enhances the affinity of PPARγ for its ligands.

DISCUSSION

A large body evidence of biochemical, structural, and genetic data have firmly demonstrated that nuclear receptors activate transcription by recruiting transcriptional coactivator proteins (3). This has led to the commonly accepted paradigm that nuclear receptor agonists function by inducing a conformation change that favors a more stable receptor-coactivator complex. The data presented here indicate that LG754 defines a new class of nuclear receptor agonist that has minimal coactivator recruitment activity and therefore minimal inherent transcriptional activity. Instead, this compound activates transcription by allosterically enhancing the ligand binding activity of its partner receptor, PPARγ. We refer to this agonist class as a "sensitizer." LG754 therefore represents the first example of a nuclear receptor-sensitizing agent.

In addition to being a PPARγ sensitizer, previous studies have demonstrated that LG754 relieves insulin resistance in vivo (18). These findings have important implications, since the molecular events that result in insulin resistance remain obscure. It is well known that PPARγ ligands reverse insulin resistance both in humans and in a variety of animal models. PPARγ agonists have the interesting property of lowering glucose in diabetic animals but not in non-diabetic animals (24). This implies that PPARγ ligands reverse or replace a deficiency that is unique to the diabetic state. It is intriguing to speculate that insulin resistance arises from a relative deficiency in endogenous PPARγ ligands and that PPARγ agonists are effective antidiabetic agents, because they correct this deficiency. In principle, this hypothesis could be directly tested by determining the levels of endogenous PPARγ ligands in normal and diabetic individuals. Several fatty acid derivatives and prostaglandins have been shown to bind to PPARγ (4, 20); however, these ligands are not specific for PPARγ, and the precise identity of the endogenous PPARγ ligand is unknown (25). Therefore, a direct quantitation of endogenous PPARγ ligand levels is not currently possible and an alternative approach is required to test this "PPARγ ligand deficiency" model. Since LG754 is a PPARγ sensitizer, this compound reverses the biological effects that result from a deficiency in PPARγ ligands. The previous demonstration that LG754 relieves insulin in db/db mice (18) provides support for the hypothesis that insulin resistance is secondary to suboptimal levels of the yet-to-be identified PPARγ ligand.

A genetic test of the "ligand deficiency" hypothesis might include the development of animals expressing PPARγ mutants with diminished ligand binding affinity. These animals would not respond to endogenous PPARγ ligands and would be predicted to develop insulin resistance. Such animals have not been described, although PPARγ-null mice have been established (26, 27). However, as PPARγ integrates both positive (endogenous ligands) and negative signals (MAP kinase) (28), the effects of chronic PPARγ ablation cannot be equated with those resulting from ligand deficiency. While appropriate animal models do not exist, several patients have been described with point mutations in PPARγ that result in defects in ligand binding and/or transactivation (29). These individuals provide insights into the pathological consequences associated with a diminished response to endogenous PPARγ ligands. Indeed, these patients develop lipodystrophy and severe insulin resistance as would be predicted by the PPARγ ligand-deficiency hypothesis (Fig. 3E).

There are a number of critical gaps in our understanding of the NIDDM-PPARγ connection. For example, PPARγ is required for adipogenesis (27, 30, 31), and its synthetic agonists increase adipose mass in vivo (24). This is unexpected, since insulin resistance worsens in most patients as fat mass in-
creases. This raises a question as to how an adipogenic agent can also act as an antiadipogenic agent (32)? Another gap is highlighted by the fact that certain rare forms of NIDDM are paradoxically associated with diminished fat mass (lipodystrophy) (33), and perhaps even more surprising is the observation that PPARG activators can effectively treat both obesity-dependent and lipodystrophic diabetes (6, 33).

The PPARG ligand deficiency hypothesis (Fig. 3E) is intriguing as it provides a rationale to close these gaps. Given the dual role of PPARG ligands in enhancing adipose mass and insulin responsiveness, I suggest that a primary defect in the synthesis or accumulation of an endogenous PPARG ligand might be the molecular event that underlies lipodystrophic diabetes. Although the identity of the endogenous PPARG ligand is unknown, it has been suggested that the transcription factor SREBP-1c (ADD1) is required to produce an endogenous ligand in adipocytes (34). The PPARG ligand deficiency hypothesis would predict that treatments which lower SREBP-1c levels should result in lipodystrophy, insulin resistance, and decreased adipogenesis. Indeed, two human immunodeficiency virus protease inhibitors (indinavir and nelfinavir) that promote lipodystrophy in vivo (35) have been shown to inhibit adipogenesis and to reduce SREBP-1 activity (36, 37). These observations provide further support for the PPARG ligand deficiency hypothesis.

In the opposing state of obesity, it is reasonable to imagine that feedback mechanisms are triggered in an attempt to limit further lipid storage. In principle, this could be accomplished by decreasing lipogenic signals such as those represented by SREBP-1. The PPAR response may provide short term benefits, a chronic deficiency in adipocytes (34). The PPAR ligand deficiency, whereas in obesity-related diabetes the deficiency would be secondary to increasing fat mass. Thus, the ligand deficiency hypothesis accounts for the paradoxical association of NIDDM with both obesity and lipodystrophy. It also explains how PPARG ligands can treat both disorders. Given the rising toll of NIDDM, these findings provide further impetus to identify and quantitate the endogenous PPARG ligand.

Acknowledgments—I thank Kevin Hollister, Eric Wang, and Karol Rostamiand for technical assistance and Richard Bergman and Gregg Van Citters for comments. [232]SB-236636 was provided by Dr. Stephen A. Smith. I am grateful to Leslie and Susan Gonda for support of research facilities and infrastructure at the City of Hope.

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