Sulfonylurea Agents Exhibit Peroxisome Proliferator-activated Receptor γ Agonist Activity*

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Sulfonylurea (SU) agents, including glimepiride and glibenclamide, are the most widely used oral hypoglycemic drugs, which stimulate insulin secretion primarily by binding to the SU receptor on the plasma membrane of pancreatic β-cells. Thiazolidinediones, such as pioglitazone and rosiglitazone, are other hypoglycemic agents that effectively improve peripheral insulin resistance through activation of peroxisome proliferator-activated receptor γ (PPARγ). In the present study, we found that glimepiride specifically induced the transcriptional activity of PPARγ in luciferase reporter assays. Glimepiride enhanced the recruitment of coactivator DRIP205 and dissociation of corepressors such as nuclear receptor corepressor and silencing mediator for retinoid and thyroid hormone receptors. In addition, glimepiride directly bound to PPARγ in a manner competitive to rosiglitazone, which is a proven ligand for PPARγ. Furthermore, in 3T3-L1 adipocytes, glimepiride stimulated the transcriptional activity of the gene promoter containing PPAR-responsive element and altered mRNA levels of PPARγ target genes including aP2, leptin, and adiponectin. Finally, glimepiride induced adipose differentiation in 3T3-F442A cells, which was known to differentiate into adipocytes in a PPARγ-dependent manner. Most effects observed with glimepiride were also seen with glibenclamide. These data strongly suggest that glimepiride and glibenclamide, both of which belong to SU agents, should have PPARγ agonist activity, whose potencies were 16–25% of the maximum level achieved by pioglitazone. Our observation that glimepiride and glibenclamide could act not only on SU receptor but also on PPARγ may give an important clue to the development of novel anti-diabetic drugs, which can enhance both insulin secretion from pancreatic β-cells and peripheral insulin sensitivity.

Hyperglycemia seen in type 2 diabetes is caused by defects in insulin secretion from pancreatic β-cells and insulin sensitivity in peripheral tissues such as liver, muscle, and fat. Thiazolidinediones (TZDs)1 are a new class of hypoglycemic agents that improve peripheral insulin resistance (1). TZDs bind and activate peroxisome proliferator-activated receptor γ (PPARγ), a key transcription factor involved in glucose and lipid metabolism, and adipose differentiation (2–5). PPARγ is highly expressed in adipose tissues and most pharmacological actions of TZDs are thought to be through the PPARγ activation in adipose cells (6, 7).

PPARγ belongs to a superfamily of nuclear receptors that regulate gene expression in response to small, lipophilic ligands (7). Several naturally occurring molecules, such as 15-deoxy-Δ12,14-prostaglandin J2 (8, 9), 9- and 13-cis-hydroxyoctadecadienoic acid (10), and lysophosphatidic acid (11), possess the agonistic activities for PPARγ. In addition to TZDs, structurally diverse synthetic compounds including N-(9-fluorenyl) methoxycarbonyl (Fmoc)-l-leucine (12) and certain nonsteroidal anti-inflammatory drugs (13) also possess the activities as PPARγ ligands. Furthermore, one of the antagonists II receptor, telmisartan, has been recently demonstrated to act as a partial agonist for PPARγ (14). Thus, one of the unique characteristics of PPARγ is that a wide range of lipophilic molecules can interact with it. Agonist ligands activate PPARγ through direct interactions with the ligand-binding domain in its C-terminal region. The rather spacious ligand-binding pocket of PPARγ is thought to potentially accommodate multiple lipophilic ligands (15).

Sulfonylurea (SU) agents have played a pivotal role in the drug therapy of type 2 diabetes patients as effective oral hypoglycemic agents for several decades. They stimulate insulin secretion primarily by binding to the SU receptor on the plasma membrane of pancreatic β-cells (16). In addition to such actions on the pancreas, several reports have pointed out that some SU agents, such as glimepiride and glibenclamide, have

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1 The abbreviations used are: TZD, thiazolidinedione; AF-2, agonist function-2; DRIP205, vitamin D receptor-interacting protein 205; FBS, fetal bovine serum; GST, glutathione S-transferase; HEK 293, human embryonic kidney 293; N-CoR, nuclear receptor corepressor; PPAR, peroxisome proliferator-activated receptor; mPPAR, mouse PPAR; PPRE, PPAR-responsive element; SU, sulfonylurea; SMRT, silencing mediator for retinoid and thyroid hormone receptors.
direct effects to potentiate or mimic the insulin action in adipocytes (17–19). However, the molecular mechanisms of such extrapancreatic effects of SU agents have not been unraveled.

The present study was designed to determine the direct effects of SU agents on PPARγ transcriptional activity in adipocytes. We found that SU agents, such as glimepiride and gliclazide, possessed agonistic activities for PPARγ and affected adipose gene expression. Our results are potentially useful for the design and development of effective novel anti-diabetic drugs that enhance both insulin secretion and insulin sensitivity.

EXPERIMENTAL PROCEDURES

Materials—[3H]Rosiglitazone (specific activity 50 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Pioglitazone was a kind gift from Takeda Chemical Industries (Osaka, Japan). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). Glimepiride was a kind gift from Aventis Pharma (Tokyo, Japan). Gliclazide, tolbutamide, chlorpropamide, and gliclazide were purchased from Sigma. Wy 14643 was purchased from Merck. GW 501516 was a kind gift from Dr. J. Sakai (Tokyo University). Plasmids—Expression plasmids encoding GAL4 (pCMX-GALA4), GAL4-chimeric reporter plasmids containing GAL4-xP3 promoter, mouse PPARα chimera protein (pCMX-GAL4-mPPARα), GAL4-mouse PPARγ chimera protein (pCMX-GAL4-mPPARγ), GAL4-mouse PPARδ chimera protein (pCMX-GAL4-mPPARδ), full-length mouse PPARγ protein (pCMX-mPPARγ), VP16 (pCMX-VP16), VP16-mouse PPARγ chimera protein (pCMX-VP16-mPPARγ), and β-galaclosidase (pCMX-β-gal) were generous gifts from Dr. David J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). The mouse PPARγ mutant construct (pCMX-ΔmPPARγ), which lacks 11 amino acids (PLLQEIYKDLY) in the C-terminal activation function-2 (AF-2) domain, was described previously (20). Expression plasmids encoding GAL4-vitamin D receptor-interacting protein 205 (DRIP205) (pCMX-GAL4-DRIP205) and GAL4-nuclear receptor corepressor (N-CoR) (pCMX-GAL4-N-CoR) were described previously (21, 22). The nuclear receptor-interacting domains of DRIP205 (amino acids 576–728) and N-CoR (amino acids 2390–2416) were described previously (27) from the 35S-Labeled nuclear receptor-interacting domain of N-CoR (27), the GST pull-down assay was performed as described previously (28).

Transfection Studies in 3T3-L1 Adipocytes—Mouse 3T3-L1 preadipocytes were cultured and induced differentiation as described previously (25). On day 4 after induction of differentiation, the media of 3T3-L1 cells were changed to OPTI-MEM (Invitrogen), and the cells were transfected with reporter plasmids containing PPRE3×3-tk-LUC reporter or human adiponectin promoters using Lipofectamine 2000 reagent (Invitrogen) as described previously (25). At 20 h after a drug treatment, luciferase reporter assays were performed using a luciferase assay system (Promega). Primer sets were the following: mouse aP2, 5′-CCG CAG ACA GGA-3′ and 5′-CTC ATG CCC TTT CAT AAA CT-3′; mouse leptin, 5′-GAT GGA CCA GAC TCT GCC AG-3′ and 5′-AGA GTG AGG CTT CCA GGA CG-3′; mouse adiponectin, 5′-GAT GGA AGG GAT GCC AC-3′ and 5′-AGA GCC GTT CCA GAG CTC AT-3′. The mRNA levels were normalized relative to the amount of cyclophilin mRNA and expressed in arbitrary units.

Analysis of Adiponectin Secretion by 3T3-L1 Adipocytes—On day 7 after induction of differentiation in 3T3-L1 adipocytes, pioglitazone, glimepiride, or gliclazide was added to the medium for 48 h. The amount of adiponectin secreted into the culture medium was measured with a mouse/rat adiponectin enzyme-linked immuno-sorbent assay kit (Otsuka Pharmaceutical, Tokushima, Japan).

Adipocyte Differentiation Assays—Mouse 3T3-F442A preadipocytes (a kind gift from Dr. H. Sakaue, Kobe University, Kobe, Japan) were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS. For differentiation, the cells (3 days after reaching confluence) were transfected with a luciferase reporter plasmid containing 1834-bp 3′-flanking region of the murine adiponectin gene. The luciferase activity was measured with a luminometer (GloMax; Promega). The mRNA levels were normalized relative to the amount of cyclophilin mRNA and expressed in arbitrary units.

RESULTS

Glimepiride and Gliclazide Are Partial Agonists for PPARγ—To elucidate the direct effects of SU agents on the transcriptional activity of PPARγ, we performed reporter assays using the GAL4-PPARγ and GAL4-responsive luciferase reporter in non adipose HEK 293 cells. The ligand-binding domain of PPARγ was fused to the DNA-binding domain of the yeast transcription factor GAL4. Because the reporter used was activated only by the exogenous GAL4-chimera receptors, the effects of endogenous receptors are eliminated. Interestingly, we found that glimepiride and gliclazide induced GAL4-PPARγ transcriptional activities; however, such increases were not seen with other SU agents, including tolbutamide, chlorpropamide, and gliclazide, at a dose of 10 μM (Fig. 1A).

Next, we examined the concentration-dependent activation of GAL4-PPARγ by SU agents in HEK 293 cells. As shown in

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Glimepiride and glibenclamide are partial agonists for PPARγ. HEK 293 cells were cotransfected with GAL4-mouse PPARγ (mPPARγ) and MH100(UAS)x4-tk-LUC reporter and treated with vehicle control (cont), or each SU agent including glimepiride (gmp), glibenclamide (gbc), tolbutamide (tb), chlorpropamide (cp), and gliclazide (gc) at 10 μM. Luciferase values were normalized by an internal β-galactosidase control and expressed as relative luciferase activity. Values are mean ± S.E. (n = 3). B, concentration-dependent activation of PPARγ by pioglitazone, glimepiride, glibenclamide, and other SU agents. HEK 293 cells were cotransfected with GAL4-mPPARγ and MH100(UAS)x4-tk-LUC reporter and treated with pioglitazone (pio), glimepiride, glibenclamide, or other SU agents at indicated doses. Luciferase values were normalized by β-galactosidase activity and expressed as fold induction relative to the vehicle control. Values are mean ± S.E. (n = 3). C, PPAR subtype selectivity of glimepiride. HEK 293 cells were cotransfected with GAL4, GAL4-mouse PPARα, GAL4-mouse PPARδ, or GAL4-mPPARγ in combination with MH100(UAS)x4-tk-LUC reporter and treated with vehicle control, a ligand for each PPAR subtype (PPARα ligand, 10 μM Wy 14643; PPARδ ligand, 10 μM GW 501516; and PPARγ ligand, 1 μM pioglitazone), or 10 μM glimepiride. Luciferase values were normalized by β-galactosidase activity and expressed as relative luciferase activity. Values are mean ± S.E. (n = 3). D, dose-response curve of displacement of [3H]rosiglitazone binding to PPARγ by pioglitazone, glimepiride, or glibenclamide. Competitive binding assays were performed as described under “Experimental Procedures.” One hundred percent binding indicates the total binding of [3H]rosiglitazone in the absence of competitors. Data represent the mean ± S.E. (n = 3).

Fig. 1B, treatment with glimepiride or glibenclamide resulted in concentration-dependent activation of GAL4-PPARγ. Glimepiride and glibenclamide activated GAL4-PPARγ up to 16 and 25% of the maximum level achieved by pioglitazone, respectively. Tolbutamide, chlorpropamide, and gliclazide also caused activation of GAL4-PPARγ at relatively higher doses than glimepiride and glibenclamide (Fig. 1B).

To examine the PPAR subtype selectivity of glimepiride, we performed reporter assays using the GAL4-PPARγ in HEK 293 cells. Wy 14643, GW 501516, and pioglitazone, known as a specific ligand for each PPAR subtype (30, 31),
activated GAL4-PPARα, GAL4-PPARβ, and GAL4-PPARγ, respectively (Fig. 1C). Interestingly, glimepiride activated GAL4-PPARγ, but not GAL4-PPARα and PPARβ (Fig. 1C). Glimepiride had no effects on the transcriptional activities of other GAL4-chimeric nuclear receptors, such as RXRα, LXRβ, and FXR (data not shown).

To clarify whether glimepiride and glibenclamide directly bind to PPARγ, we performed competitive binding assays using GST-full-length PPARγ and [3H]rosiglitazone. The displacement of [3H]rosiglitazone by pioglitazone was seen, and the IC50 value was 3.0 μM (Fig. 1D). Glimepiride and glibenclamide were also concentration-dependent, and IC50 values were 27 and 7.6 μM, respectively (Fig. 1D). These data strongly suggest that both glimepiride and glibenclamide activate PPARγ through the direct association and that they could be considered as partial agonists for PPARγ.

**Glimepiride Increases the Interaction of PPARγ with Cofactors—**Upon ligand binding, nuclear receptors undergo conformational changes that result in AF-2 domain-dependent dissociation of corepressors and recruitment of coactivators (32). We examined the effect of glimepiride on a PPARγ AF-2 deletion mutant by cotransfecting full-length PPARγ or mutant PPARγ (ΔAF-2) and PPREx3-tk-LUC receptor. Glimepiride as well as pioglitazone activated full-length PPARγ. However, glimepiride-dependent activation of PPARγ was completely abolished by truncation of the AF-2 domain (Fig. 2A). To confirm the expression of mutant PPARγ (ΔAF-2), we performed the Western blot analysis. Full-length PPARγ was detected in the expected size, with both anti-PPARγ antibodies recognizing the N terminus (H-100) and C terminus (E-8) of PPARγ. On the other hand, the ΔAF-2 PPARγ was detected only with the H-100 antibody, not with the E-8 antibody (Fig. 2A).

Next, we examined the effect of glimepiride on the interaction of PPARγ and the coactivator DRIP205, also known as PPAR-binding protein (33, 34), or corepressor N-CoR by mammalian two-hybrid assays in HEK 293 cells. The assays were performed using full-length PPARγ fused to the transactivation domain of herpesvirus VP16 protein and the nuclear receptor-interacting domain of DRIP205 or N-CoR fused to the DNA-binding domain of GAL4 for detection of ligand-dependent cofactor recruitment or detachment (35). Both pioglitazone and glimepiride markedly increased the transcriptional activity by cotransfection of VP16-PPARγ, GAL4-DRIP205, and GAL4-responsive LUC reporter (Fig. 2B). These results suggest that glimepiride induces the association of PPARγ with DRIP205. On the other hand, cotransfection of VP16-PPARγ with GAL4-N-CoR resulted in activation of the reporter without ligands. The reporter activity was markedly inhibited in the presence of pioglitazone or glimepiride (Fig. 2C). Similar inhibitory effects by pioglitazone and glimepiride were also seen upon interaction of PPARγ with SMRT, another corepressor (data not shown). These results suggest that glimepiride induces the dissociation of PPARγ from N-CoR or SMRT.

We also verified the direct effect of glimepiride on interaction of full-length PPARγ with DRIP205 using GST pull-down assays. As shown in Fig. 2D, [35S]-labeled PPARγ slightly bound to GST-DRIP205 (lane 7), and this binding was augmented by pioglitazone (lane 8). Similarly, glimepiride induced the association of labeled PPARγ with GST-DRIP205 in a dose-dependent manner (Fig. 2D, lanes 9–11). The binding between GST alone and labeled PPARγ was not observed in the absence or presence of ligands (Fig. 2D, lanes 2–6). These data suggest that direct binding of glimepiride to PPARγ induces the association of PPARγ with DRIP205. Furthermore, we examined the direct effect of glimepiride on the interaction of full-length PPARγ with N-CoR. GST-PPARγ strongly bound to [35S]-labeled N-CoR in the absence of ligands (Fig. 2E, lane 7). Both glimepiride and pioglitazone induced the dissociation of GST-PPARγ from labeled N-CoR (Fig. 2E, lanes 8–11). The binding between GST alone and labeled N-CoR was not observed in the absence or presence of ligands (Fig. 2E, lanes 2–6). These data suggest that direct binding of glimepiride to PPARγ induces the dissociation of PPARγ from N-CoR.

**Glimepiride and Glibenclamide Induce PPAR-dependent Transcriptional Activities in 3T3-L1 Adipocytes—**To investigate the effects of glimepiride and glibenclamide on PPAR-dependent transcriptional activity in adipocytes, we performed luciferase reporter assays using the PPREx3-tk-LUC in 3T3-L1 adipocytes. Glimepiride significantly induced the activity of the PPRE reporter in adipocytes in a dose-dependent fashion (Fig. 3A). Glibenclamide also activated the PPRE reporter in adipocytes as effectively as glimepiride (Fig. 3A). These results suggest that both glimepiride and glibenclamide should activate endogenous PPARγ in adipocytes. Next, we examined the effect of glimepiride on the transcriptional activity of the adiponectin gene in 3T3-L1 adipocytes by reporter as-
sues. We demonstrated previously that the expression of adiponectin gene is up-regulated by PPARγ activation (25). As shown in Fig. 3B, glimepiride as well as pioglitazone significantly increased the transcriptional activity of the wild-type adiponectin promoter in adipocytes. As reported previously (25), transfection of the PPRE-mutated adiponectin promoter markedly reduced the transcriptional activity in the basal condition, and no further induction was seen by treatment with pioglitazone. Interestingly, glimepiride-dependent activation of the adiponectin promoter was also completely abolished by mutation of PPRE (Fig. 3B). These results indicate that glimepiride activates the adiponectin promoter in adipocytes via a PPAR-dependent mechanism.

Glimepiride and Glibenclamide Enhance Adiponectin Production in Adipocytes—We investigated the effects of glimepiride on mRNA expression of known PPARγ target genes, aP2 and leptin, in differentiated 3T3-L1 adipocytes. As reported previously (26, 37), pioglitazone significantly increased aP2 mRNA level and reduced leptin mRNA level in adipocytes (Fig. 4, A and B). Similarly, glimepiride significantly altered both aP2 and leptin mRNA levels (Fig. 4, A and B).

Next, we examined the effects of glimepiride on mRNA expression and secretion of adiponectin in differentiated 3T3-L1 adipocytes. As reported previously (38), pioglitazone increased both the mRNA expression and secretion of adiponectin (Fig. 4, C and D). Interestingly, treatment with glimepiride significantly increased adiponectin mRNA level in adipocytes (Fig. 4C). Furthermore, glimepiride dose-dependently stimulated adiponectin secretion into the medium (Fig. 4D). In addition, we examined the secretion of adiponectin in glibenclamide-treated adipocytes. Glibenclamide enhanced adiponectin secretion as well as glimepiride (Fig. 4D). These results suggest that both glimepiride and glibenclamide enhance the production of adiponectin, which is an insulin-sensitizing hormone, via PPARγ activation in adipocytes.

Glimepiride and Glibenclamide Stimulate Adipose Differentiation—PPARγ agonists are known to promote the maturation of preadipocytes into adipocytes (4). To further characterize the profile of glimepiride and glibenclamide, we examined their effects on differentiation of 3T3-F442A preadipocytes. 3T3-F442A preadipocytes are known to exhibit PPARγ-dependent adipose differentiation (39). Incubation with glimepiride as well as pioglitazone markedly stimulated preadipocyte differentiation, as indicated by the staining of lipids with Oil red O (Fig. 5A). Glibenclamide also resulted in lipid accumulation, similar to glimepiride (data not shown).

Finally, we examined the effects of glimepiride and glibenclamide on induction of adipose differentiation marker genes, aP2 and adiponectin. Both glimepiride and glibenclamide significantly induced the mRNA expression of these genes in 3T3-F442A cells, similar to pioglitazone (Fig. 5, B and C). These results suggest that both glimepiride and glibenclamide stimulate adipose differentiation via PPARγ activation.

**DISCUSSION**

The major findings of the present study were that glimepiride 1) specifically induced the transcriptional activity of PPARγ in HEK 293 cells, 2) enhanced the recruitment of coactivator DRIP205 and dissociation of corepressors such as N-CoR and SMRT, 3) directly bound to PPARγ in a competitive manner to rosiglitazone, 4) stimulated the transcriptional activity of the gene promoter containing PPRE and altered the mRNA levels of PPARγ target genes in 3T3-L1 adipocytes, and 5) induced adipose differentiation of 3T3-F442A cells. Most of the effects observed with glimepiride were also seen with glibenclamide. These results strongly suggest that glimepiride and glibenclamide, both of which are SU anti-diabetic agents, can act as agonists for PPARγ, whose potencies were 16–25% of pioglitazone. Our results provide a new aspect of SU agents as PPARγ agonists. We observed that tolbutamide, chlorpropo-
mide, and gliclazide also had similar effects on the transcriptional activity of PPARγ, although with weaker potencies than glimepiride and glibenclamide. Based on these results, one cannot exclude the possible contribution of the SU-related structure itself to the PPARγ activation.

Our results showed that SU agents are partial agonists for PPARγ. Several compounds have been reported as partial agonists for PPARγ (14, 40). For example, GW0072 is equipotent with a full agonist in detachment of the corepressor N-CoR. However, recruitment of coactivators, such as CBP and SRC-1, is less compared with TZD (40). We observed that glimepiride induced both recruitment of DRIP205 and detachment of N-CoR and SMRT as effectively as pioglitazone. Therefore, the lower maximum level of PPARγ transactivation by glimepiride could be due to disability in recruitment of other coactivators than DRIP205 or detachment of other corepressors.

In clinical studies, Tsunekawa et al. (41) indicated that glimepiride increased plasma adiponectin levels in type 2 diabetic patients, whereas our group and other investigators previously reported that PPARγ agonists elevated plasma adiponectin levels in humans (58, 42). Thus, the augmenting effect of glimepiride on plasma adiponectin levels in human subjects may be partly accounted for by its PPARγ agonist activity. Hyperglycemia in type 2 diabetes is the consequence of defects in insulin secretion from pancreatic β-cells and insulin sensitivity in peripheral tissues. Therefore, to develop effective pharmacological agents for type 2 diabetes, we believe that it is important to improve insulin sensitivity in addition to increasing plasma insulin concentrations. SU agents have been the most widely used hypoglycemic agents for type 2 diabetes, because they effectively lower blood glucose by stimulating pancreatic insulin secretion. On the other hand, TZDs, PPARγ agonists, exhibit powerful hypoglycemic effects by improving peripheral insulin resistance. According to the pharmacoki- netic studies, after glimepiride was orally administered at 1 and 8 mg, which are effective dosages for lowering blood glucose in patients with type 2 diabetes, peak plasma concentrations (Cmax) were 103.2 and 550.8 µg/liter (about 0.2 and 1.1 µM), respectively (43). The doses of glimepiride in our experiments that exerted PPARγ agonist activity were at least 10-fold higher than those required for lowering blood glucose clinically. Our observation that glimepiride and glibenclamide could act not only on SU receptor but also on PPARγ may be helpful for the design and development of novel antidiabetic drugs, which could potentially enhance both insulin secretion and insulin sensitivity.

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