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The glucagon-like peptide 1 receptor agonist enhances intrinsic peroxisome proliferator-activated receptor γ activity in endothelial cells

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ABSTRACT

Recent studies have suggested glucagon-like peptide-1 (GLP-1) signaling to exert anti-inflammatory effects on endothelial cells, although the precise underlying mechanism remains to be elucidated. In the present study, we investigated whether PPARγ activation is involved in the GLP-1-mediated anti-inflammatory action on endothelial cells. When we treated HUVEC cells with 0.2 ng/ml exendin-4, a GLP-1 receptor agonist, endogenous PPARγ transcriptional activity was significantly elevated, by approximately 20%, as compared with control cells. The maximum PPARγ activity enhancing effect of exendin-4 was observed 12 h after the initiation of incubation with exendin-4. As H89, a PKA inhibitor, abolished GLP-1-induced PPARγ enhancement, the signaling downstream from GLP-1 cross-talk must have been involved in PPARγ activation. In conclusion, our results suggest that GLP-1 has the potential to induce PPARγ activity, partially explaining the anti-inflammatory effects of GLP-1 on endothelial cells. Cross-talk between GLP-1 signaling and PPARγ activation would have major impacts on treatments for patients at high risk for cardiovascular disease.

1. Introduction

Glucagon-like peptide (GLP)-1, which is one of the incretin peptides released from the intestine in response to ingesting food, has been shown to improve glycemic control in type 2 diabetic patients by augmenting insulin secretion from β-cells [1]. Thus, these agents are widely used for treating patients with type 2 diabetes. In addition to its insulin-secreting activity, GLP-1 reportedly has a number of beneficial effects, such as β-cell proliferation [2], reducing the plasma glucagon concentration [3], a postprandial glucose-lowering effect via delayed gastric emptying [4], myocardial protection [5] and attenuating endothelial dysfunction [6]. Some of these extrapancreatic effects are extremely beneficial to patients with type 2 diabetes, who are at high risk for developing atherosclerosis [7]. Recent studies have suggested GLP-1 receptor agonists to exert anti-inflammatory effects on endothelial cells. For example, GLP-1 receptor agonists inhibited TNFα-induced upregulation of NADPH oxidase, NF-κB activation and apoptosis in endothelial cells [8]. All of these effects would lead to the prevention of atherosclerosis development. However, the precise underlying mechanism remains to be elucidated.

Thiazolidinediones (TZDs), insulin-sensitizing agents used for treating type 2 diabetic patients, are a well-known exogenous ligand for the peroxisome proliferator-activated receptor γ (PPARγ). PPARγ activation leads to the inhibition of intracellular serine/threonine kinases, such as JNK, resulting in reduced IRS-1 serine phosphorylation, i.e., one of the main causes of insulin resistance [9]. In the PROactive study [10], the TZD pioglitazone was demonstrated to reduce the risk of macrovascular events in type 2 diabetes. To date, a number of studies have focused on the mechanism by which TZD, i.e., PPARγ activation, reduces atherosclerosis. The most plausible explanation is that PPARγ is abundantly expressed, not only in adipose tissue, but also in the vascular endothelium and macrophages [11]. Furthermore, PPARγ activation was demonstrated to inhibit transcription factors, such as NF-κB, resulting in atherosclerosis prevention via suppression

Abbreviations: GLP-1, glucagon-like peptide-1; HUVECs, human umbilical vein endothelial cells; PKA, protein kinase A; IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-kappa B; TNFα, tumor necrosis factor α; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; NADPH, nicotinamide adenine dinucleotide phosphate; NOX-1, nicotinamide adenine dinucleotide phosphate oxidase-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PPRE, peroxisome proliferator-activated receptor response element; HOMA-R, homeostasis model assessment as an index of insulin resistance; PAI-1, plasminogen activator inhibitor 1.

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of the expressions of cytokines and adhesion molecules in endothelial cells [12–14].

When we compare these biological effects of GLP-1 with those of TZDs, it is apparent that these agents have shared properties, i.e., anti-inflammatory effects on endothelial cells. In the present study, we hypothesized that PPARγ activation is involved in the GLP-1-mediated anti-inflammatory action on endothelial cells and thus aimed to explore this possibility. Using HUVEC, an endothelial cell line, we measured PPARγ transcriptional activity in the presence of exendin-4, a GLP-1 receptor agonist. We obtained evidence that this GLP-1 receptor agonist has the potential to induce PPARγ activity, partially explaining the anti-inflammatory effects of GLP-1 on endothelial cells. Our findings may provide a novel insight into the effects of current therapies for patients with type 2 diabetes.

2. Materials and methods

2.1. Cell culture and chemicals

HUVECs (human umbilical vein endothelial cells), purchased from Takara Bio, Inc., were maintained in Endothelial Cell Basal Medium 2 (EBM-2) (Takara Bio, Inc, Shiga, Japan) at 37 °C in 10% CO_2. Cells at more than 90% confluence by passages 3–5 were used in the current study. Recombinant TNFα (Wako Pure Chemical Industries, Ltd., Osaka, Japan), forskolin (Wako Pure Chemical Industries, Ltd.), H89 (Seikagaku Co., Tokyo, Japan), pioglitazone (Takeda Co., Tokyo, Japan), GW9662 (Millipore, Temecula, CA, USA), and exendin-4 (Sigma-Aldrich, Co., St. Louis, MO, USA) were purchased and used for the experiments. The indicated concentrations of chemicals were added 12 h before cell lysate preparation.

2.2. Antibodies and Western blotting

Western blotting was performed as previously described [15]. Briefly, after incubation with the indicated chemicals, HUVECs were washed with ice-cold phosphate buffered saline (PBS), lysed in ice-cold RIPA buffer (Millipore, Temecula, CA, USA), and then centrifuged at 14,000×*g* for 10 min at 4 °C. The resulting supernatants, including tissue protein extracts, were then subjected to SDS–PAGE. In the immunoprecipitation experiments, cell lysates were incubated with anti-whole nuclear factor κB (NF-κB) polyclonal antibody (Santa Cruz Biotechnology, Inc, Dallas, TX, USA), and protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were then used to precipitate the immune complexes. The beads were washed three times with lysis buffer and the immunoprecipitated proteins were separated from the beads by boiling in Laemmli buffer. The beads were removed by centrifugation, and the supernatants were subjected to SDS–PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with the indicated antibody. Commercial antibodies against ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Inc.), and against phospho-NF-κB (Santa Cruz Biotechnology, Inc.), were used as primary antibodies. After blotting with the indicated secondary antibody, detection was performed using an ECL chemiluminescent kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The bands were then scanned and analyzed with National Institute of Health Image software. Protein band intensities under basal conditions were set as 1 for the purpose of normalization.

2.3. RNA preparation and real time PCR

Total RNA was isolated from HUVECs with Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized from the purified RNA using a reverse transcriptase kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. For quantitative analysis of VCAM-1 and NADPH oxidase-1 (NOX-1) mRNA, we conducted real-time PCR using an ABI PRISM Model 7000 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primer sets and probes for human VCAM-1 (Assay ID; Hs00365486_m1), NOX-1 (Assay ID; Hs00246589_m1), PPARγ (Assay ID; Hs01115513_m1) and, as an internal control, GAPDH (Assay ID; Hs01922876_u1) were purchased.

2.4. Measurement of PPARγ transcriptional activity

Subconfluent HUVECs were co-transfected with adenoviruses expressing either PPRE(−)-Luc reporter or the PPRE(+)-Luc reporter gene and Rluc reporter vectors as previously described [16]. After HUVECs had been incubated with EBM-2 containing the adenoviruses for 2 h at 37 °C, the growth medium was added. Experiments were performed 48 h after adenovirus transfection. Chemicals were added to the medium 12 h before the experiments. Two days after transfection, a luciferase assay was performed according to the manufacturer's instructions (Promega, Fitchburg, WI, USA).

2.5. Statistical analysis

Data are presented as means ± SDs. Log transformation of continuous variables was used when needed to satisfy distributional requirements for parametric tests. Differences in clinical characteristics were assessed by employing the paired Student's *t* test and a *P* value <0.05 was considered statistically significant. Statistical analyses were performed using Stat View software (Version 5.01; SAS Institute, Cary, NC, USA).

3. Results

3.1. Expressions of NOX-1 and adhesion molecules in the presence of exendin-4 or pioglitazone

To compare the effects of TZD and GLP-1 on HUVEC, we first investigated the transcriptional expressions of NOX-1 and VCAM-1, which both appear to be regulated by PPARγ agonists as previously described [17,18]. As shown in Fig. 1A, both NOX-1 and VCAM-1 expressions were slightly but significantly inhibited by the incubations with a GLP-1 receptor agonist, exendin-4, while these expressions were strongly suppressed during incubation with pioglitazone. As the translational expressions of these genes were so low that it was difficult to compare their protein levels by Western blotting, we next incubated HUVEC with TNFα and examined the translational expressions of ICAM-1 and VCAM-1, which were up-regulated by prior incubation with TNFα. When HUVEC were treated with 1 μM pioglitazone, the expressions of ICAM-1 and VCAM-1 were both strikingly decreased, to less than half of the levels obtained by incubation with TNFα alone (Fig. 1B, left panel). On the other hand, with exendin-4 treatment, expressions of ICAM-1 and VCAM-1 were both inhibited to a smaller extent than with pioglitazone (Fig. 1B, right panel).

3.2. Effects of GW9662 (PPARγ inhibitor) on the exendin-4 induced suppressions of adhesion molecules and NF-kB phosphorylation

To examine the mechanisms underlying the decreased expressions of adhesion molecules by exendin-4, we co-incubated HUVEC with a PPARγ inhibitor, GW9662. As shown in Fig. 2A, incubation with GW9662 in addition to TNFα and exendin-4, partially eliminated the exendin-4-induced suppressions of ICAM-1 (left panel) and VCAM-1 (right panel). These results suggest that

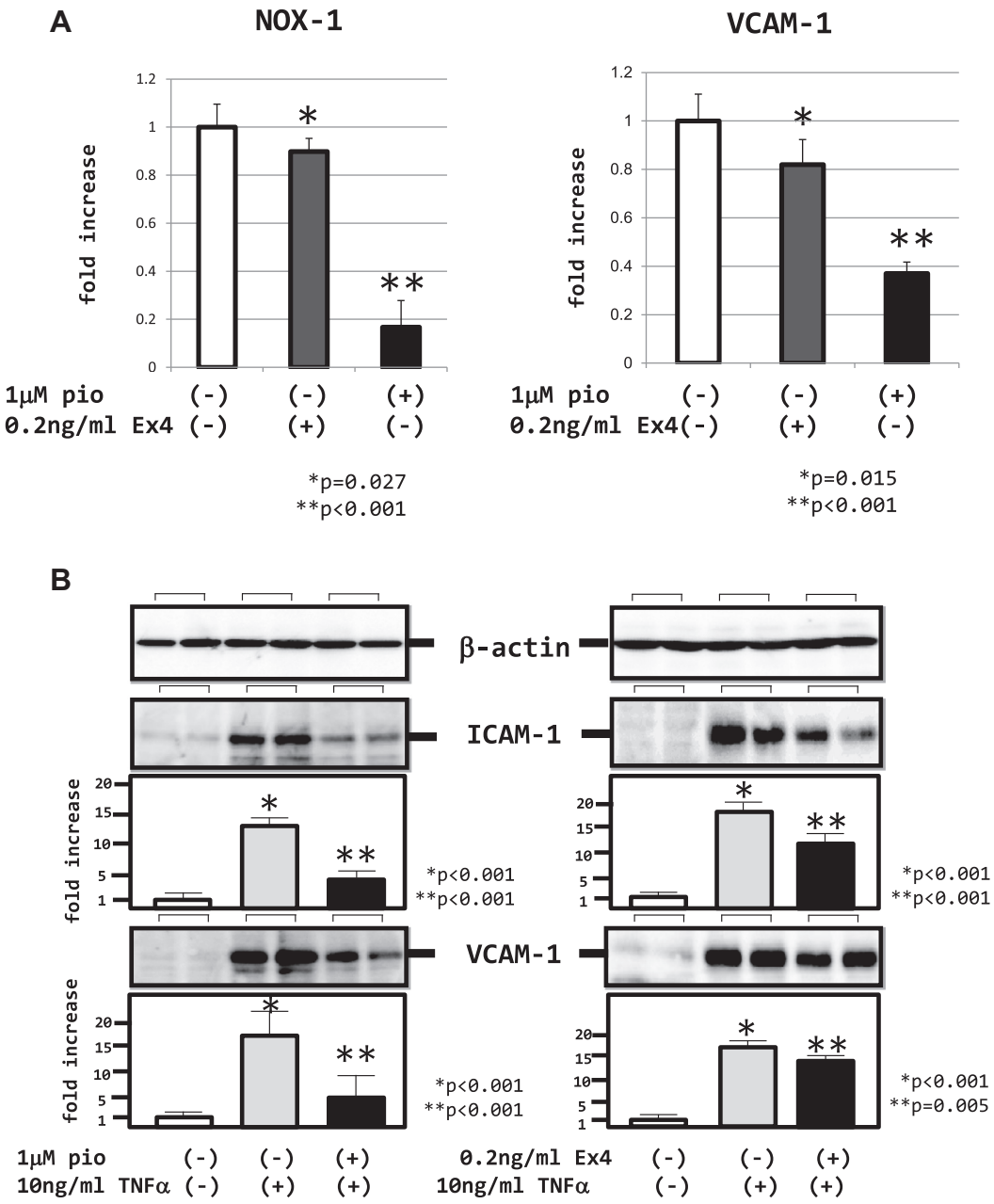


Fig. 1. (A) Expressions of NOX-1 and VCAM-1 mRNA in HUVEC were analyzed by quantitative real time PCR, during incubation with the indicated concentration of exendin-4 or pioglitazone. Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals ($n = 6$). (B) Effects of exendin-4 or pioglitazone on the TNFα-induced expressions of ICAM-1 and VCAM-1 proteins in HUVEC were analyzed by Western blot using anti-β-actin (upper panel) or anti-ICAM-1 (left middle panel) or anti-VCAM-1 (right middle panel) antibody. Representative data from three independent experiments are presented. *Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals ($n = 6$). **Significant difference ($P < 0.05$) relative to cells incubated with only TNFα ($n = 6$).

PPARγ activation might be involved in the actions of GLP-1 in HUVEC, which are endothelial cells. As previously demonstrated in skeletal muscle cells, PPARγ activation strongly suppresses cytokine-induced transcriptions of NF-κB dependent genes [19]. We thus investigated whether NF-κB inactivation is involved in the suppression of GLP-1-induced adhesion molecules. As shown in Fig. 2B, TNFα-induced NF-κB phosphorylation was significantly inhibited by exendin-4 incubation, while this effect was reversed by GW9662. These results indicate pioglitazone and GLP-1 activation to have similar mechanisms of action, at least in terms of inhibiting the NF-κB targeted adhesion molecules in endothelial cells.

3.3. Induction of PPARγ activity by incubation with exendin-4

As previously described [16], transcription reporter assays were used to determine the effects of the indicated concentrations of exendin-4 and 1 μM pioglitazone on the transcriptional activity of endogenous PPARγ in HUVEC (Fig. 3A). A marked increase (3.73 ± 0.37-folds) in transcription was observed after 12 h treatment with 1 μM pioglitazone as a positive control. When we examined the effects of exendin-4 on PPARγ-mediated transcriptional activity, it was found to be significantly increased by 1.21 ± 0.22-fold at the 0.2 ng/ml dose of exendin-4, which would be a therapeutic concentration in diabetic patients receiving

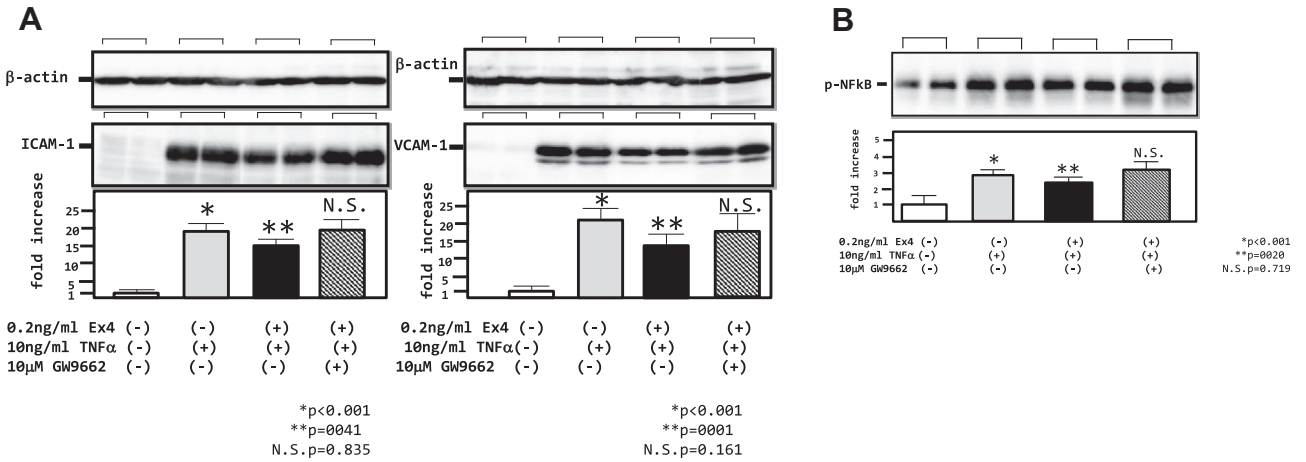
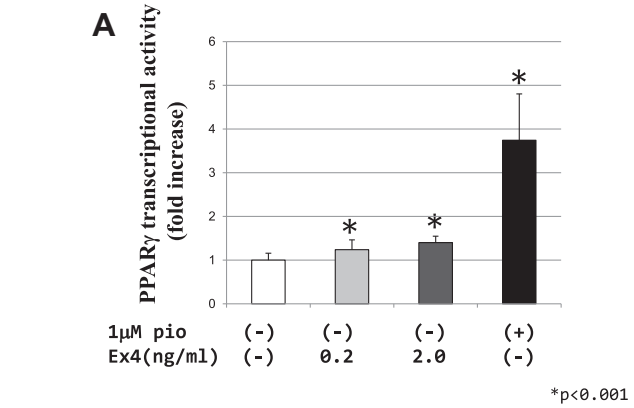


Fig. 2. (A) Effects of GW9662, a PPARγ inhibitor, on the exendin-4 induced suppressions of ICAM-1 or VCAM-1 in HUVEC were analyzed by Western blot using anti-β-actin (upper panel) or anti-ICAM-1 (left middle panel) or anti-VCAM-1 (right middle panel) antibody. (B) Effects of exendin-4 on the NF-κB phosphorylation in HUVEC were analyzed by Western blot using anti-phospho NF-κB antibody (upper panel). Representative data from three independent experiments are presented. *Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals ($n = 6$). **Significant difference ($P < 0.05$) relative to cells incubated with only TNFα ($n = 6$). N.S.; not significant relative to cells incubated with only TNFα.



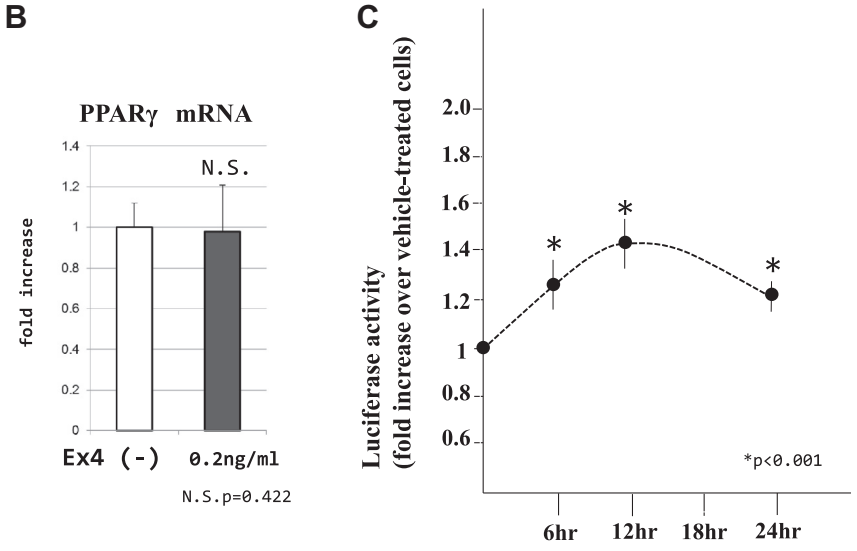


Fig. 3. (A) HUVEC were incubated with the indicated concentrations of exendin-4 in the presence or absence of pioglitazone. Extracts from HUVEC, analyzed by dual luciferase assay as previously described [16]. Data from four independent experiments (each $n = 6$) were analyzed. *Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals. (B) PPARγ mRNA levels of HUVEC were analyzed by quantitative real time PCR ($n = 6$). N.S.; not significant relative to cells incubated with no chemicals. (C) Time course of PPARγ activity in the presence of 2 ng/ml exendin-4 is presented. Data from four independent experiments (each $n = 6$) were analyzed. *Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals.

exendin-4 treatment [20]. This effect was concentration dependent. Based on the lack of significant changes in PPARγ amounts in the presence of 0.2 ng/ml exendin-4 (Fig. 3B), the PPARγ enhancing activity observed with exendin-4 was not attributable to an increased amount of PPARγ but rather to greater enhancement of the intrinsic PPARγ activity. The time course of the exendin-4 effect on PPARγ activity is shown in Fig. 3C. The PPARγ transcriptional activity increase induced by pioglitazone was detected after 3 h and had nearly peaked by 12 h of treatment. The maximum PPARγ activity-enhancing effect was observed 12 h after the initiation of exendin-4 incubation.

3.4. Effects of forskolin or H89 on the exendin-4 induced PPARγ activity

Next, we examined whether PKA activation, which is a major downstream signal of GLP-1, is involved in exendin-4 induced PPARγ enhancing activity. We first endeavored to confirm that PKA activation increased PPARγ activity in HUVEC. As shown in Fig. 4 and 12 h incubation with 100 nM Forskolin, a PKA activator, up-regulated PPARγ transcriptional activity by 2.1-fold as previously described [21–23]. When HUVEC were incubated with both exendin-4 and H89, a PKA inhibitor, exendin-4-induced PPARγ enhancing activity was partially abolished, suggesting that the exendin-4 induced PPARγ enhancing effect was exerted via enhanced PKA activity at a point downstream from GLP-1 signaling.

4. Discussion

In our present study, we directly demonstrated the PPARγ-enhancing activity of a GLP-1 receptor agonist in endothelial cells and also showed this agent to inhibit TNFα-induced expressions of adhesion molecules via attenuation of NF-κB phosphorylation. Our results provide evidence that a GLP-1 receptor agonist induces PPARγ activity via PKA activation, a major downstream component of GLP-1 signaling. The incubation with 0.2 ng/ml exendin-4 for 6 h employed in our experiments is equivalent to the exendin-4 exposure received in the treatments given to type 2 diabetic patients [24]. Thus, our observations are physiologically relevant to the evaluation of therapy using this agent. Several studies have already shown PPARγ to be activated by PKA activators [21–23]. Though the precise mechanism underlying PPARγ activation by PKA activators remains to be elucidated, it is possible that PKA stabilizes

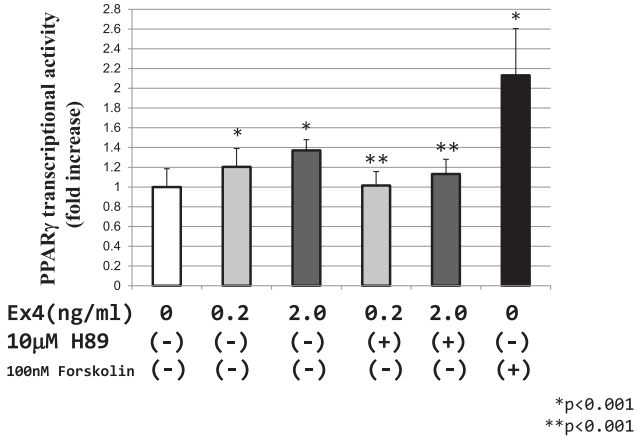


Fig. 4. Effects of forskolin, PKA activators or H89, a PKA inhibitor, on exendin-4 induced PPARγ activity were examined. HUVEC were incubated with the indicated concentrations of exendin-4 in the presence or absence of 10 μM H89. Cells treated with 100 nM forskolin are presented as a positive control. Data from four independent experiments (each $n = 6$) were analyzed. *Significant difference ($P < 0.05$) relative to control cells in the absence of the tested chemicals. **Significant difference ($P < 0.05$) relative to corresponding cells incubated without H89.

binding of PPARγ molecules to PPRE, as demonstrated by gel retardation experiments [21]. One report indicated that PKA signaling pathways are required for adipogenesis, i.e., PPARγ activation [22]. This hypothesis is strongly supported by the requirement of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, which stimulates PKA by raising the intracellular cAMP level, for efficient differentiation of 3T3-L1 fibroblasts into adipocytes.

Apart from PKA, many natural and synthetic compounds reportedly activate PPARγ. Among them, a specific subset of angiotensin II type 1 receptor blockers was reported to induce PPARγ activity [25]. We also previously demonstrated glimepiride, a sulfonylurea agent, to exert a PPARγ-enhancing pleiotropic effect [23]. As these two agents are widely used for the treatment of obese or type 2 diabetic patients, it is very significant that these agents have an insulin-sensitizing PPARγ action. A number of studies including our previous trials [26,27] examined whether the PPARγ-enhancing activities confirmed by employing *in vitro* experiments were clinically exerted, resulting in the amelioration of insulin resistance as assessed by HOMA-R.

As PPARγ is abundantly expressed in adipose tissue and the beneficial effects of PPARγ activators were not observed in lean subjects [26,27], the main target organ of PPARγ activators is apparently adipose tissue. Provided that GLP-1 has a PPARγ-enhancing activity as observed in the present study, GLP-1 as well as PPARγ ligands, would presumably exert insulin-sensitizing effects. In fact, a GLP-1 receptor agonist was previously reported to have an insulin-sensitizing effect in muscle and adipose tissues [28,29]. Recently, liraglutide, a GLP-1 receptor agonist, was reported to promote pre-adipocyte proliferation and adipogenesis [30]. Thus, we hypothesized that GLP-1 exerts these actions via PPARγ activation and endeavored to examine whether a GLP-1 receptor agonist stimulates PPARγ activity in 3T3-L1 adipocytes. However, in our preliminary experiment, we detected no PPARγ-enhancing activity when 3T3-L1 adipocytes were incubated with a GLP-1 receptor agonist. As GLP-1 receptors are not as abundantly expressed in adipocytes as in endothelial cells [31], these different characteristics, which HUVEC and 3T3-L1 adipocytes exhibited in terms of their responses to GLP-1, might partially be explained by the differences in their GLP-1 receptor expression levels.

When vascular endothelial cells were incubated with high glucose or TNFα, increased expressions of adhesion molecules [13], PAI-1 [32] and NADPH oxidase [12] were observed. Oxidative stress derived from NADPH oxidase is a critical mediator promoting atherosclerosis [33]. Thus, these molecules are recognized as triggers of endothelial dysfunction or arteriosclerosis, possibly providing a persuasive explanation for why obesity and hyperglycemia apparently predispose patients to cardiovascular disease (CVD) [8,12,13]. PPARγ activators exert their beneficial effects, besides reducing serum glucose levels, by attenuating NF-κB phosphorylation, translocation and binding activity to DNA in vascular endothelial cells, thereby reducing NF-κB targeted proteins, such as adhesion molecules, and NADPH oxidase [12]. GLP-1 receptor agonists were also reported to have effects similar to those of PPARγ activators in endothelial cells. For example, GLP-1 receptor agonists were revealed to reduce the expressions of PAI-1 [34] and adhesion molecules [8]. However, the mechanism by which GLP-1 exerts such beneficial effects on endothelial cells remains unknown. Our *in vitro* results provide the first evidence contributing to clarification of this mechanism. However, the limitation of this study is that we cannot clinically demonstrate this mechanism, because the atherosclerosis preventing benefits of employing a GLP-1 receptor agonist cannot be discriminated between PPARγ activation and improving glycemic control.

In conclusion, our results suggest that GLP-1 has the potential to induce PPARγ activity in HUVEC, which may explain the

GLP-1-induced anti-inflammatory actions observed in vascular endothelial cells. As PPARγ activation is definitely associated with the prevention of arteriosclerosis development, i.e., a reduced risk for CVD, our findings may provide major insights into the mechanism of GLP-1 based therapy for type 2 diabetes.

Acknowledgments

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