PPARγ Agonists Ameliorate Endothelial Cell Activation via Inhibition of Diacylglycerol–Protein Kinase C Signaling Pathway
Role of Diacylglycerol Kinase

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Subject—Peroxisome proliferator-activated receptor (PPAR)-γ agonists are emerging as potential protectors against inflammatory cardiovascular diseases including atherosclerosis and diabetic complications. However, their molecular mechanism of action within vasculature remains unclear. We report here that PPARγ agonists, thiazolidinedione class drugs (TZDs), or 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2) were capable of activating diacylglycerol (DAG) kinase (DGK), resulting in attenuation of DAG levels and inhibition of protein kinase C (PKC) activation. The PPARγ agonist-induced DGK was completely blocked by a dominant-negative mutant of PPARγ, indicating an essential receptor-dependent action. Importantly, the suppression of DAG-PKC signaling pathway was functional linkage to the anti-inflammatory properties of PPARγ agonists in endothelial cells (EC), characterized by the inhibition of proinflammatory adhesion molecule expression and adherence of monocytes to the activated EC induced by high glucose. These findings thus demonstrate a novel molecular action of PPARγ agonists to suppress the DAG-PKC signaling pathway via upregulation of an endogenous attenuator, DGK. (Circ Res. 2004;94:1515-1522.)

Key Words: PPARγ ▪ diacylglycerol kinase ▪ protein kinase C ▪ vascular inflammation ▪ diabetic complications

The recognition of inflammatory process in the development of atherosclerotic cardiovascular diseases including diabetic vascular complications has significantly impacted on clinical and basic approaches to the prevention and treatment of these diseases.1,2 Among these approaches, activators of the ligand-activated nuclear transcription factor, peroxisome proliferator-activated receptor-γ (PPARγ), have emerged as useful agents to protect against vascular inflammation. PPARγ is originally identified as a master regulator in adipogenesis and glucose homeostasis (reviewed in3). PPARγ is also expressed in vascular cells and exhibit anti-inflammatory properties within the vessel wall.4 Clinical observations have suggested that the thiazolidinedione (TZD) class of antihyperglycemic drugs, such as troglitazone, rosiglitazone, and pioglitazone, a set of PPARγ agonists, have beneficial effects on vasculature in addition to their insulin-sensitizing action.5,6 However, the precise effects of PPARγ agonists on vascular cells and the molecular mechanisms underlying their anti-inflammatory action remain unclear.

The integrity of vascular endothelial cells (EC) is fundamental for normal homeostasis of the vessel wall, particularly for maintaining the uninterrupted circulation of leukocytes, which is responsible for the anti-inflammatory phenotype of the endothelium.7 The anti-inflammatory phenotype of EC is maintained by a balance between positive and negative cellular signals in response to the environmental stimuli. In various disease states, such as atherosclerosis and diabetes, multiple or individual risk factors damage this phenotype through deregulation of signal transduction pathways, often rendering the induction of lipid second messengers such as diacylglycerol (DAG). DAG serves as an allosteric activator of the conventional and novel protein kinase C (PKC) isoforms that mediate many cellular functions including cell growth, activation, and differentiation.8 The DAG-PKC signaling pathway has been implicated in the pathogenesis of diabetic vascular diseases and insulin-resistance.9,10 Inhibition of this pathway by specific inhibitors of PKC, exemplified by the PKCβ inhibitor, was demonstrated to ameliorate the dysfunction of vasculature in diabetic animals,11 providing a new strategy for the treatment of diabetic vascular diseases. Inhibition of the DAG-PKC pathway can also be achieved by diacylglycerol kinase (DGK) that functions via...
decreasing intracellular DAG levels by phosphorylation of DAG yielding phosphatidic acid. DGK is thus regarded as an endogenous attenuator of the DAG-PKC pathway. In the present study, we have examined this endogenous attenuation mechanism in EC and found that the PPARγ agonists were capable of increasing DGKα production and DGK activity resulting in suppression of the DAG-PKC signaling pathway. Consequently, this inhibition was connected to the PPARγ agonists’ anti-inflammatory effect characterized by an inhibition of the proinflammatory adhesion molecule induction and adherence of monocytes to the activated EC induced by high glucose. These findings demonstrate that inhibition of DAG-PKC pathway through upregulation of DGK is a novel molecular mechanism by which PPARγ agonists ameliorate EC activation and protect against vascular inflammation.

Materials and Methods

Cell Cultures

Human umbilical vein cells (HUVEC) were isolated and cultured on gelatin-coated culture flasks in DMEM medium containing 20% fetal calf serum (FCS), endothelial growth supplement (Cambrex, Walkersville, Md), and heparin as described previously. Bovine aortic endothelial cells (BAEC) were harvested from calf aorta by methods previously described and maintained in DMEM supplemented with 10% FCS. ECs were used between passage 2 to 6. For the present study, we have examined this endogenous attenuation of the DAG-PKC pathway. In the present study, we have examined this endogenous attenuation of the DAG-PKC pathway.

Plasmids and Transfection

The human wild-type PPARγ1 cDNA and the double mutant PPARγ2A/A3 (gift from Dr F. Sakane, Sapporo Medical University, Japan), DGKα (Santa Cruz Biotechnology, Calif), FLAG (M2; Sigma, Clayton, Australia), and Actin (CHEMICON, Calif), respectively. Expression of cell-surface adhesion molecules was measured as fluorescence intensity by use of a Coulter Epics Profile XL flow cytometer.

Adherence of U937 Cells to EC

U937 cells (CRL 1593.2; American Type Culture Collection, Manassas, Va) were grown in RPMI-1640 medium (Invitrogen) containing 10% FCS and coloric labeled with 0.2 mg/mL MTT in the medium for 30 minutes at 37°C. The cells were then collected by low-speed centrifugation and resuspended at a density of 2x10⁶ cells/mL in medium without FCS. HUVECs were seeded into 24-well plates and cultured with NG or HG medium for 3 days after confluence. After washing twice with warm RPMI-1640 medium, the MTT-labeled U937 cell suspension (100 µL/well) was added and incubated for 30 minutes at 37°C. Nonadherent cells were removed by rinsing the plates 3 times with phosphate-buffered saline, and the number of adherent cells were counted under microscopy with at least 6 fields per well culture analyzed.

Measurement of DGK Activity

DGK activity was determined in cell lysates as previously described. Briefly, cell lysates were incubated with 1,2-dioleoylglycerol in the presence of [γ-32P]ATP. Thereafter, the generation of [γ-32P]PA by endogenous DGK was determined by its separation using TLC as a solvent system consisting of chloroform/methanol/4 mol/L ammonium hydroxide [9:7:2 (v/v/v)]. Bands corresponding to radiolabeled PA were quantified by using the Phospholimager (Molecular Dynamics).

Determination of total DAG Levels

Total lipids were extracted from cells exposed to NG or HG for 3 days. The amount of DAG mass was determined by its conversion into [γ-32P]PA by Escherichia coli DGK in the presence of [γ-32P]ATP as previously described. DAG levels were then corrected to the protein levels assayed by Bio-Rad reagents.

Reverse-Transcription Polymerase Chain Reaction and Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from HUVEC using Trizol (Invitrogen) in combination with RNeasy RNA kit (Qiagen). First-strand cDNA was synthesized from 1 μg of RNA, using 1 μmol/L of poly A primer of the sequence AAGCAGTGTTAAACCGGGAGAAGTATTTTTTTTTTTTTTTTTT with Omniscript RT Kit (Qiagen) following the manufacturer’s instructions. The sequences of polymerase chain reaction (PCR) primers were used were forward GGCTAGCGGAGATTCCAGCA and reverse CATCTCCAGACCCAGACAGA for DGKa; and forward GCCAAAATGCTGACCCACACAAA and reverse CTAGCTAGGAGGGCAAAGGAA for a housekeeping gene, cyclophilin. For transcript quantification purposes, real-time PCR was performed. Then 1 µL of the reverse-transcriptase product was used in a 25 µL volume reaction containing 200 µmol/L of each dNTP, 5 pmol of each primer, 1.5 µmol/L MgCl2, 0.2× SYBR Green I (Molecular Probes), and 1.25 U of AmpliTaq Gold (Applied Biosystems). Amplification was performed using Rotor Gene-3000 (Corbett Research) programmed as 94°C for 12 minutes followed by 40 cycles of (94°C for 20 seconds, 57°C for 20 seconds, 72°C for 40 seconds). A standard curve of DGKa amplification was generated by using known concentrations of the full DGKa sequence. Variability in the initial quantities of cDNA was normalized to the internal control, cyclophilin. A negative control was included in each set of experiments. Melting curve analysis was performed to enhance specificity of the amplification reaction, and the ROTorgene software was used to compare the amplification in the experimental samples during the log-linear phase to the standard curve.

Immunoblotting

Cell lysates were equalized to the same amount of proteins and resolved on 10% polyacrylamide-SDS gels followed by transfer to a nitrocellulose membrane. Thereafter, immunoblotting was performed with the antibodies against DGKa (Gift from Dr F. Sakane, Sapporo Medical University, Japan), DGKβ, PKCα (Santa Cruz Biotechnology, Calif), FLAG (M2; Sigma, Clayton, Australia), and Actin (CHEMICON, Calif), respectively.

Assay of PKC Activity and Translocation

PKC activity was assayed in situ as described previously. Briefly, cells were seeded in 24-well plates and exposed to NG or HG for 3 days. After the indicated treatments, total PKC activity was determined in the permeabilized cells in the presence of 10 µmol/L [γ-32P]ATP (5000 cpm/pmol) and 200 µmol/L PKC-specific peptide substrate (RKRTLRL). The activity was then quantified by scintillation counting and normalized with total protein levels. For PKCβ translocation analysis, transfected BAEC with stable overexpression of enhanced green fluorescent protein-fused PKCβ1 (PKCβ1-EGFP) were grown in NG or HG medium for 1 week, then plated onto fibronectin-coated 8-well microscope slides and incubated for
24 to 48 hours under the same culture conditions. The cells were washed once with serum-free DMEM containing NG or HG and then incubated for 4 hours in the presence or absence of 1 μmol/L or 10 μmol/L ciglitazone. Cells stimulated with VEGF were incubated with 10 ng/mL VEGF for 5 minutes. After the treatments, cells were immunostained for F-actin using rhodamine phalloidin. Epifluorescence microscopic analysis was performed using either 100× oil-immersion or 40× objective on an Olympus BX-51 microscope system, acquired to a Cool Snap FX charge-coupled device camera (Photometrics). Images were then analyzed with V++ software (Digital Optics, Auckland, New Zealand). For immunoblot analysis of PKCβ translocation, cytosolic and membrane fractions were isolated as described previously\(^\text{17}\) from the treated PKCβ-transfected BAEC.

### Results

**PPARγ Agonists Ameliorate EC Activation**

Because the induced expression of adhesion molecules is a phenotypic hallmark of EC activation and is critical for proinflammatory responses, we examined the effect of PPARγ agonists on the production of VCAM-1, ICAM-1, and E-selectin in HUVEC exposed to high glucose. After the glucose level was increased from 5.5 to 22 mmol/L in the media for 3 days, cell surface expression of VCAM-1, ICAM-1, and E-selectin were significantly increased by \(\sim\)3-fold, 2-fold, and 4-fold in cultured HUVEC, respectively (Figure 1). Neither mannitol nor l-glucose at 22 mmol/L had significant effect on the adhesion molecule expression (data not shown), indicating a specific effect of high glucose on EC caused by the surplus cellular metabolites of D-glucose rather than osmotic stress. Interestingly, the high-glucose–induced adhesion molecule expression was completely inhibited after treatment with the PPARγ agonists ciglitazone, troglitazone, or 15d-PGJ\(_2\) at a concentration of 2 μmol/L (Figure 1). This concentration used herein is reflective of the known affinity of the ligands binding to PPARγ,\(^\text{18}\) suggesting a receptor-dependent effect. Investigating the functional consequences of this observation, EC exposed to 22 mmol/L glucose resulted in significant increases in adherence of leukocytes to the EC (Figure 2). Remarkably, administration of 2 μmol/L ciglitazone profoundly reduced the number of leukocytes adhering to high-glucose–stimulated EC (Figure 2), suggesting a role for PPARγ agonists in protection against high-glucose–mediated EC activation.

**The Effect of PPARγ Agonists on EC Requires DGK Activity**

It has been recognized that activation of DAG-PKC signaling pathway plays a critical role in high-glucose–mediated dysfunction of vasculature including EC activation in diabetes.\(^\text{9}\) We thus examined a possible role of DAG-PKC pathway in the action of PPARγ agonists. As shown in Figure 3, high-glucose–induced expression of VCAM-1 was significantly attenuated by a PKC–specific inhibitor, GF-109203X. In contrast, PMA, an exogenous activator of PKC, significantly enhanced adhesion molecule expression, suggesting an involvement of DAG-PKC pathway in the regulation of adhesion molecule expression. However, although ciglitazone effectively blocked the high-glucose–induced VCAM-1 expression (Figure 3A), the PPARγ agonists were unable to...
PPARγ Agonists Upregulate DGKα Production

Time-course analysis showed that a significant increase in DGK activity induced by TZDs or 15d-PGJ2 appeared after treatment for at least 4 hours (Figure 4C). In addition, the effect of PPARγ agonists was abrogated by pretreatment with a transcription inhibitor, actinomycin-D (Figure 4A), suggesting that PPARγ-dependent transcriptional activities are required for the increased DGK activity. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis showed that treatment of cells with the PPARγ agonists for 4 hours resulted in significant increases in the mRNA levels of DGKα (Figure 5A), but not DGK-β or DGK-ζ isoforms (data not shown). The increased DGKα mRNA levels were quantified by real-time PCR analysis, showing ~5-fold to 8-fold increases in the mRNA levels after the treatment with PPARγ agonists (Figure 5B). Furthermore, the immunoblotting analysis revealed marked increases in DGKα protein levels in the cells treated with TZDs or 15d-PGJ2 in a dose-dependent manner (Figure 5C). These data demonstrate a role for PPARγ agonists in the regulation of DGKα production. To verify whether these PPARγ agonists’ effects are mediated by the receptors, we used a dominant-negative PPARγ mutant, PPARγL468A/E471A, which has been shown to effectively silence the PPARγ-dependent gene transcriptions.15 Neither TZDs nor 15d-PGJ2 were able to induce DGκα production in the cells expressing PPARγL468A/E471A, whereas overexpression of the wild-type receptors enhanced the PPARγ agonist-induced DGκα expression (Figure 5D), indicating the action of PPARγ agonists principally dependent on the nuclear receptors.

PPARγ Agonists Inhibit Activation of the DAG-PKC Pathway

Given the ability of PPARγ agonists to upregulate DGK, we sought to define the consequent effects on the DAG-PKC signaling pathway. Consistent with previous reports,17,20 EC exposed to high glucose for 3 days resulted in significant increases in total DAG levels and PKC activities in comparison with the cells exposed to 5.5 mM/L glucose (Figure 6A and 6B). Treatment of cells with 2 μmol/L TZDs or 15d-PGJ2 significantly reduced the high-glucose-induced increases in DAG levels by 81.2%, 10.7%, and 61.4%, respectively (Figure 6A). Consequently, the high-glucose-induced PKC activity was reversed nearly to basal levels by the PPARγ agonists (Figure 6B). Among the various PKC isoforms in vascular EC, PKCβ isoform has been shown to be preferentially activated by high glucose.21 In agreement with this, the transfected BAEC with stable

inhibit the PMA-induced adhesion protein expression (Figure 3B), indicating that the PPARγ agonists had no direct effect on PKC activity. Interestingly, pretreatment with a specific inhibitor of DGK, R59949,19 completely reversed the inhibitory effect of ciglitazone on high-glucose–induced expression of VCAM-1 (Figure 3A). By contrast, R59949 had no influence on the inhibitory effect of GF-109203X (Figure 3A), and R59949 alone had no effect on the adhesion molecule expression (data not shown). Therefore, a possible role of DGK in the PPARγ agonists’ action was then examined. Treatment of HUVEC with TZDs or 15d-PGJ2 resulted in significant increases in DGK activity in a dose-dependent manner, reaching a maximum effect (>2-fold increases) at ~20 μmol/L (Figure 4A and 4B). Even at a concentration as low as 2 μmol/L, the PPARγ agonists were capable of significantly increasing DGK activity by 40% (Figure 4B). As a control, fenofibrate, a PPARα agonist, had no effect on the DGK activity (data not shown), indicating a specific effect of PPARγ agonists. Collectively, these data suggest that DGK may be a potential molecular target for the action of PPARγ agonists in protection of EC against activation.

Figure 3. DGK activity is involved in the action of PPARγ agonists. VCAM-1 expression was measured in (A) HUVEC exposed to 5.5 mM/L NG or 22 mM/L glucose (HG) for 3 days, or (B) treated with PMA (50 ng/mL) for 4 hours in the presence of GFX (5 μmol/L), ciglitazone (2 μmol/L), R59949 (5 μmol/L), or vehicle alone for 24 hours. Data are mean±SD from 1 experiment (n=3) and representative of 3 independent experiments. *P<0.01, R59949 versus control.

Figure 4. PPARγ agonists increase DGK activity. DGK activity was measured in HUVEC with the following treatments. A, Cells were treated with 20 μmol/L ciglitazone, troglitazone, 15d-PGJ2, or vehicle for 4 hours in the absence (Nil) or presence of 1 μmol/L actinomycin D (AcD). B, Cells were treated with an increasing concentration of ciglitazone or 15d-PGJ2 for 4 hours. C, Cells were treated with the agonists (20 μmol/L) for the indicated time. Values in (A) and (C) are mean±SEM (n=6). Data in (B) are mean of duplicates and representative of 3 independent experiments. *P<0.01 and **P<0.05 treated versus nil.
overexpression of EGFP-tagged PKCβ exposed to high glucose resulted in a significant translocation of EGFP-PKCβ from cytosol to plasma membrane (Figure 7A and 7C). However, this translocation was dramatically inhibited by pretreatment with ciglitazone at a concentration as low as 1 μmol/L. To further verify the inhibition of PKCβ activation by PPARγ agonists, we examined the effect of PPARγ agonists on VEGF-induced PKC activation, because VEGF has been demonstrated to activate PKC though PLCγ activation and DAG generation.22 Again, the translocation of PKCβ induced by VEGF was also markedly inhibited by ciglitazone (Figure 7B and 7C). By contrast, PMA-promoted PKCβ translocation was not influenced by the PPARγ agonists (Figure 7C), suggesting the effect of PPARγ agonists specifically targeting the upstream of PKC activation (ie, DGK) rather than PKC itself.

**Discussion**

In this report, we show a novel mechanism by which PPARγ agonists, such as TZD antidiabetic drugs and 15d-PJ2, protect...
against EC activation and vascular inflammation. We found that PPARγ agonists were capable of attenuating the activation of DAG-PKC signal transduction pathway via upregulation of DGK activity, leading to the inhibition of high-glucose–induced EC activation.

Induction of adhesion molecules on the EC surface and the interaction between EC and blood cells are critical for the pathogenesis of atherosclerotic cardiovascular diseases including diabetic complications.7 For instance, the expression of VCAM-1, ICAM-1, or E-selectin has been seen in atherosclerosis-prone regions and over fatty streaks and is likely, at least in part, to be responsible for the recruitment of monocytes to these areas.23,24 Atherogenic stimuli such as native or oxidized low-density lipoproteins stimulated adhesion protein expression,25 and protective agents such as high-density lipoproteins inhibited this.26 Hyperglycemia or high glucose was also capable of inducing hyperadhesion of leukocyte to the endothelium both in vitro and in vivo.27,28 Consistent with these reports, we found that HUVEC exposed to high glucose for 3 days resulted in significant increases in cell-surface expression of VCAM-1, ICAM-1, and E-selectin and consequently enhanced adherence of leukocytes to the activated EC. This thus represents an ideal model for the study of high-glucose–induced EC activation.

PPARγ agonists such as TZDs and 15d-PGJ2 have been reported to inhibit the expression of adhesion molecules in cytokine-activated EC, albeit with some inconsistencies because of different laboratories using different cell lines.29,30 The inhibitory effect of PPARγ has been recently demonstrated by enforced expression of a constitutively active mutant of PPARγ in EC, resulting in significant suppression of adhesion molecule expression,31 revealing an essential PPARγ receptor-dependent mode of action. In agreement with this finding, we found that the high-glucose–induced expression of VCAM-1, ICAM-1, and

Figure 7. PPARγ agonists suppress PKCβ translocation. A, Epifluorescent microscopy shows translocation of PKCβ-EGFP in BAEC stably expressing PKCβ-EGFP exposed to 5.5 mmol/L (NG) or 22 mmol/L glucose (HG) and treated for 4 hours with or without 1 μmol/L ciglitazone. Actin staining was shown in middle panels. The images of PKCβ-EGFP and actin staining were merged in right panels (100× objective). B, The PKCβ-transfected BAEC were treated for 4 hours with or without 1 μmol/L Cigl followed by stimulation of VEGF (10 ng/mL) for 5 minutes, and epifluorescent microscopic imaged (40×). C, The cytosolic and membrane fractions were isolated from the treated PKCβ-transfected BAEC and immunoblotted with anti-PKCβ1 antibodies.
E-selectin was completely inhibited by both TZDs and 15d-PGJ2 at 2 μmol/L (Figure 1), within the range of known affinity of the ligands binding to the receptors, suggesting the role for PPARγ receptors. Consequently, adhesion of leukocytes to the activated EC was significantly reduced by the PPARγ agonists, further indicating the anti-inflammatory effect of PPARγ on EC.

The finding that PPARγ agonists-induced reduction of adhesion molecule expression was blocked by R59949, a DGK-specific inhibitor, suggests a potential role of DGK in the action of PPARγ agonists. In support of this notion, we found that the PPARγ agonists were strong inducers of DGK activity as documented by an in vitro kinase activity assay (Figure 4) and intracellular DAG level determination (Figure 6). The observations of a delayed increase in DGK activity and the ability of a transcriptional inhibitor to block the elevated DGK activity suggest that the PPARγ agonist-induced increases in DGK activity may result from an increase in the production of DGK. The increased DGK protein levels could cause changes in the enzyme subcellular localization that facilitates the enzymatic activation and/or enhances the availability of substrate, leading to increases in the DGK activity. Indeed, it has been reported that enforced overexpression of DGKα per se caused increases in DGK activity and attenuation of DAG signaling. Demonstrating the effect of PPARγ agonists on DGK production, both mRNA and protein levels of DGKα, but not DGK-β or DGK-ζ isoforms, were significantly increased in EC treated with TZDs or 15d-PGJ2 (Figure 5). Remarkably, the PPARγ agonist-induced production of DGKα was completely abolished by the dominant-negative mutant, PPARγ<sub>ΔΔα</sub>, suggesting that the effect depends on the receptor-mediated transcriptional activity. Analysis of genome sequences of human DGKα shows that at least 2 PPAR-responsive elements are present in the putative promoter region of the gene, further supporting the role of PPARγ in the regulation of DGKα expression. Additional studies are required to identify the direct effect of PPARγ in DGKα gene regulation.

DGK is a well-conserved lipid kinase that phosphorylates DAG to yield phosphatidic acid and is therefore a potent endogenous terminator of DAG-PKC signaling. To date, 9 DGK isoenzymes have been identified in mammals. Although our findings suggest that DGKα is a key isoform of DGK to be upregulated by PPARγ activators in human EC, the effect of PPARγ on other isoenzymes needs further investigation. As consequences of the increased DGK activity, PPARγ agonists profoundly attenuated DAG levels and inhibited PKC activation reflected in the decrease of total PKC activities and inhibition of PKC intracellular translocation in the high-glucose–treated EC (Figures 6 and 7). It was noted that at a concentration as low as 2 μmol/L, the PPARγ agonists induced a 40% increase in DGK activity and a nearly complete inhibition of high-glucose–induced PKC activity, suggesting an upstream inhibition of the signaling cascades. By contrast, PMA-stimulated PKC activity was not influenced by the PPARγ agonists, supporting the view that PPARγ agonist-induced inhibition of DAG-PKC signaling pathway is initiated by the elevated DGK activity.

Within the family of PKC, at least 7 isoforms represent major downstream targets for DAG, including the conventional and novel PKC subfamilies. Among these isoforms PKCβ has been shown to be preferentially linked to the pathogenesis of cardiovascular diseases, especially in diabetic vascular complications. In vascular cells including EC, PKCβ can be activated by high glucose or VEGF, known factors that contribute to the development of vascular lesions in diabetes. By using stably EGFP-PKCβ-transfected EC, we are able to convincingly show a translocation of PKCβ to the plasma membrane in response to high glucose or VEGF stimulation, which was consistent with previous data from membrane fractionation experiments. Remarkably, either the high-glucose-induced or the VEGF-induced translocation of PKCβ was blocked by the PPARγ agonists (Figure 7). It is interesting to note that VEGF-activated PKC is mediated by DAG generation on PLCγ activation, whereas high glucose induces DAG production mainly through the de novo synthesis pathway. Both sources of DAG-promoted PKC activation were attenuated by the PPARγ agonists, indicating a general broad effect of PPARγ on the termination of DAG-PKC signaling.

It has been recently recognized that PKC is not the only molecular target of DAG. Other proteins, such as RasGRP, Unc-13, protein kinase D, and chimaeras, bear Cl domains that bind to DAG, and these proteins can be activated by DAG. However, phosphatidic acid, the product of DAG phosphorylation on DGK activation, has also emerged as a potential second messenger, with several candidate target proteins including Raf-1 kinase, PKCζ, phosphatidylinositol 4-phosphate 5-kinase, and protein tyrosine phosphatases (reviewed in ). Thus, the biological action of the PPARγ agonist-induced DGK activity and DAG attenuation possibly involve various signaling pathways. For instance, PPARγ agonists have been shown to activate PKCζ and enhance insulin action in adipocytes. Wakino et al have recently reported that PPARγ agonists were capable of increasing protein tyrosine phosphatase activity leading to inhibition of PKCδ activation and p21<sup>WAF1</sup> turnover in vascular smooth muscle cells. It will be of interest to test whether these effects of PPARγ agonists are mediated via the upregulation of DGK resulting in attenuation of DAG and elevated phosphatidic acid levels.

As a transcription factor, PPARγ regulates the expression of numerous genes that are involved in the process of vascular inflammation including cytokines, chemokines, and adhesion molecules. However, the observed anti-inflammatory effect of PPARγ often vary according to the agonists used and are not always consistent with their capacity to bind to the receptors. Recent studies have suggested an ability of PPARγ to regulate other transcription factors and their signaling, such as NF-κB, and C/EBPβ, which could account for the effect of PPARγ on the gene regulation. In this respect, the PPARγ agonist-induced upregulation of DGK and attenuation of DAG-PKC pathway as described in this study demonstrate a novel signaling pathway in coupling PPARγ activators to the inhibition of proinflammatory gene expression, and as such PPARγ agonists may serve as potent anti-inflammatory regents for the prevention and treatment of atherosclerotic diseases and diabetic vascular lesions.

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References


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