—Original—

Evaluation of Teneligliptin Effects on Transcriptional Activity of PPARγ in Cell-Based Assays

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Background: The antidiabetic drug teneligliptin is a novel dipeptidyl peptidase-4 (DPP-4) inhibitor with a thiazolidine-specific structure. This study aimed to investigate whether teneligliptin can activate PPARγ directly and/or indirectly in cell-based assays.

Methods: Promoter assays using the reporter construct driven under the control of the SV40 promoter and the PPAR response element (PPRE) were performed. Luciferase activity was measured after a 3-day incubation of vector-transduced cells with various concentrations of teneligliptin.

Results: Treatment of the cells with 50 μM teneligliptin significantly transactivated a reporter gene. The presence of the PPARγ antagonist, GW9662, did not affect the activation of PPRE-reporter expression by teneligliptin.

Conclusion: We found that teneligliptin could increase PPARγ activity in cell-based assays irrespective of the PPARγ ligand-binding domain. (J Nippon Med Sch 2018; 85: 95–101)

Key words: thiazolidinedione, incretin, ligand, adipogenesis, promoter assay

Introduction

PPARγ, a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of ligand-dependent nuclear receptors[1,2], plays a dominant role in adipose cell differentiation[3]. It regulates downstream adipocyte-related genes such as the family of the basic region-leucine zipper transcription factor C/EBPα and the adipocyte-specific fatty acid-binding protein gene FABP4 (aP2) to promote adipogenesis[1]. Upon binding numerous biological ligands such as polyunsaturated fatty acids[4], prostanoids[5,6] and oxidized fatty acids, PPARγ becomes activated. Thiazolidinedione (TZD) antidiabetic drugs such as troglitazone, pioglitazone and rosiglitazone have been studied as PPARγ agonist ligands[7]. The TZDs can lower circulating glucose and free fatty acid (FFA) levels and improve insulin sensitivity in humans[8] through increasing the transcriptional activity of PPARγ. They also decrease levels of inflammatory mediators such as tumor necrosis factor-α, plasminogen activator inhibitor-1, and interleukin-6, and also increase adiponectin production from adipose tissue.

A different therapeutic target of type II diabetes mellitus (T2DM) is the gut hormone incretin that controls glucose homeostasis, enhances insulin secretion and inhibits glucagon secretion. Glucagon-like peptide-1 (GLP-1), one of the incretin members, is secreted from L cells in the intestine as a response to meals but is then rapidly degraded by dipeptidyl peptidase-4 (DPP-4). Thus, the specific inhibition of DPP-4 can extend the duration of GLP-1 action. Teneligliptin is an antidiabetic drug that acts as a DPP-4 inhibitor that lowers the postprandial glucose level in patients with T2DM[9]. Teneligliptin also suppresses adipocyte hypertrophy and hepatic steatosis in mice fed with a high-fat diet[10].

Only teneligliptin has a thiazolidine-like structure among the DPP-4 inhibitors administered to help manage

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T2DM[11]. Since teneligliptin is functionally and structurally similar to TZDs, we investigated whether teneligliptin can activate PPARγ in cell-based assays.

Materials and Methods

Chemicals

Teneligliptin powder was donated by Mitsubishi Tanabe Pharma. Rosiglitazone, pioglitazone and GW9662 were purchased from Sigma-Aldrich. Teneligliptin was dissolved in distilled water to prepare a 10-mM stock solution. Rosiglitazone and pioglitazone were dissolved in ethanol. GW9662 was dissolved in DMSO. Polyethylenimine "MAX" (PEI "MAX")[12] (Polysciences Inc.) was dissolved in distilled water to a final concentration of 1 mg/mL. The pH was adjusted to 7.0 with 1 N NaOH.

Plasmid Constructs

Oligonucleotides PPRE_UP_MluI-BglII, 5′-cgcgtgacgTGGGTCAAAGGTCAttcagtcgaca-3′ and PPRE_LP_MluI-BglII, 5′-GATCtgtcgactgaaTGACCTTTGACCCAcgtca-3′ were annealed to prepare a double-stranded DNA fragment containing the single PPARγ response element (PPRE, upper case)(**Fig. 1A**). The PPRE fragment was inserted into the *Bgl* II site of the pGL3-Promoter vector (Promega) to generate a PPARγ-responsive luciferase reporter construct, designated pGL3-Promoter-PPRE (**Fig. 1B**). We overexpressed PPARγ2 without an epitope tag using a cDNA clone (MC201042) harboring full-length mouse *Pparγ2* cDNA in pCMV6-Kan/Neo (OriGene). Other cDNAs encoding mouse RXRα, PGC-1α, PGC-1β, PGC-2, SRC1, SRC3 and p300 were amplified from a 3T3-L1 cDNA library using appropriate primer sets (**Table 1**). The PCR products were inserted into the expression vector pcDNA3.1/Hygro (Thermo). A CREB binding protein (CREBP) overexpression construct was generously gifted from Richard H. Goodman, M.D., Ph.D., Oregon Health and Science University.

Transfection and Luciferase Assay

NIH3T3 cells were obtained from the Japan Health Science Foundation, Health Science Research Resources Bank, and maintained in Dulbecco's Modified Eagle Medium with high glucose and L-glutamine (Thermo) containing 10% fetal bovine serum (Sigma-Aldrich) and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For the luciferase reporter assay, we seeded NIH3T3 cells that stably express *Renilla* luciferase in 96-well black clear-bottom plates (Corning), and used them for the luciferase reporter assays when they reached 95% confluence. The cells were co-transfected with pGL3-Promoter-PPRE (60 ng) and plasmid DNA mix (60 ng) containing PPARγ2, RXRα, and CBP overexpression constructs (20 ng each) in 6 μL of Hanks' Balanced Salt Solution and 0.24 μL of PEI "MAX" per well. Teneligliptin (final concentration, 10 or 50 μM) or water was added to the wells at 30 minutes after transfection. GW9662 (final concentration, 1, 5 or 10 μM) was added to the culture medium with teneligliptin (final concentration, 10 or 50 μM). The cells were harvested three days later, and luciferase assays were performed using the Dual-luciferase reporter assay system (Promega)(n=8). Luminescence was measured for 10 seconds using a Varioskan Flash (Thermo). The values were normalized to internal *Renilla* luciferase activities.

Adipocyte Differentiation

ST2 cells were obtained from the Japan Health Science Foundation, Health Science Research Resources Bank (Osaka, Japan), and maintained in RPMI1640 (Thermo) containing 10% fetal bovine serum (Sigma-Aldrich) and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in 96-well black clear-bottom plates and incubated until they reached 100% confluence. The culture medium was then changed to RPMI1640 containing 0.025 μM dexamethasone (DEX), 50 μM isobutylmethyxanthine (IBMX), 0.1 μM insulin with teneligliptin (1.56 or 12.5 μM), rosiglitazone (0.1 μM) or pioglitazone (1.25 μM). We estimated adipocyte differentiation by staining intracellular triglycerides with AdipoRed assay reagent (Lonza) at day 9 after initiating differentiation (n = 8).

Results

Effects of Teneligliptin on PPRE-Reporter Activity

Since teneligliptin is functionally and structurally similar to TZDs, we investigated whether teneligliptin can affect transcriptional activity of PPARγ using cell-based reporter assays. We co-transfected a reporter construct containing 1xPPRE (**Fig. 1A**) with a mouse PPARγ2 overexpression vector and the PPARγ-associated transcription factors, RXRα and CBP, into NIH3T3 cells. The nucleotide sequence of PPRE was determined based on previous reports[3,13–16]. Luciferase activity was measured after a 3-day incubation with various concentrations of teneligliptin. Relative luciferase activity increased dose-dependently in the presence of teneligliptin (**Fig. 1B**). Teneligliptin significantly activated PPRE-reporter expression at 50 and 100 (data not shown) μM. Relative luciferase activity did not significantly increase in negative control cells without PPARγ2 overexpression even in the presence of 50 μM teneligliptin (**Fig. 1B**). Thus,

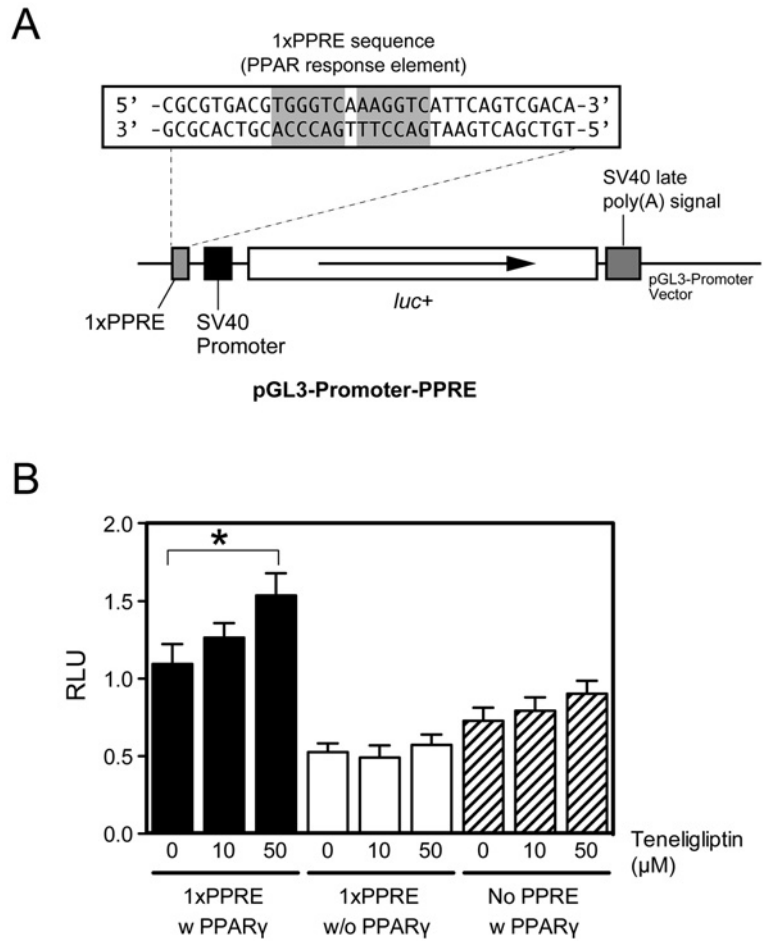

Fig. 1 **A**, Sequence of PPRE in pGL3-Promoter vector used for reporter assay. Consensus sequence of PPAR responsive element (1xPPRE) was designed based on referenced publications. Shaded nucleotides indicate direct repeat sequences separated by a nucleotide. **B**, Effects of teneligliptin on PPRE-reporter activity. NIH3T3 cells were transfected with luciferase reporter construct pGL3-Promoter-PPRE, under control of a SV40 promoter and 1xPPRE sequence and a PPARγ2 overexpression construct. Teneligliptin (50 μM in culture medium) significantly transactivated luciferase reporter gene downstream of 1xPPRE. No significant increase was evident in negative control cells transfected with a reporter construct harboring 1xPPRE but without PPARγ2 overexpression (1xPPRE, w/o PPARγ) or a reporter construct harboring no PPRE but with PPARγ2 overexpression (no PPRE, w PPARγ) at any tested dose of teneligliptin (n=8). *P<0.01.

teneligliptin appeared to activate PPRE-regulated expression although the dose exceeded that of TZDs (nanomolar range) and the concentration of teneligliptin used in the clinical therapy (submicromolar range).

Effect of Transcription Coregulators on Teneligliptin-Induced Transactivation of PPRE-Reporter Construct

PPARγ activity is regulated by many common transcription coregulators including the nuclear receptors CBP/p300, SRC1 and PGC-1α. To determine whether these coregulators modify the transactivation of PPRE-reporter expression induced by teneligliptin, we co-transfected a coregulator expression vector with a reporter and PPARγ2 expression constructs. **Figure 2A** shows that 10 and 50 μM teneligliptin increased PPRE-reporter activity in the cells that overexpressed only PPARγ2 (pcDNA, empty vector)(p<0.01). The activation effect of teneligliptin was lost in cells overexpressing PGC-1α, PGC-1β, PGC-2 or CREBP; reporter activity did not significantly differ between 0 and 50 μM. The overexpression of SRC1, SRC3 and p300 caused a similar

Table 1 Primer sequences of transcription factors

mRXRa_UP1	5′-CACCATGGACACCAAACATTTCCTGCCGCTCGACTTC-3′
mRXRa_LP1	5′-CTAGGTGGCTTGATGTGGTGCCTCCAGCATCTC-3′
mPGC1a_UP1	5′-CACCATGGCTTGGGACATGTGCAGCCAAGACTCTGTA-3′
mPGC1a_LP1	5′-TTACCTGCGCAAGCTTCTCTGAGCTTCCTTCAGTAAA-3′
mSRC-1_UP1	5′-CACCATGAGTGGCCTTGGGGACAGTTCATCAGAC-3′
mSRC-1_LP1	5′-CTAGTCTGTAGTCACCACAGAGAAGAACTCTTCTGTCTTCTTGTC-3′
mSRC-3_UP1	5′-CACCATGAGTGGACTAGGCGAAAGCTCTTTGGAT-3′
mSRC-3_LP1	5′-TCCCACTAGGGAGATGTCAGCAGTATTTCTGAT-3′
mPGC1b_UP1	5′-CACCATGGCGGGGAACGACTGCGGCGCGCTGCTGGATGAAGA-3′
mPGC1b_LP1	5′-TCAATGCAGGCTCTGCTGGGCCTCTTTCAGT-3′
mPGC2_UP1	5′-CACCATGGCGGCGCCGCAGCCCAGCCAGGACCCCCAAAG-3′
mPGC2_LP1	5′-CAGGCTCCGCCGCTCAGCCGGTGATG-3′

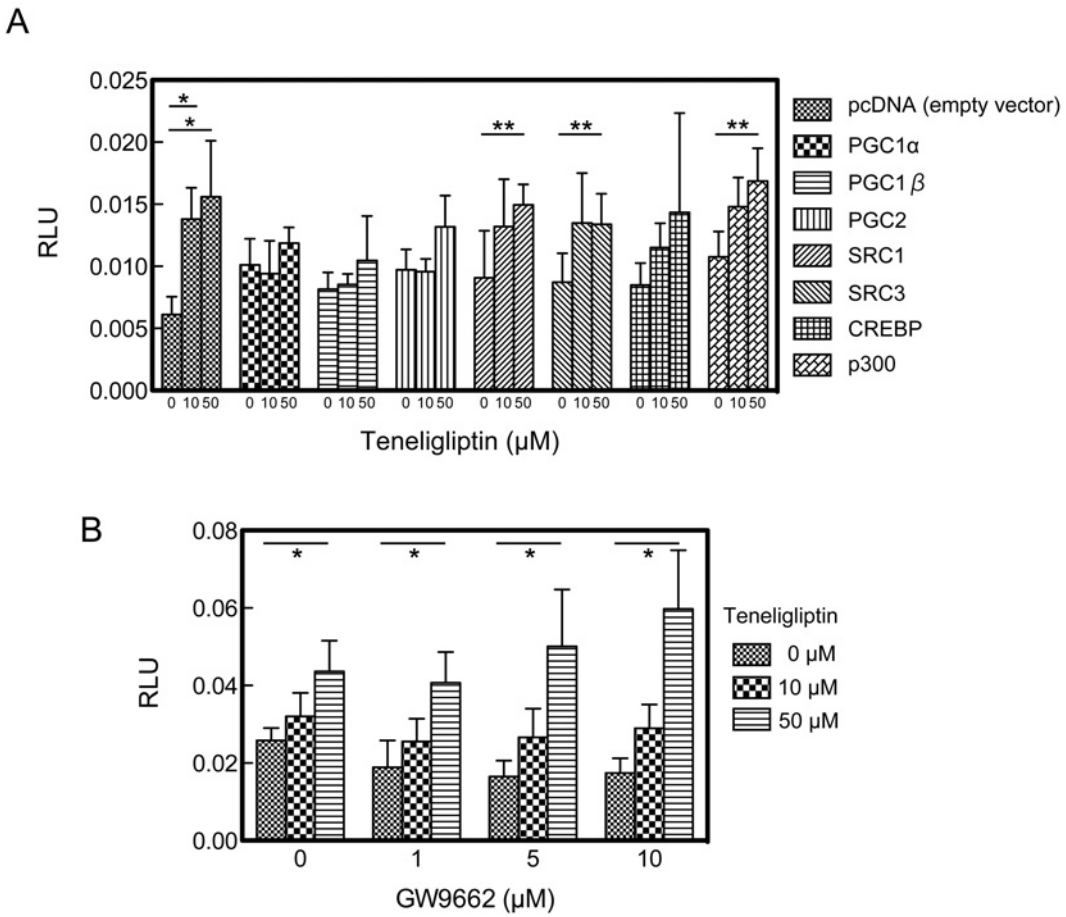

Fig. 2 **A**, Effects of transcription coregulators on transactivation of PPRE-reporter construct. A luciferase reporter construct containing 1xPPRE was co-transfected with constructs that express PPARγ2 and transcription coregulators such as PGC1α or SRC1. Adding teneligliptin into culture medium at 10 or 50 μM of positive-control cells overexpressing only PPARγ2 resulted in a significant activation of reporter gene expression (pcDNA, empty vector), whereas suppression or no effect on transactivation was found in cells overexpressing either transcription coregulators (n=4). **P*<0.01, ***P*<0.05. **B**, Effects of GW9662, PPARγ antagonist, on PPRE-reporter transactivation induced by teneligliptin. GW9662 (1−10 μM) did not affect PPRE-reporter transactivation induced by teneligliptin (10 and 50 μM) (n=8). **P*<0.01.

amount of reporter gene upregulation at only 50 μM compared with that of untreated control cells. Thus, transcription coregulators used here slightly suppressed or did not affect the reporter gene upregulation by teneligliptin.

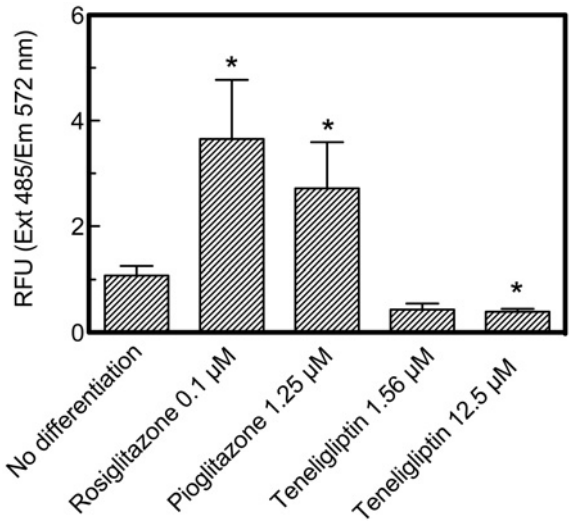

Fig. 3 Effects of teneligliptin on adipogenesis of ST2 cells. Teneligliptin (1.56 and 12.5 μM) did not induce or rather slightly suppressed adipogenesis. Adipogenesis is evident in cells cultured in medium containing the PPARγ agonist ligands rosiglitazone or pioglitazone, compared with that of cells cultured in medium containing no agonist (n=8). *P<0.01.

Effect of PPARγ Antagonist, GW9662, on Transactivation of PPRE-Reporter Construct by Teneligliptin

The activity of the ligand-dependent transcription factor PPARγ is inhibited in the presence of antagonistic agents. GW9662 is an irreversible PPARγ antagonist that blocks PPARγ activation by TDZs at the nanomolar level[17]. To determine whether the transactivation of PPRE-reporter expression by teneligliptin is caused by the association of teneligliptin with the ligand-binding domain of PPARγ, we incubated NIH3T3 cells expressing the reporter gene and PPARγ2 with various doses of GW 9662 and teneligliptin. As shown in **Figure 2B**, doses of GW9662 between 1−10 μM did not inhibit the activation of PPRE-reporter expression by teneligliptin (P<0.01).

Effect of Teneligliptin on Adipogenesis

The finding that teneligliptin stimulated PPRE-reporter transactivation in NIH3T3 cells indicated that it is a potential PPARγ activator. During the adipocyte differentiation, PPARγ is upregulated and plays a pivotal role in the adipogenic signaling cascade[18,19]. To determine whether teneligliptin can promote adipogenic differentiation, we cultured the mouse mesenchymal stem-cell line, ST2, that can differentiate into adipocytes upon specific stimulation[20] in differentiation medium containing DEX, IBMX, insulin and teneligliptin. Teneligliptin did not induce (1.56 μM) or rather slightly suppressed adipogenesis (12.5 μM, P<0.01) in ST2 cells whereas rosiglitazone and pioglitazone significantly enhanced the differentiation into adipocytes (P<0.01) (**Fig. 3**).

Discussion

Teneligliptin is a DPP-4 inhibitor that is administered to patients with T2DM to control fasting and postprandial glucose levels. Teneligliptin also prevents obesity and obesity-related manifestations in mice with obesity induced by a high-fat diet[21]. One of the main reasons for this might be increased energy expenditure because the total O_2 consumption was increased by 22% in mice dosed with teneligliptin and fed with a high-fat diet. Teneligliptin could not increase the expression levels of receptors that increase energy expenditure such as PPARs, estrogen receptors, and thyroid receptors[21]. The present study found that teneligliptin can affect genes that are regulated by PPARγ-binding to PPREs (**Fig. 1B**), whereas the activation of PPREs by teneligliptin was not attenuated by GW9662, an irreversible PPARγ antagonist (**Fig. 2B**). One possible explanation is that teneligliptin associates with various sites in PPARγ other than the ligand-binding domain. We speculate that teneligliptin binds to PPARγ and causes a conformational change, which enhances its transcriptional activity. The activation of PPARγ by teneligliptin might contribute to an increase in whole-body energy expenditure[21] without DPP-IV inhibition[22], as seen in the thiazolidinedione activation of PPARγ[21,23,24] by inducing the expression of uncoupling protein (UCP)-2 in adipose tissue[25–27] and UCP-3 in muscle[28]. This might be a unique feature of teneligliptin as it has not been identified in other DPP-IV inhibitors such as sitagliptin and linagliptin.

Teneligliptin did not promote but rather suppressed the adipogenesis of ST2 cells (**Fig. 3**). Teneligliptin decreases uric acid secretion in rats fed with a high-fat diet and in 3T3-L1 adipocytes by reducing xanthine dehydrogenase (XDH) activity[29]. The activity of XDH is transiently induced during the 3T3-L1-cell differentiation into adipocytes[30]. Furthermore, knockdown of xanthine oxidoreductase, which has the enzymatic activity of either XDH or xanthine oxidase, causes a decrease in uric acid levels and consequently inhibits the mRNA-upregulation and transcriptional activity of PPARγ during adipogenesis[30]. Therefore, the suppression of ST2-cell differentiation into adipocytes might be due to reduced activity of XDH caused by teneligliptin, although PPARγ, which is essential to the initiation of adipogenesis, was probably partially activated by an association between teneligliptin and a non-ligand-binding domain of PPARγ.

In conclusion, we found that teneligliptin could increase PPARγ activity in cell-based assays irrespective of the PPARγ ligand-binding domain, whereas it inhibits adipocyte differentiation.

Acknowledgements: We are grateful to Ms. Sawako Sato (Saitama Medical University) for her technical assistance.

Acknowledgements of grant support: This study was supported by JSPS KAKENHI (https://www.jsps.go.jp/english/index.html) Grant number 25460299 and 26461367.

Conflict of Interest: The authors have no conflicts of interest regarding the publication of this paper.

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(Received, November 11, 2017)

(Accepted, December 12, 2017)