Nitric Oxide Production and Regulation of Endothelial Nitric-oxide Synthase Phosphorylation by Prolonged Treatment with Troglitazone

EVIDENCE FOR INVOLVEMENT OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) γ-DEPENDENT AND PPARγ-INDEPENDENT SIGNALING PATHWAYS

Received for publication, August 26, 2003, and in revised form, October 10, 2003 Published, JBC Papers in Press, October 30, 2003, DOI 10.1074/jbc.M309451200

Du-Hyong Cho, Yoon Jung Choi, Sangmee Ahn Jo, and Inho Jo‡

From the Department of Biomedical Sciences, National Institute of Health, 5 Nobuk-dong, Eunpyung-gu, Seoul 122-701, Korea

Recently, peroxisome proliferator-activated receptor γ (PPARγ) ligands have been reported to increase endothelial NO, but the signaling mechanisms involved are unknown. Using troglitazone, a PPARγ ligand known as an antidiabetic compound, we investigated the molecular mechanism of its effect on NO production in bovine aortic endothelial cells. Troglitazone increased endothelial NO production in a dose- and time-dependent manner with no alteration in endothelial nitric-oxide synthase (eNOS) expression. The maximal increase (−3.1-fold) was achieved with 20 μM troglitazone treatment for 12 h, and this increase was accompanied by increases in the expression of vascular endothelial growth factor (VEGF) and its receptor, KDR/Flk-1, and in Akt phosphorylation. Analysis with antibodies specific for each phosphorylated site demonstrated that troglitazone (20 μM treatment for 12 h) significantly increased both the phosphorylation of Ser1179 of eNOS (eNOS-Ser1179) and the dephosphorylation of eNOS-Ser116 but did not alter eNOS-Thr497 phosphorylation. Treatment with anti-VEGF antibody to scavenge the increased VEGF induced by troglitazone partially inhibited troglitazone-stimulated NO production. This was accompanied by the attenuation of troglitazone-stimulated increases in the phosphorylation of Akt and eNOS-Ser1179 with no alteration in eNOS-Ser116 dephosphorylation. We also found that bisphenol A diglycidyl ether, a PPARγ antagonist, partially inhibited troglitazone-stimulated NO production with a concomitant reduction in VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation but with no alteration in eNOS-Ser116 dephosphorylation induced by troglitazone. Taken together, our results demonstrate that prolonged treatment with troglitazone increases endothelial NO production by at least two independent signaling pathways: PPARγ-dependent, VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation and PPARγ-independent, eNOS-Ser116 dephosphorylation.

Troglitazone, the first thiazolinedione (TZD) 1 compound synthesized, was used clinically as an oral antidiabetic drug to improve insulin resistance in patients with type 2 diabetes mellitus (DM) (1, 2). However, it was later withdrawn from the market because of fatal hepatic injury (3). The effects of this drug are known to be mediated, in part, through its binding to peroxisome proliferator-activated receptor γ (PPARγ) (4), a member of the nuclear hormone receptor superfamily. Because people with type 2 DM often have at least one cardiovascular disease risk factor (5), troglitazone is likely to be implicated in the pathophysiology of both cardiovascular disease and DM. Troglitazone has been reported to lower blood pressure (6), increase insulin sensitivity, and correct hyperinsulinemia (7, 8), although the results are conflicting (7). Furthermore, another TZD drug, pioglitazone, attenuated vasoconstriction in vitro (9) and reduced L-type currents in rat vascular smooth muscle cells (VSMC) (10). Recently, troglitazone has also been reported to inhibit microvascular endothelial cell proliferation (11).

Endothelial nitric-oxide synthase (eNOS) is an enzyme essential to the maintenance of cardiovascular integrity by producing NO in vivo, a key molecule with multiple functions, including vasodilation, and many antiatherogenic properties (12). Therefore, the dysregulation of eNOS is thought to contribute to the pathogenesis of certain vascular diseases, such as atherosclerosis and hypertension (13). eNOS is regulated not only at the level of expression (14, 15) but also nongenomically by subcellular targeting (16), protein-protein interactions (17), fatty acylation (18), and phosphorylation (19). Recently, specific sites of phosphorylation have been identified; among these sites, Ser1179 (eNOS-Ser1179; bovine sequence) and eNOS-Thr497 are the most studied. The phosphorylation of eNOS-Ser1179 reduces the Ca2+-calmodulin dependence of the enzyme (20) and increases the rate of electron flux from the reductase domain to the oxygenase domain (21), thereby increasing NO production (22–24). This phosphorylation is mediated by several specific protein kinases, including protein kinase B (Akt), AMP-activated protein kinase, calmodulin-dependent kinase II, and protein kinase A (20, 25–28). In contrast, the phosphorylation of eNOS-Thr497 decreases eNOS activity by increasing

1 The abbreviations used are: TZD, thiazolidinedione; DM, diabetes mellitus; PPARγ, peroxisome proliferator-activated receptor γ; VSMC, vascular smooth muscle cells; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; VEGF, vascular endothelial growth factor; BADGE, bisphenol A diglycidyl ether; BAEC, bovine aortic endothelial cells; PKC, protein kinase C; 15d-PGJ2, 15-deoxy-

Δ12,14-prostaglandin J2.
Ca2+-calmodulin dependence (20, 27, 29). Phosphatases such as protein phosphatase 1 and protein phosphatase 2B increase the dephosphorylation of eNOS-Thr497, resulting in an increase in NO production (19, 27). Another phosphorylation site, eNOS-Ser1179, has also been reported, and its dephosphorylation by protein phosphatase 2B increases eNOS activity (30). Recently, two other sites, eNOS-Ser116 and eNOS-Ser1177, were also identified as phosphorylation targets of protein kinase A and Akt, respectively (31). However, the roles of these protein kinases and phosphatases as signaling molecules for eNOS phosphorylation and dephosphorylation at several potential sites are dependent on the experimental conditions used, such as the presence of agonists, the endothelial cell type, and treatment time. For example, vascular endothelial growth factor (VEGF) (32) and fluid shear stress (25) stimulated Akt-dependent eNOS-Ser1179 phosphorylation at an earlier time, but they also stimulated protein kinase A-dependent eNOS-Ser635 phosphorylation at a later time (33). In contrast to VEGF and shear stress, which cause no dephosphorylation of eNOS-Thr497, 8-bromo-cAMP rapidly dephosphorylates eNOS-Thr497. All of these findings suggest that the activity of eNOS in cells may be controlled through a coordinated regulation of, and interaction between, several protein kinases and phosphatases.

Most studies of troglitazone have focused on the inhibition of cytokine-induced NO production via a decrease in the expression of inducible nitric-oxide synthase (iNOS) in adipocytes (34) and VSMC (35). However, troglitazone were recently shown to increase endothelial NO production (37). However, no detailed mechanism underlying this increase has yet been reported. Together with the previous observation that TZDs in vivo increased the expression of VEGF, a well known agonist for endothelial NO production (38), these results prompted us to characterize the molecular mechanism underlying the troglitazone-stimulated increase in endothelial NO production. Our current data demonstrate, for the first time, that troglitazone increases NO production by at least two independent signaling pathways: PPARγ-dependent, VEGF-KDR/Flik-1-Akt-mediated eNOS-Ser1179 phosphorylation and PPARγ-independent, eNOS-Ser116 dephosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Troglitazone was obtained as a gift from Sankyo Co. (Tokyo, Japan), and bisphenol A diglycidyl ether (BADGE) was obtained from Sigma. Antibodies against eNOS and Akt were purchased from Santa Cruz Biotechnology (La Jolla, CA), respectively. Antibodies against Akt phosphorylated at Ser473 (p-Akt-Ser473) and p-eNOS-Ser1179 were obtained from Cell Signaling Technology (Beverly, MA), and those against p-eNOS-Thr497 and p-eNOS-Ser1177 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Troglitazone for RNA extraction and SuperScript™ II RNase H− reverse transcription were obtained from Invitrogen. Recombinant rat (rTag) DNA polymerase was purchased from Takara Bio Medicals (Shiga, Japan), and collagenase (type 2) was purchased from Worthington Biochemical Corporation (Freehold, NJ). Minimal essential medium, Dulbecco’s phosphate-buffered saline, newborn calf serum, penicillin and streptomycin, G418, trypsin-EDTA solution, and MDCK cells were purchased from Invitrogen. All other chemicals were of the purest analytical grade.

**Cell Culture and Drug Treatments**—Bovine aortic endothelial cells (BAEC) were isolated exactly as described previously (39) and maintained in minimal essential medium supplemented with 5% newborn calf serum at 37 °C under 5% CO2 in air. The endothelial cells were confirmed by their typical cobblestone configuration when viewed by light microscopy and by a positive indirect immunofluorescence test for von Willebrand factor VIII. The cells between passages 5 and 9 were used for all experiments. When BAEC were grown to confluence, the cells were further maintained for the indicated times in minimal essential medium supplemented with 0.5% newborn calf serum containing various concentrations of troglitazone. In some experiments, the cells were co-treated with either anti-VEGF antibody (200 μg/ml) or BADGE (5 μM).

**RNA Extraction and Semi-quantitative Reverse Transcription-PCR**—After treatment with troglitazone for the indicated times, the culture medium was removed, and total cellular RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. A reverse transcription reaction was performed with 2 μg of total RNA in a final volume of 40 μl using 20 pmol of oligo(dT)12-18 in the presence of 200 units of SuperScript™ II RNase H− reverse transcriptase (Invitrogen). Subsequent PCR amplification of cDNA encoding VEGF was carried out in a total volume of 20 μl containing 0.5 units of rTaqDNA polymerase (Invitrogen) and 10 pmol of each primer. Primer sequences were as follows: for VEGF (475 bp), forward 5′-ACCAAGAAAGGGAGCA-GAAA-3′, reverse 5′-GGAACTTGTCTGACTGACACA-3′. Amplification of cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (494 bp) was performed for semi-quantitative normalization using the following primers: forward, 5′-ACCAAGTCATCCTGGCATTACAC-3′, and reverse, 5′-TCCACCACCTTGTGCTGTA-3′. The amplified fragments were separated on a 2% agarose gel containing ethidium bromide and visualized with an image analyzing device (Vilber Lourmat, France) under UV illumination. The bands on the images were quantitated with the image analyzing software, ImageJ (National Institutes of Health, Bethesda, Washington, D.C.).

**Western Blot Analysis**—For Western blot analysis, the cells treated with troglitazone in the absence or presence of various chemicals were lysed with ice-cold Dulbecco’s phosphate-buffered saline and lysed in wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM Na-pyrophosphate, 1 mM NaVO4) containing 1% Protease Inhibitor Mixture (Roche Applied Science). The protein concentrations were determined with the BCA protein assay kit (Sigma). Equal quantities of protein (30 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibody directed against VEGF (1:5000), KDR/Flik-1 (1:1000), Akt (1:4000), eNOS (1:4000), p-Akt-Ser473 (1:4000), p-eNOS-Ser1177 (1:1000), p-eNOS-Thr497 (1:2000), or p-eNOS-Ser116 (1:4000), followed by the corresponding secondary antibody and finally developed using ECL reagents (Amersham Biosciences).

**Measurement of NO Release**—NO production by BAEC was measured as nitrite (a stable metabolite of NO) concentration in cell culture supernatants, as described in many previous studies (41), with minor modifications. Briefly, after cells were treated with troglitazone for the indicated times in the absence or presence of various chemicals, the culture medium was changed to Krebs’ solution (pH 7.4; 1.5 mM CaCl2, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, and 11.1 mM glucose, and was equilibrated for 1 h at 37 °C. At the end of the incubation, 200 μl of each supernatant (in Krebs’ solution) was carefully transferred to a 96-well plate, with the subsequent addition of 100 μl of Griess reagent (50 μl of 1% sulfanilamide containing 5% phosphoric acid and 50 μl of 0.1% N-(1-naphthyl)ethylenediamine). After color development at room temperature for 10 min, the absorbance was measured on a microplate reader at a wavelength of 520 nm. Each sample was assayed in triplicate wells. A calibration curve was plotted using known amounts of sodium nitrate solution. With this protocol, the measured values represent the amounts of NO produced by the cells during the 1-h incubation in Krebs’ solution, following troglitazone treatment of a specified duration in the absence or presence of various chemicals. Therefore, subsequent NO production was solely dependent on eNOS activity at the end of these treatments.

**Statistical Analysis**—All results are expressed as the means ± standard deviation (S.D.), with n indicating the number of experiments. Statistical significance was determined by Student’s t test for two points. All differences were considered significant at a p value of < 0.05.

**RESULTS**

**Troglitazone Increases NO Production in BAEC with No Alteration in eNOS Expression**—Troglitazone increased NO production by BAEC in a dose- and time-dependent manner, as shown in Fig. 1. The maximal increase in NO levels (3.1 ± 0.42-fold of the control) was observed with 20 μM troglitazone treatment for 12 h. Longer incubation (24 h) of cells with 20 μM troglitazone caused no further increase. Therefore, all of the
Troglitazone Increases NO Production in BAEC by Increasing Either eNOS-Ser1179 Phosphorylation or eNOS-Ser116 De-phosphorylation—Because phosphorylation of eNOS-Ser1179 is a major mechanism for VEGF-mediated increase in NO production (43, 44), we examined whether troglitazone increases NO production by the stimulation of the phosphorylation of this residue. Troglitazone (20 μM) increased eNOS-Ser1179 phosphorylation in a time-dependent manner, as shown in Fig. 3, suggesting a potential role for troglitazone in eNOS-Ser1179 phosphorylation. The maximal increase (2.4 ± 0.53-fold of the control) was observed with the 12-h treatment. With longer incubation (24 h) of the cells with troglitazone, however, a slight but significant attenuation was observed (1.8 ± 0.17-fold of the control). Furthermore, using antibodies specific to p-eNOS-Thr497 and p-eNOS-Ser116, we also observed that troglitazone significantly stimulated the dephosphorylation of eNOS-Ser116 in a time-dependent manner (Fig. 3), whereas the phosphorylation levels of eNOS-Thr497 were not altered by troglitazone treatment for up to 12 h. It should be noted that eNOS-Ser116 dephosphorylation by troglitazone occurred at an earlier time point (6 h) than eNOS-Ser1179 phosphorylation and that a slight but significant increase in the phosphorylation of eNOS-Thr497 was also observed with longer exposure (24 h).

Our current results, together with previous findings of a positive role for both eNOS-Ser1179 phosphorylation and eNOS-Ser116 dephosphorylation in increasing NO production (30, 32), indicate that troglitazone-dependent coordinated changes in the phosphorylation levels of eNOS at these two different residues are primarily involved in the troglitazone-stimulated NO increase in BAEC.

Troglitazone-induced Increases in eNOS-Ser1179 Phosphorylation and eNOS-Ser116 Dephosphorylation Are Mediated by at Least Two Separate Signaling Pathways—We examined whether either the phosphorylation or dephosphorylation of eNOS at the Ser1179 or Ser116 residues, respectively, is the consequence of a troglitazone-stimulated VEGF–KDR/Flk-1-mediated signaling pathway. Co-treatment with anti-VEGF antibody and troglitazone significantly reversed the eNOS-Ser1179 phosphorylation induced by troglitazone, whereas no alteration in the phosphorylation levels of either eNOS-Thr497 or eNOS-Ser116 was observed, as shown in Fig. 4 (A–C). Furthermore, anti-VEGF antibody also completely reversed the troglitazone-stimulated increase in the phosphorylation of Akt, an intermediate signaling molecule that mediates VEGF-eNOS-Ser1179 phosphorylation (Fig. 4D). Troglitazone also stimulated Akt phosphorylation in a time-dependent manner, showing a very similar pattern to the phosphorylation of eNOS-Ser1179 induced by troglitazone (data not shown). These results, together with the finding that anti-VEGF antibody partially attenuates troglitazone-stimulated NO increase (Fig. 2D), suggest that troglitazone increases NO production in BAEC in part by increasing VEGF–KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation. In contrast, because anti-VEGF antibody did not alter the level of phosphorylation of
eNOS-Ser116 (Fig. 4) and eNOS-Ser116 dephosphorylation has a potential role in the troglitazone-stimulated increase in NO production, we hypothesize that the VEGF-KDR/Flk-1-Akt-mediated pathway is not an upstream signaling pathway for eNOS-Ser116 dephosphorylation.

This hypothesis was further examined using BADGE, a PPARγ antagonist (45, 46). BADGE (5 μM) partially (40%) attenuated the troglitazone-stimulated increase in NO production, as shown in Fig. 5A. Furthermore, BADGE completely blocked KDR/Flk-1 expression stimulated by troglitazone (Fig. 5B). Like anti-VEGF antibody, BADGE significantly attenuated the phosphorylation of Akt and eNOS-Ser1179 induced by troglitazone but not the phosphorylation of eNOS-Thr497 and eNOS-Ser116 (Fig. 5C–F). These data further support the premise that an increase in eNOS-Ser1179 phosphorylation, but not in eNOS-Ser116 dephosphorylation, is the consequence of troglitazone as a PPARγ agonist. Taken together, our data clearly show that the troglitazone-stimulated increase

FIG. 2. Troglitazone increases the expressions of VEGF mRNA, VEGF protein, and its receptor, KDR/Flk-1, and anti-VEGF antibody attenuates the NO production elicited by troglitazone. BAEC were prepared and treated with 20 μM troglitazone for the indicated times, as described in the legend to Fig. 1. The cell lysates were prepared, and the amount of p-eNOS was measured by Western blot analysis with antibodies specific for eNOS phosphorylated at Ser1179, Thr497, or Ser116. The nitrocellulose membranes were reprobed with antibody detecting total eNOS to monitor equal loading of the samples. Densitometry was performed to quantitate the phosphorylated bands, and graphs show the mean fold increases above control ± S.D., as described in legend to Fig. 1 (n = 5–8). In BAEC, troglitazone increased dephosphorylation of eNOS-Ser116 at the earliest time point (6 h), followed by phosphorylation of eNOS-Ser1179 at 12 h and phosphorylation of eNOS-Thr497 at 24 h. Whereas troglitazone continuously reduced the phosphorylation of eNOS-Ser116 over the entire experimental period (for 24 h), the pattern of increasing eNOS-Ser1179 phosphorylation by troglitazone was reversed after 12 h.

eNOS-Ser116 (Fig. 4C) and eNOS-Ser116 dephosphorylation has a potential role in the troglitazone-stimulated increase in NO production, we hypothesize that the VEGF-KDR/Flk-1-Akt-mediated pathway is not an upstream signaling pathway for eNOS-Ser116 dephosphorylation.

This hypothesis was further examined using BADGE, a PPARγ antagonist (45, 46). BADGE (5 μM) partially (40%) attenuated the troglitazone-stimulated increase in NO production, as shown in Fig. 5A. Furthermore, BADGE completely blocked KDR/Flk-1 expression stimulated by troglitazone (Fig. 5B). Like anti-VEGF antibody, BADGE significantly attenuated the phosphorylation of Akt and eNOS-Ser1179 induced by troglitazone but not the phosphorylation of eNOS-Thr497 and eNOS-Ser116 (Fig. 5C–F). These data further support the premise that an increase in eNOS-Ser1179 phosphorylation, but not in eNOS-Ser116 dephosphorylation, is the consequence of troglitazone as a PPARγ agonist. Taken together, our data clearly show that the troglitazone-stimulated increase

generate mRNA was used for semi-quantitative normalization (A and B). The expression of VEGF and its receptor, KDR/Flk-1 (C), was measured by Western blot analysis using specific antibodies, as described in the legend to Fig. 1. NO production by troglitazone was measured in the absence or presence of anti-VEGF antibody (200 μg/ml) (D). The data are presented as fold increases above control ± S.D. Statistically significant at p < 0.05 (* and #) and p < 0.01 (** and ##) (n = 7–12). In BAEC, troglitazone increased VEGF mRNA in a dose- and time-dependent manner (C). Anti-VEGF antibody significantly attenuated the troglitazone-stimulated increase in NO production (D), suggesting that troglitazone increases NO production in part by a VEGF-KDR/Flk-1 signaling pathway. DMSO, dimethyl sulfoxide.
in NO production is mediated by two independent signaling pathways: PPARγ-dependent, VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation and PPARγ-independent, eNOS-Ser116 dephosphorylation.

**DISCUSSION**

Recently, a single study showed that the PPARγ ligands 15d-PGJ2 and ciglitazone increase NO release in endothelial cells (36). The mechanisms underlying this effect of PPARγ ligands remain undefined. However, the authors of that report suggested that PPARγ ligands regulate the expression of another target gene rather than eNOS itself, which promotes NO release, particularly because 15d-PGJ2 increased NO production but decreased eNOS protein expression. In this study, we have further characterized this molecular mechanism and shown for the first time that troglitazone, another PPARγ ligand, increases NO production in endothelial cells by the molecular activation of eNOS through alterations in its phosphorylation status.

Two lines of evidence in this study suggest that there are two independent signaling pathways that may account for troglitazone-stimulated NO production: PPARγ-dependent, VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation and PPARγ-independent, eNOS-Ser116 dephosphorylation. Firstly, we found that only VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation was inhibited by anti-VEGF antibody, resulting in a partial attenuation of troglitazone-stimulated NO production. However, anti-VEGF antibody did not alter eNOS-Ser116 dephosphorylation. Secondly, BADGE, a PPARγ antagonist, also partially attenuated troglitazone-stimulated NO increase by suppressing the signaling pathway responsible for VEGF-KDR/Flk-1-Akt-mediated eNOS-Ser1179 phosphorylation but did not alter eNOS-Ser116 dephosphorylation.

It has previously been reported that TZDs up-regulate VEGF mRNA in VSMC (47) and blood VEGF in patients with type 2 DM (42). These results are reproduced reasonably closely in our current study using BAEC. Furthermore, the up-regulation of VEGF protein by troglitazone coincides with the up-regulation of its mRNA, suggesting that troglitazone increases VEGF expression, perhaps by regulating its transcription. Nonetheless, it seems likely that the effect of troglitazone on VEGF mRNA expression may be mediated by unknown transcription factor(s) rather than by a direct binding of this drug and its receptor complex to the promoter region of the VEGF gene, because VEGF mRNA is devoid of a PPARγ response element in its promoter (48–51). In this regard, several transcription factors and co-activators such as activator protein-1, cAMP response element binding protein/p300, hypoxia inducible factor-1, and stimulatory protein-1 were reported to be involved in the PPARγ-activated increase in VEGF mRNA expression (49). Up-regulation of the VEGF receptor, KDR/Flk-1, was also observed in response to chronic troglitazone treatment, to our

FIG. 4. Anti-VEGF antibody reverses troglitazone-stimulated increases in eNOS-Ser1179 and Akt-Ser473 phosphorylation but does not alter the phosphorylation status of eNOS-Thr497 or eNOS-Ser116. BAEC were prepared and treated with 20 μM troglitazone for 12 h, as described in the legend to Fig. 1, in the absence or presence of anti-VEGF antibody (200 μg/ml). After treatment, the cell lysates were prepared, and eNOS and p-eNOS were analyzed as described in the legend to Fig. 3. The expression of Akt and p-AktSer^{473} was measured by Western blot analysis using antibodies specific for those proteins. The data are presented as described in the legend to Fig. 1 (n = 5–8). In BAEC, co-treatment with anti-VEGF antibody significantly blocked the troglitazone-stimulated increase in eNOS-Ser^{1179} phosphorylation (A) and completely blocked the increase in Akt-Ser^{473} phosphorylation (D), but it did not alter the phosphorylation status of eNOS-Thr^{497} (B) or eNOS-Ser^{116} (C). These results, together with the finding that anti-VEGF antibody partially inhibited the troglitazone-stimulated increase in VEGF mRNA expression (49), indicate that troglitazone increases NO production in part by VEGF-Akt-mediated eNOS-Ser^{1179} phosphorylation, which is independent of eNOS-Ser^{116} dephosphorylation. DMSO, dimethyl sulfoxide.
knowledge for the first time. The mechanism underlying this up-regulation remains unclear, but it is speculated that the increase in VEGF induced by troglitazone may in turn induce the expression of its receptor (52, 53). At present, it is reasonable to infer that the activation of Akt, as a downstream signaling molecule of VEGF, follows the VEGF up-regulation elicited by troglitazone treatment. However, it should be noted that chronic exposure to troglitazone may also utilize the same Akt-dependent mechanism to exert its cellular effect, such as an alteration in Akt phosphorylation but not in Akt expression, a phenomenon observed during acute exposure to various agonists (25). Further studies using other signaling molecules are required to generalize this concept. The activation of the VEGF signaling pathway by troglitazone, together with the partial inhibition of troglitazone-stimulated NO production by anti-VEGF antibody, clearly suggests that the increase in VEGF

Fig. 5. BADGE reverses troglitazone-stimulated increases in eNOS-Ser^{1179} and Akt-Ser^{473} phosphorylation but does not alter the phosphorylation status of eNOS-Thr^{497} or eNOS-Ser^{116}. BAEC were prepared and treated with 20 μM troglitazone for 12 h, as described in the legend to Fig. 1, in the absence or presence of BADGE (5 μM). After treatment, the cell lysates were further processed as described in the legend to Fig. 4. The data are presented as described in the legend to Fig. 1 (n = 5–8). In BAEC, co-treatment with BADGE partially blocked the troglitazone-stimulated increase in NO production (A) and the increase in eNOS-Ser^{1179} (C) and Akt-Ser^{473} phosphorylation (F). Similarly, BADGE also completely blocked the troglitazone-stimulated increase in KDR/Flik-1 expression (B). In contrast, BADGE did not alter the phosphorylation status of eNOS-Thr^{497} (D) or eNOS-Ser^{116} (E), suggesting that troglitazone increases NO production, in part, by PPARγ-dependent, VEGF-KDR/Flik-1-Akt-mediated eNOS-Ser^{1179} phosphorylation and PPARγ-independent, eNOS-Ser^{116} dephosphorylation. DMSO, dimethyl sulfoxide.
expression induced by troglitazone may account, at least in part, for troglitazone-stimulated NO production. However, unlike previous studies that showed a significant up-regulation of eNOS protein by chronic VEGF treatment in human umbilical vein endothelial cells (44) and bovine adrenal cortex endothelial cells (54), we were unable to detect any up-regulation of eNOS expression (Fig. 2). The reasons for these apparently conflicting results are unclear but may be related to the cell type studied, i.e. BAEC as opposed to either human umbilical vein endothelial cells or bovine adrenal cortex endothelial cells. Alternatively, it may also be speculated that the amount of VEGF increase elicited by troglitazone under our experimental conditions was insufficient to cause an increase in eNOS expression.

We found that BADGE partially blocked troglitazone-stimulated phosphorylations of Akt and eNOS at the Ser1179 residue in troglitazone-stimulated cells, whereas it did not alter the phosphorylation of eNOS-Ser116. Furthermore, a higher dose (10 μM) of BADGE even stimulated troglitazone-induced eNOS-Ser116 phosphorylation (data not shown). This result clearly suggests that an alteration in the phosphorylation status of eNOS-Ser116 by troglitazone is not PPARγ-dependent, whereas VEGF-KDR/Flik-1-Akt-mediated eNOS-Ser1179 phosphorylation is PPARγ-dependent. Although the effects of troglitazone on various cellular functions are predominantly mediated by PPARγ, it has also been suggested that troglitazone can exert PPARγ-independent effects. For example, 15d-PGJ2, inhibited IkB kinase, thus interrupting NF-kB signaling by PPARγ-independent signaling pathways (55, 56). Furthermore, troglitazone inhibited protein kinase C (PKC) activity in a PPARγ-independent manner in VSMC (57). These data, together with an earlier finding that PKC inhibitor blocks the phosphorylation of eNOS-Ser116 (30), suggest a potential role for PKC in troglitazone-induced eNOS-Ser116 phosphorylation in BAEC. Therefore, possible interactions between the regulation of PKC activity and troglitazone should be assessed in future to clarify this issue.

As shown in all of the figures in this study, a temporally distinct phosphorylation pattern of eNOS exists at any given time at these three residues. It should be noted that the observed NO levels are dependent on the total phosphorylation status of eNOS at these residues and at other known eNOS residues as well. For example, using this concept, the absence of any further increase in NO production induced by troglitazone with 24 h of treatment (compared with 12 h) (Fig. 1B) might be attributable to the combined effects of both decreased phosphorylation at eNOS-Ser1179 and eNOS-Ser116 and increased phosphorylation at eNOS-Thr497. Although PKC (29) and AMP-activated protein kinase (20) regulate the phosphorylation status of eNOS-Ser116 and eNOS-Thr497, it is unclear at present whether these enzymes also play a role in troglitazone-induced eNOS phosphorylation at a later time. One important outcome of the time course study of the troglitazone-stimulated increase in NO production is that the phosphorylation and dephosphorylation of eNOS at these three residues display different kinetics. The earliest response to troglitazone was eNOS-Ser116 phosphorylation, followed by eNOS-Ser1179 phosphorylation and eNOS-Thr497 phosphorylation. Our current data show that NO production is stimulated by troglitazone via changes in eNOS phosphorylation. This offers a novel and potentially unifying mechanism for the reduction in blood pressure elicited by TZD drugs (9). In contrast to our findings, it has been also reported that TZD inhibits NO in VSMC by the suppression of iNOS expression (34, 35). However, in this study, we were unable to detect iNOS expression in BAEC (data not shown), suggesting that the eNOS-dependent mechanism proposed in this study accounts for the ability of troglitazone to modulate NO production in endothelial cells. Our results, together with previous finding that troglitazone stimulates the suppression of iNOS, suggest that the overall vascular protective effects of troglitazone on lipid metabolism, inflammation, and vasodilation may be attributable to the coordinated actions of two vascular cells, endothelial cells and VSMC, through the regulation of eNOS and iNOS, respectively. Furthermore, our data also suggest that care should be exercised in the use of troglitazone in patients with type 2 DM carrying retinopathy or cancer, because NO in endothelial cells is implicated in stimulating angiogenesis (58).

In summary, the most important and original finding of this study is that chronic treatment with troglitazone increases NO production by at least two independent pathways: PPARγ-dependent, VEGF-KDR/Flik-1-Akt-mediated eNOS-Ser1179 phosphorylation and PPARγ-independent, eNOS-Ser116 dephosphorylation. A better understanding of the mechanisms involved in troglitazone-induced eNOS regulation may help to provide new strategies to modify the pathophysiology of cardiovascular diseases, such as hypertension and atherosclerosis, as well as type 2 DM.
A. T., and Kemp, B. E. (2001) J. Biol. Chem. 276, 17625–17628
30. Kou, R., Greif, D., and Michel, T. (2002) J. Biol. Chem. 277, 29669–29673
31. Michell, B. J., Harris, M. B., Chen, Z. P., Ju, H., Venema, V. J., Blackstone, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) J. Biol. Chem. 277, 42344–42351
32. Gelinas, D. S., Bernatchez, P. N., Rollin, S., Bazan, N. G., and Siros, M. G. (2002) Br. J. Pharmacol. 137, 1021–1030
33. Michell, B. J., Harris, M. B., Chen, Z. P., Ju, H., Venema, V. J., Blackstone, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) J. Biol. Chem. 277, 42344–42351
34. Gelinas, D. S., Bernatchez, P. N., Rollin, S., Bazan, N. G., and Sirois, M. G. (2002) Br. J. Pharmacol. 137, 1021–1030
35. Boo, Y. C., Hwang, J., Sykes, M., Michell, B. J., Kemp, B. E., Lum, H., and Jo, H. (2002) Am. J. Physiol. 283, H1819–H1828
36. Dobashi, K., Asayama, K., Nakane, T., Kodera, K., Hayashibe, H., and Nakazawa, S. (2000) Life Sci. 67, 2093–2101
37. Calnek, D. S., Mazzella, L., Roser, S., Roman, J., and Hart, C. M. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 52–57
38. Baba, T., Shimada, K., Neugebauer, S., Yamada, D., Hashimoto, S., and Watanabe, T. (2001) Diabetes Care 24, 953–954
39. Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999) Biochem. Biophys. Res. Commun. 263, 257–262
40. Emoto, M., Anno, T., Sato, Y., Tanabe, K., Okuya, S., Tanizawa, Y., Matsutani, A., and Oka, Y. (2001) Diabetes 50, 1166–1170
41. He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B., and Caldwell, R. B. (1999) J. Biol. Chem. 274, 25130–25135
42. Hoef, J. D., Menninger, C. J., Ziehe, M., and Granger, H. J. (1998) Am. J. Physiol. 274, H1054–H1058
43. Wright, H. M., Clish, C. B., Mikami, T., Hauser, S., Yanagi, K., Hiramatsu, R., Serhan, C. N., and Spiegelman, B. M. (2000) J. Biol. Chem. 275, 1873–1877
44. Zander, T., Kraus, J. A., Grommes, C., Schlegel, U., Feinbinder, D., Klockgether, T., Landreth, G., Koenigschnknecht, J., and Heneka, M. T. (2002) J. Neurochem. 81, 1052–1060
45. Yamakawa, K., Hosui, M., Koyama, H., Tanaka, S., Fukumoto, S., Morii, H., and Nishizawa, Y. (2000) Biochem. Biophys. Res. Commun. 271, 571–574
46. Shima, D. T., Kuroki, M., Deutsch, U., Ng, Y. S., Adams, A. P., and D’Amore, P. A. (1996) J. Biol. Chem. 271, 3877–3883
47. Zander, T., Kraus, J. A., Grommes, C., Schlegel, U., Feinbinder, D., Klockgether, T., Landreth, G., Koenigschnknecht, J., and Heneka, M. T. (2002) J. Neurochem. 81, 1052–1060
48. Yoshi, M., Itoh, H., Tanaka, T., Chun, T. H., Doi, K., Fukunaga, Y., Sawada, N., Yamahita, J., Masatsugu, K., Saito, T., Sakuguchi, S., Sone, M., Yamahara, K. I., Yurugi, T., and Nakao, K. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 560–566
49. Fauconnet, S., Lascombe, I., Chabannes, E., Adessi, G. L., Desvergne, B., Wahli, W., and Bittard, H. (2002) J. Biol. Chem. 277, 23534–23543
50. Niimi, Y., Mochida, S., Matsu, A., Inoue, M., and Fujiwara, K. (2001) Hepatol. Res. 21, 261–267
51. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. (2000) Nature 403, 103–108
52. Straas, D. S., Pascual, G., Li, M., Welch, J. S., Riote, M., Hsiang, C. H., Sengenathan, L. L., Ghosh, G., and Glass, C. K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4844–4849
53. Hattori, Y., Akimoto, K., and Kasai, K. (2000) Biochem. Biophys. Res. Commun. 273, 1144–1149
54. Babi, S., Teichert-Kuliszewska, K., Zhang, Q., Jones, N., Dumont, D. J., and Stewart, D. J. (2003) Am. J. Pathol. 162, 1927–1936

Up-regulation of NO Production by Troglitazone