Troglitazone Increases IL-1β Induced Cyclooxygenase-2 and Inducible Nitric Oxide Synthase Expression *via* Enhanced Phosphorylation of IκBα in Vascular Smooth Muscle Cells from Wistar-Kyoto Rats and Spontaneously Hypertensive Rats

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Peroxisome proliferator-activated receptor gamma (PPARγ) agonists of the thiazolidinedione class are widely used for the treatment of type 2 diabetes subjects due to their ability to improve insulin resistance. Troglitazone and ciglitazone belong to the PPARγ agonists of thiazolidinediones. We report here that troglitazone but not ciglitazone increased IL-1β induced cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in vascular smooth muscle cell (VSMC) from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Potentiated expression of COX-2 and iNOS by troglitazone was inhibited by MG-132, a specific inhibitor of inhibitory factor κB (IκB) activation. Troglitazone treatment of these cells also resulted in a dose-dependent increase in IL-1β induced IκBα phosphorylation. These data suggest that troglitazone is capable of increasing IL-1β induced COX-2 and iNOS expression through an IκBα dependent mechanism in VSMC from WKY and SHR.

Key words troglitazone; cyclooxygenase-2; inducible nitric oxide synthase; spontaneously hypertensive rat; Wistar-Kyoto rat

Troglitazone, one of the thiazolidinediones (TZDs), has been reported to improve insulin resistance in type 2 diabetic patients.[1,2] The mechanisms of TZDs are unknown, but it is reported that TZDs bind to a specific ligand-activated nuclear receptor, the peroxisome proliferator-activated receptor γ (PPARγ).[3] Activation of PPARγ upregulates the gene expression process that is involved in the control of glucose and lipid metabolism.[4] PPARγ is also a key factor in adipogenesis and plays an important role in cell cycle regulation, differentiation and inflammation.[5,6] Troglitazone and ciglitazone are potent and selective ligands for PPARγ. These compounds are used to improve insulin resistance and hyperglycemia, since correction of insulin resistance constitutes a major therapeutic target.[7,8] Because many of the therapeutic benefits of PPARγ ligands are attributed to their antiinflammatory effects, an investigation of the different effect between each PPARγ ligands in regulating inflammatory responses should allow for better understandings of PPARγ ligands.[9,10] However, troglitazone was withdrawn due to severe liver toxicity.[11]

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were required for inflammation in response to various stimuli including IL-1β and lipopolysaccharide (LPS) in the vascular smooth muscle cell (VSMC).[12,13] This mechanism contributes to pathophysiological conditions in which elevated levels of cytokines are evident and various inflammatory effects of COX-2 and iNOS have been described including an increase in vascular permeability, cytotoxicity, and tissue damage.[14,15]

For the antiinflammatory effect of PPARγ ligands, several studies have investigated the effects of TZDs on expression of COX-2.[16,17] Recent studies with different cell types have demonstrated that the effect of TZDs on COX-2 expression is cell-type specific.[18,19] Furthermore, it is not clear how TZDs regulate IL-1β induced COX-2 and iNOS expression in 2 different blood pressure (BP) rat models such as Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). VSMC from SHR proliferate faster as compared with normotensive control rats (WKY).[20] IL-8/CXCL8 expression in thoracic aorta tissue and VSMC in SHR were significantly higher than in WKY.[21]

To evaluate the antiinflammatory property of TZDs, we compared the effects of troglitazone and ciglitazone on IL-1β induced COX-2 and iNOS expression and IκBα signalings in VSMC. Furthermore, we also compared antiinflammatory effects in VSMC form WKY and SHR, since these 2 rat models showed different pathophysiologic responses including BP, VSMC proliferation, and inflammatory process. The aim of this study is to investigate the potential of troglitazone and ciglitazone to modulate COX-2 and iNOS expression and its underlying mechanisms in VSMC from WKY and SHR.

MATERIALS AND METHODS

Cell Culture Aortic VSMC were derived from thoracic aorta of 9 weeks old male WKY and SHR by primary explant culture techniques. Briefly, excised thoracic aortas were minced into small pieces and washed by HBSS (Sigma, St. Louis, U.S.A.). These pieces were plated onto a culture dish containing 50% fetal bovine serum (FBS) (Gibco BRL, Grand Island, U.S.A.) and antibiotics-antimycotics (penicillin 100 U/ml, amphotericin 2.5 μg/ml and streptomycin 100 μg/ml) in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, U.S.A.). Outgrowthed cells were plated and grown in DMEM supplemented with 10% FBS and antibiotics-antimycotics. Cells were maintained in a humidified 95% O_2–5% CO_2 incubator. Media were changed two or three times a week. Cells at passage between 2 and 10 were used for this study.

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Western Blotting Confluent VSMC grown in media supplemented with 10% FBS were treated with IL-1β and/or ciglitazone and troglitazone (Cayman Chemical, Ann Arbor, U.S.A.). After stimulation, cells were washed with phosphate buffered saline (PBS) and harvested by scraping. Protein extracts (12.000 g supernatant) in the PRO-PREP (Intron Biotechnology, Korea) were separated by electrophoresis (30 μg protein per lane) on 10% polyacrylamide gels in the presence of sodium dodecylsulphonate and then transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). The loading and transfer of equal amounts of protein in each lane was verified by staining the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid). After extensive washing with distilled water to remove the protein stain, blots were blocked with 5% non-fat dry milk in PBS. The immobilized protein was visualized by subsequent incubation with a primary antibody (COX-2, iNOS, IκBα and *p*-IκBα obtaind from Santa Cruz Biothechnology, Inc., Santa Cruz, U.S.A.) according to each experiment and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody followed by staining with the enhanced chemiluminescence (ECL) technique developed by NEN life science.

Reverse Transcriptation-Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using Trizol (Invitrogen, Carlsbad, U.S.A.). RNA concentrations were calculated from absorbance at 260 and 280 nm. For reverse transcription, the following components were added to the reaction vials: 5 μg of total RNA, both sense and antisense primer, 10× first-strand buffer, RNase inhibitor, dNTPs, and MMLV-RT in a total volume of 25 μl. The vials were incubated for 60 min at 42 °C, thereafter reverse transcription was terminated by heating at 95 °C for 5 min. The PCR was carried out with 5 μl of RT reaction mixtures, 10× Taq DNA polymerase buffer, dNTPs, both sense and antisense primer, Taq DNA polymerase, and DEPC-treated water with a total volume of 25 μl. The samples were placed in a GeneAmp PCR system 2400 (Perkin Elmer, Shelton, U.S.A.) which was programmed as follows: Pre-PCR; 94 °C 1 min, PCR (30 cycles) Denaturation; 94 °C 1 min, Annealing; 60 °C 1 min, Extension; 72 °C 1 min, Post-PCR; 72 °C 10 min. The PCR products (10 μl) were size-fractionated by agarose (1.5%) gel electrophoresis, stained with ethidium-bromide, and visualized by use of an ultraviolet transilluminator. The sequence of the two iNOS-specific primers was 5-ATGCCTTGCCCCTGGAAGTTTCTC-3 (sense) and 5-CCTCTGATGGTGCCATCGGGCATCTG-3 (antisense), and the predominant cDNA amplification product was predicted to be 800 bp in length. The sequence of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers was 5-GTCATGAGCCCTTCCACGATGC-3 (sense) and 5-AATCTACTGGCGTCTTTCACC-3 (antisense), and the predominant cDNA amplification product was predicted to be 300 bp in length. RT-PCR of GAPDH served as a positive control.

Statistical Analysis Results are expressed as mean± S.E.M. of at least 3 experiments. Comparisons of the means of the two groups were performed by the unpaired *t* test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

TZDs show tissue-specific effect for inflammatory response. They reduced COX-2 expression in prostate cells.[18] However, TZDs increased COX-2 expression in monocyte.[19] Compared to ciglitazone, troglitazone had a significant impact on many of the pathways monitored *in vitro* although no major perturbation was detected *in vivo* for toxocity.[22] In the present study, we wanted to investigate the different impacts of ciglitazone and troglitazone on inflammatory response in VSMC. In addition, we tried to compare these TZDs effects in VSMC from inflammatory prone SHR and control rats

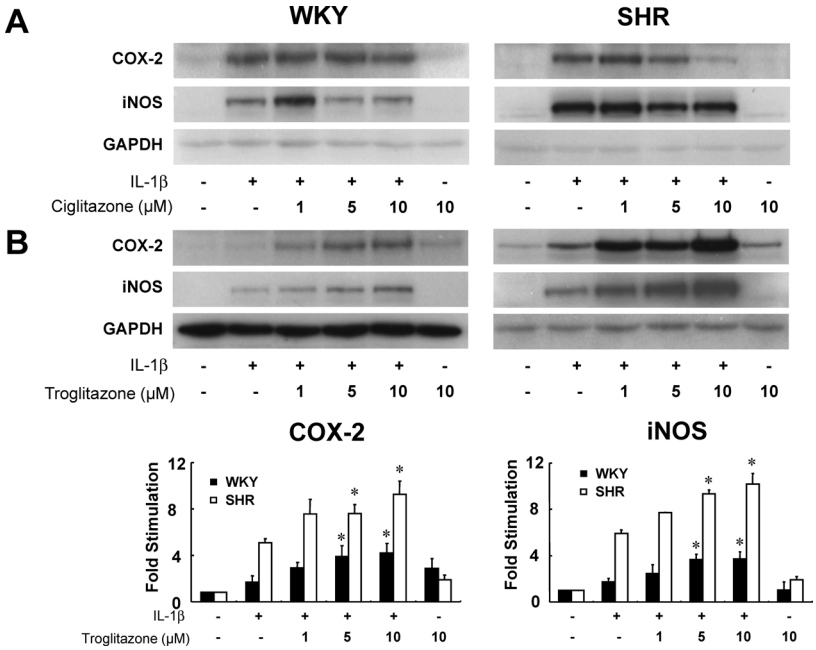

Fig. 1. Troglitazone Increased IL-1β Induced Expressions of COX-2 and iNOS Protein in VSMC

VSMC from WKY and SHR were pretreated with indicated concentrations of ciglitazone (A) and troglitazone (B) for 3 h, then activated with IL-1β 10 ng/ml for 12 h. The lysates were analyzed by SDS-PAGE and reacted with specific antibodies for COX-2, iNOS, and GAPDH. Representative blots were shown. Values are expressed as mean±S.E.M. ($n=3$) $*p<0.05$, compared to IL-1β treatment.

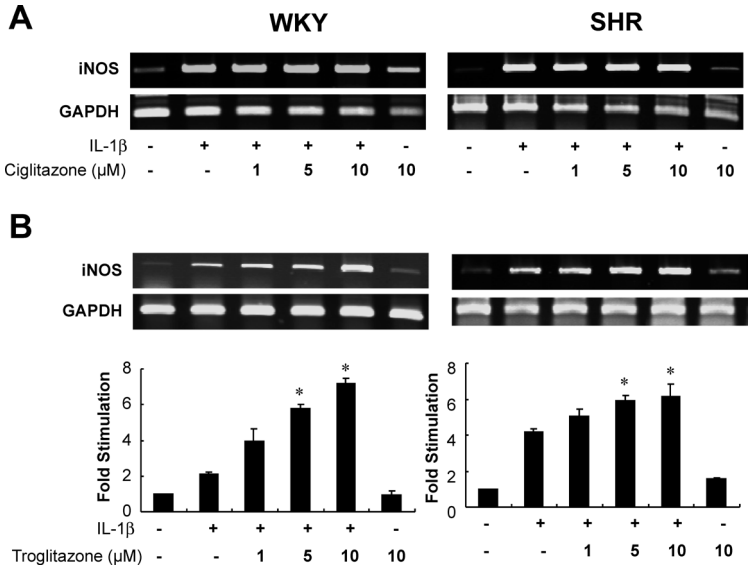

Fig. 2. Troglitazone Increased IL-1β Induced iNOS mRNA Expression in VSMC

VSMC from WKY and SHR were pretreated with indicated concentrations of ciglitazone (A) and troglitazone (B) for 3 h, then activated with IL-1β 10 ng/ml for 12 h. iNOS mRNA expression was analyzed by RT-PCR. RNA was extracted with a Trizol agent, and RT-PCR was performed using specific primers. The image is representative of three independent experiments. Values are expressed as mean±S.E.M. (n=3) $*$ $p<0.05$, compared to IL-1β treatment.

(WKY).

We investigated the effect of TZDs on IL-1β induced COX-2 and iNOS protein expressions in VSMC from WKY and SHR. We found that troglitazone, but not ciglitazone, potentiated the expression of COX-2 and iNOS in VSMC from WKY and SHR. Troglitazone pretreatment elicits a significant increase in COX-2 and iNOS protein expression induced by IL-1β. This increase showed dose-dependency. Although these increases were observed in both WKY and SHR, there was a more profound increase in the VSMC from SHR (Figs. 1A, B). To investigate whether troglitazone affects IL-1β induced iNOS mRNA expression, we tried RT-PCR analysis. iNOS mRNA expression in the absence or presence of troglitazone is shown in Fig. 2. Troglitazone markedly increased the abundance of iNOS mRNA in a dose-dependent manner, whereas ciglitazone did not alter the abundance of iNOS mRNA in VSMC from WKY and SHR. In order to identify the molecular target of troglitazone, the investigation proceeded with a blockade of IκB degradation by MG-132. IκB degradation has been implicated in COX-2 and iNOS expression by various stimuli in VSMC.[23] We pretreated with MG-132 30 min prior to troglitazone treatment. As shown in Fig. 3, MG-132 blocked the effect of troglitazone on IL-1β induced COX-2 and iNOS protein expression, and this effect also showed in IL-1β induced iNOS mRNA expression. Moreover, troglitazone elicited a dose-dependent increase in IL-1β induced IκBα phosphorylation in VSMC from WKY and SHR (Fig. 3C). Although troglitazone has been reported to exert PPARγ dependent as well as PPARγ independent effects, we suppose that troglitazone increases transcription of iNOS mRNA in VSMC, since VSMC has a PPARγ mediated up-regulation of gene expression mechanism.[24,25] VSMC are considered useful for the evaluation of mechanisms underlying the antiinflammatory effect of TZDs.[26]

Results from this study strongly indicate a proinflammatory mechanism that troglitazone enhance IL-1β induced

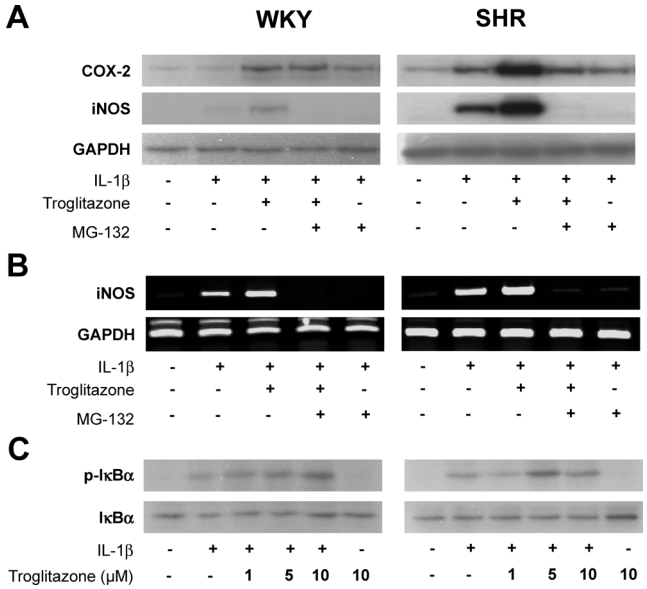

Fig. 3. Troglitazone Increased Phosphorylation of IκBα in VSMC

(A) The role of MG-132 on troglitazone enhanced COX-2 and iNOS protein expression in VSMC. We pretreated cells with MG-132 (10 μM) 30 min prior to troglitazone (10 μM) treatment, then followed by activation with IL-1β 10 ng/ml for 12 h. COX-2 and iNOS expression were analyzed by Western blot, respectively. The blot is representative of three independent experiments. (B) MG-132 abrogated troglitazone enhanced iNOS mRNA expression in VSMC. Representative RT-PCR images were shown. (C) Troglitazone increased phosphorylation of IκBα in a dose-dependent manner. VSMC were pretreated with indicated doses of troglitazone for 3 h, then activated with IL-1β 10 ng/ml for 12 h. Phosphorylated IκBα and IκBα were determined by Western blot analysis. Representative blots were shown.

IκBα phosphorylation in VSMC. The difference in inflammatory potency of SHR and WKY dose not affect inflammatory signaling of ciglitazone and troglitazone.

In conclusion, these results suggest that troglitazone exerts a proinflammatory effect in VSMC that is mediated by increased phosphorylation of IκBα. These observations may contribute to a better understanding of the proinflammatory

effects of troglitazone in VSMC.

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