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European Journal of Pharmacology 407 (2000) 227–235

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Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells

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Received 23 March 2000; received in revised form 10 July 2000; accepted 29 September 2000

Abstract

Thiazolidinediones, activators of peroxisome proliferator-activated receptor (PPAR)γ, have been reported to induce apoptosis in many types of cells. In the present study, we investigated the effects of thiazolidinediones, troglitazone, and pioglitazone on the cell growth of vascular smooth muscle cells, and identified a specific effect of troglitazone in addition to PPARγ activation. Subconfluent rat culture vascular smooth muscle cells were treated with or without PPARγ activators, troglitazone (1–30 μM), or pioglitazone (1–30 μM) for 72 h. After treatment, cell viability was significantly reduced by troglitazone in concentration of 5–30 μM but not by pioglitazone. Vascular smooth muscle cells appeared to float and shrink 48 h after treatment with 20 μM of troglitazone. In situ DNA labeling showed that the nuclei of these cells were positively stained, and genomic DNA extracted from the cells showed nucleosomal laddering. Messenger RNA expression levels of *c-myc*, *p21*, *bax*, *bcl-2*, and *bcl-x* were not changed by the treatment with troglitazone. In contrast, along with the induction of vascular smooth muscle cell apoptosis, both the mRNA and protein expression levels of p53 and Gadd45 markedly increased in response to troglitazone. These results strongly suggest that troglitazone can induce vascular smooth muscle cell apoptosis and that this effect is caused primarily by activation of the p53 and Gadd45 pathway but not by PPARγ activation.

Keywords: Apoptosis; Smooth muscle cell, vascular; p53; Gadd45; Troglitazone; Pioglitazone

1. Introduction

The proliferation and migration of vascular smooth muscle cells are critical factors in the progression of atherosclerosis and the development of restenosis after percutaneous transluminal angioplasty (Schwartz, 1997). Recently, vascular smooth muscle cell apoptosis has been identified in these lesions, suggesting that vascular smooth muscle cell apoptosis as well as vascular smooth muscle cell proliferation are involved in the etiology or pathogenesis of vascular remodeling (Isner et al., 1995; Geng and Libby, 1995; Igase et al., 1999).

The thiazolidinediones, novel insulin-sensitizing agents, have been shown to significantly reduce hyperinsulinemia in insulin-resistant animals and humans (Lee et al., 1994; Iwamoto et al., 1991; Nolan et al., 1994). Another functionally important property of thiazolidinediones is their ability to inhibit the growth and proliferation of vascular smooth muscle cells (Dubey et al., 1993; Law et al., 1996; Goetze et al., 1999). One of the thiazolidinedione analogues, pioglitazone, inhibits the growth of vascular smooth muscle cells stimulated by insulin, epidermal growth factor, or serum (Dubey et al., 1993). Another analogue, troglitazone, suppresses the growth and migration of vascular smooth muscle cells induced by basic fibroblast growth factor or platelet-derived growth factor-BB (Law et al., 1996). Furthermore, thiazolidinediones have recently been reported to induce apoptosis in many types of cells, including endothelial cells (Bishop-Bailey and Hla, 1999), monocyte-derived macrophages (Chinetti et al., 1998), choriocarcinoma cells (Keelan et al., 1999), and gastric

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00758-5

cancer cells (Takahashi et al., 1999). These effects have been thought to be produced primarily by the activation of peroxisome proliferator-activated receptor (PPAR)γ. PPAR belongs to a family of ligand-activated transcriptional factors and is composed of three members, PPARα, δ, and γ. Each PPAR member is specifically activated by each different ligand. The activation of PPAR is regulated by this restrictive ligand-receptor binding system, and therefore thiazolidinedione analogues are thought to be one of the specific ligands for PPARγ. However, recent reports have demonstrated that trogitazone has its own specific functions in addition to PPARγ activation (Hattori et al., 1999; Ikeda and Watanabe, 1998; Wang et al., 1999; Sunaga et al., 1999; Ishizuka et al., 1998). Troglitazone, but not pioglitazone, has an inhibitory effect on platelet aggregation or cholesterol biosynthesis that is not due to PPARγ activation (Wang et al., 1999; Ishizuka et al., 1998). Another troglitazone-specific action is an effect on the excitability, action potential configurations, and the membrane currents of ventricular myocytes (Ikeda and Watanabe, 1998).

In this study, we have evaluated the direct effects of a PPARγ activator, either pioglitazone or troglitazone, on vascular smooth muscle cell proliferation, and have also demonstrated that troglitazone-induced vascular smooth muscle cell apoptosis occurs through a pathway independent of PPARγ.

2. Materials and methods

2.1. Materials

Troglitazone and pioglitazone were kindly provided by Sankyo Pharmaceutical (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. These agents were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1% in a culture medium. Caspase-3/7 inhibitor [Acetyl-L-Aspartyl-L-Glutamyl-L-Valyl-L-Aspart-1-al (Ac-DEVD-CHO)] was purchased from the Peptide Institute (Osaka, Japan). [α-^{32}P]dCTP (110 TBq/mmol) was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Affinity-purified polyclonal antibodies against p53 and Gadd45 were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA)

2.2. Cell culture

Rat vascular smooth muscle cells were isolated from the thoracic aortas of 10-week-old male Sprague–Dawley rats (Charles River Japan, Kanagawa, Japan) by a previously described method (Kitami et al., 1995). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, and were maintained at 37°C with atmospheric air and 5% CO_2. Vascular smooth muscle cells were passaged every 4–7 days, and all experiments were performed on cells at 3–10 passages from primary culture. In the cells, PPARγ expressions at protein and mRNA levels were confirmed by Western and Northern blotting, respectively (data not shown). Experimental protocols were approved by the Committee of Animal Experimentation at Ehime University School of Medicine, and performed in accordance with the Recommendations from the Declaration of Helsinki and the NIH Guide for the Care and Treatment of Laboratory Animals.

2.3. Cell viability measurement

Using a cell proliferation reagent WST-1 (4-[3(4-lodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1, 3-benzen disulfonate) (Boehringer Mannheim, Mannheim, Germany), cell viability was assessed according to the manufacturer's specification (Okura et al., 1998). Vascular smooth muscle cells were seeded onto 96-well tissue culture plates at a concentration of 1×10^4 cells/well in 100 μl of a 10% fetal calf serum-containing DMEM, and were cultured for 48 h until reaching 70–80% confluency. The culture medium was changed to a 0.1% fetal calf serum containing DMEM, and cells were cultured for an additional 24 h. The cells were then treated with troglitazone (1–30 μM) or pioglitazone (1–30 μM), and were cultured for 3 days. Control cells were cultured in a 0.1% fetal calf serum-containing medium alone. After treatment, 10 μl of WST-1 was added to each well, and the culture plates were incubated for 1 h at 37°C. Absorbency was measured at 450 nm with a reference wavelength at 690 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan Bichromatic Labsystems; Helsinki, Finland), and cell viability was presented as the relative cell viability in reference to the viability of control cells, which was set as 100%. To examine the effects of caspase inhibitor on cell viability, Caspase-3/7 inhibitor, Ac-DEVD-CHO, was added to the culture medium at a final concentration of 10 μM and incubated for 24 h.

2.4. Morphological examination

Subconfluent vascular smooth muscle cells were cultured in a 0.1% fetal calf serum-containing DMEM for 24 h, and then treated in freshly prepared a 0.1% fetal calf serum-containing medium with troglitazone (20 μM) or pioglitazone (20 μM) for an additional 48 h. Cell morphology was monitored by phase-contrast microscopy during an incubation of 48 h.

2.5. DNA fragmentation analysis

Genomic DNA was isolated from vascular smooth muscle cells after treatment with 20 μM troglitazone or piogli-

tazone for 48 h according to a previously described method (Okura et al., 1998). Briefly, cells were lysed in a DNA extraction solution and were incubated with 100 μg/ml of proteinase K (Ambion, Austin, TX, USA) for 16 h at 37°C. After incubation, the same volume of phenol was added to cell lysates and mixed gently for 6 h. The mixture was centrifuged, and the upper aqueous phase was incubated with 5 μg/ml RNase A (Sigma, St. Louis, MO, USA) for 1 h at 37°C, and the following treatment with phenol/chloroform (1:1, v/v) was carried out. After ethanol precipitation, DNA was dissolved in water and electrophoresed on a 1.5% agarose gel.

2.6. In situ DNA labeling analysis

To detect DNA fragmentation in situ, nick end-labeling was performed using an ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA) (Okura et al., 1998). After treatment with 20 μM troglitazone for 48 h, vascular smooth muscle cells cultured on a chamber slide were fixed with 0.25% glutaraldehyde and were treated with 0.3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. The cells were then exposed by deoxyribonucleotide transferase with digoxygenin-conjugated dUTP for 1 h at 37°C. After washing, end-labeled DNA fragments were reacted with peroxidase-conjugated anti-digoxigenin antibody for 30 min at room temperature, and were finally detected with chromogen 3,3′-diaminobenzidine (Wako, Osaka, Japan).

2.7. Messenger RNA measurement by RT-PCR

To measure the mRNA expression levels of *c-myc*, *p21*, *p53*, *gadd45*, *bax*, *bcl-2*, and *bcl-x*, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously (Okura et al., 1991). After treatment with or without troglitazone (20 μM) for 24 h, total cellular RNA was isolated from vascular smooth muscle cells according to the manufacturer's instructions with ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of RNA was reverse-transcribed into the first-strand cDNA with oligo-dT primers using a 1st-strand™ cDNA synthesis kit (Clontech, Palo Alto, CA, USA), and the synthesized cDNA was subjected to PCR amplification. The PCR conditions were as follows: denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 60 s for 25 cycles. The mRNA level of glyceralaldehyde 3-phosphate dehydrogenase (G3PDH) was used as an internal control. The oligonucleotide sequences of the PCR primers used herein were as follows. Forward and reverse primers for rat *c-myc* were: 5′-CCCCTCAGTGGTCTTCCCCTAC-3′ and 5′-TGTTCTCGCCGTTTCCTCAGTA-3′; for rat *p21*, 5′-ATGACTGAGTATAAACTTGTGG-3′ and 5′-TCACATGACTATACACCTTGTC-3′; for rat *p53*, 5′-ATGGAGGATTCACAGTCGGA-3′ and

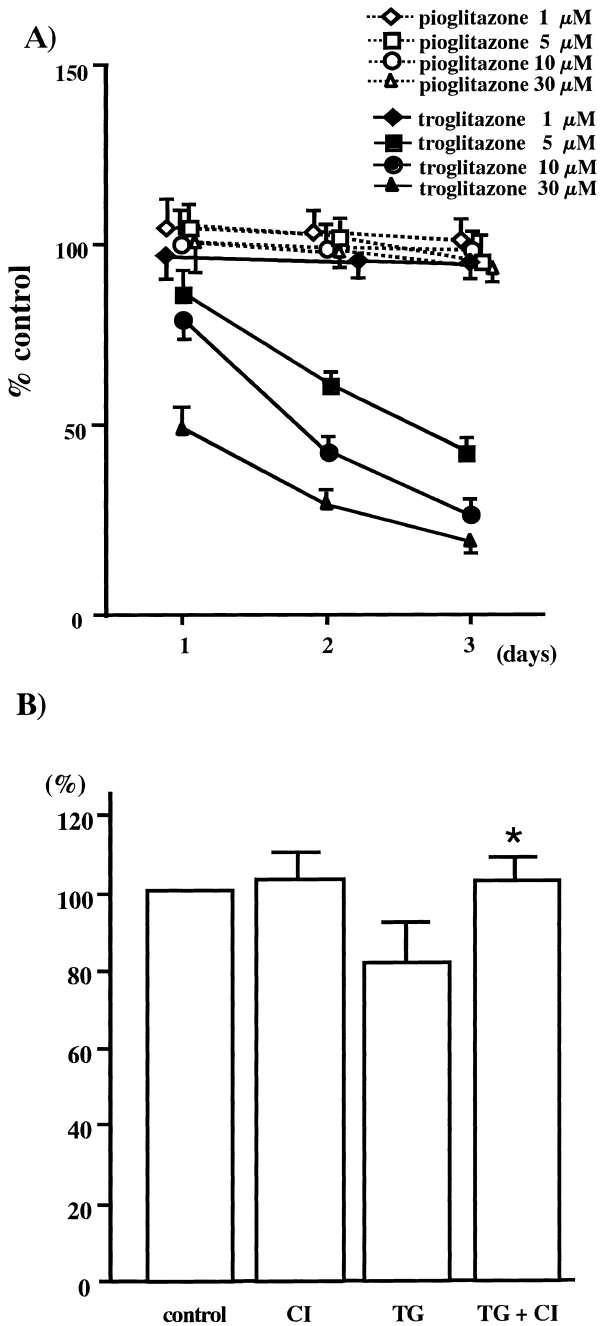

Fig. 1. (A) Vascular smooth muscle cell viability after treatment with troglitazone or pioglitazone. Vascular smooth muscle cells were treated with various concentrations of troglitazone or pioglitazone for the indicated time. After incubation of the WST-1 reagent (0.1 volume of culture medium) for 1 h at 37°C, the absorbencies (A450–A690) were measured by an ELISA reader. The cell viability was shown as a relative viability in reference to that of control cells, which was set as 100%. The results represent the means ± S.D. of six separate assays. (B) Effect of caspase-3/7 inhibitor on the reduction of cell viability by troglitazone. Caspase-3/7 inhibitor (10 μM) reversed the reduction of vascular smooth muscle cell viability by troglitazone (10 μM). The results represent the means ± S.D. of six separate assays. $^{*}P < 0.01$, significant difference between troglitazone treatment (TG) and troglitazone treatment + caspase-3/7 inhibitor (TG + CI). CI indicated the treatment with caspase-3/7 inhibitor alone.

5′-TCAGTCTGAGTCAGGCCCCA-3′; for rat *gadd45*, 5′-ATGACTTTGGAGGAATTCTCGG-3′ and 5′-TCACCGTTCGGGGAGATTAATC-3′; for rat bax, 5′-GACACCTGAGCTGACCTTGG-3′ and 5′-GAGGAAGTCCAGTGTCCAGC-3′; for *bcl-2*, 5′-ATGGCGCAAGCCGGGAGAAC-3′ and 5′-TCACTTGTGGCCCAGGTATG-3′; for *bcl-x*, 5′-ATGTCTCAGAGCAACCGGGA-3′ and 5′-TCACTTCCGACTGAAGAGTG-3′; and for G3PDH, 5′-TGGAGTCTACTGGCGTCTTC-3′ and 5′-CAAAGGTGGAGGAATGGGAG-3′. The PCR products were electrophoresed on a 1.4% agarose gel and were visualized with ethidium bromide staining.

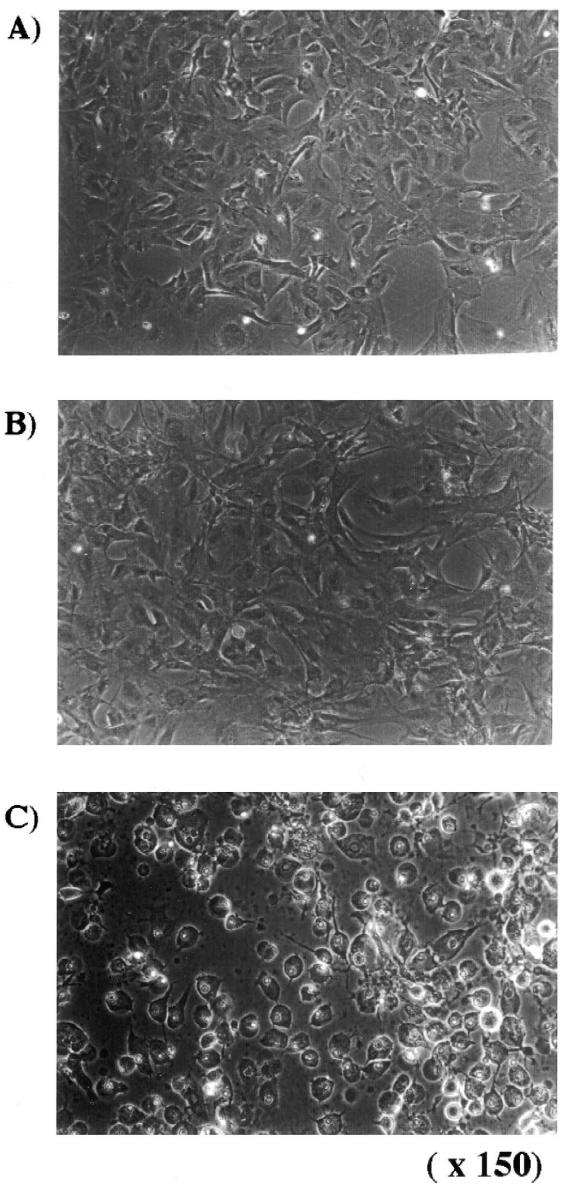

Fig. 2. Apoptotic features of troglitazone-induced cell death. After treatment with medium containing (A) 0.1% fetal calf serum alone as a control, (B) pioglitazone (20 μM), or (C) troglitazone (20 μM) for 48 h, vascular smooth muscle cells were monitored by phase-contrast microscopy (original magnification: ×150).

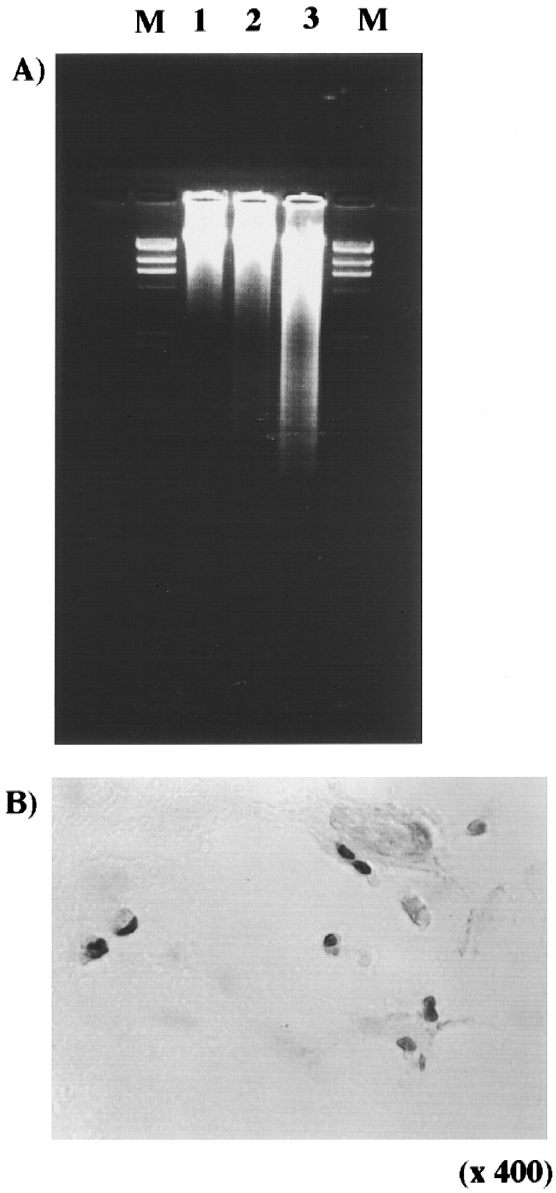

Fig. 3. (A) Gel electrophoresis of internucleosomal DNA fragmentation. Genomic DNA were extracted from vascular smooth muscle cells treated with medium containing 0.1% fetal calf serum alone (lane 2) as a control, pioglitazone (20 μM) (lane 3), or troglitazone (20 μM) (lane 4) for 48 h. DNA size makers are shown in lanes 1 and 5. (B) Identification of apoptotic cells by in situ nick DNA end-labeling. After treatment with 20 μM troglitazone for 48 h, vascular smooth muscle cells in a chamber slide were stained by in situ DNA labeling.

2.8. Northern blotting

To prepare the cDNA probes for *p53* and *gadd45*, RT-PCR products obtained from the preceding experiment were isolated and subcloned into Bluescript KS (+) II plasmid vector (Stratagene, La Jolla, CA, USA). After the entire sequence of each clone was determined by sequencing, an inserted cDNA fragment was used as a probe for Northern blotting. Each probe was labelled with [α-^{32}P]dCTP using a random primer method, and 20 μg of

total cellular RNA was used for the analysis. In this experiment, RNA was isolated from the vascular smooth muscle cells after treatment with 30-μM troglitazone for 0–24 h, and Northern blotting was performed by a previously described procedure (Okura et al., 1998).

2.9. Western blotting

Western blotting was carried out by a previously described method (Fukuoka et al., 1999). After treatment with 30-μM troglitazone for 0–24 h, vascular smooth muscle cells were lysed in a buffer containing 50 mM Tris–HCl (pH8.0), 150 mM NaCl, 2 mM phenyl methyl sulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1% Triton X-100. After centrifugation, the supernatant was transferred, and protein concentrations were determined by a Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA, USA). The cell lysate (50 μg) was applied on a 12.5% Ready-gel (Bio-Rad Laboratories) and was electrophoresed at 38 mA. Proteins in the gel were transferred to poly-vinylidene difluoride (PVDF) membranes (Immun-Blot™ PVDF membrane; Bio-Rad Laboratories) by electroblotting for 1 h at 100 V. The membranes were incubated with a diluted antibody against p53 or Gadd45 for 1 h at room temperature, and then incubated with a diluted secondary antibody against goat or rabbit immune globulin G (IgG) (Santa Cruz Biotech.) for 1 h at room temperature, respectively. After washing, immunoreactive proteins were finally visualized by an enhanced chemiluminescence detection system (ECL Western blotting analysis system; Amersham Pharmacia Biotech), and the ensuing autoradiography.

2.10. Statistical analysis

An analysis of variance with Bonferroni–Dunn post-hoc analysis was used to analyze differences between the two experimental groups. All data are expressed as means ± S.D., and the statistical significance is defined as $P < 0.05$.

3. Results

3.1. Direct effect of thiazolidinediones on vascular smooth muscle cell viability

After treatment with various concentrations of troglitazone or pioglitazone, the vascular smooth muscle cell viability was determined by a WST assay. Although 1 μM of troglitazone did not alter the cell viability, 5 − 30 μM troglitazone reduced the cell viability. In contrast, similar to the control, pioglitazone did not alter the viability in any concentration (Fig. 1A). Fig. 2 shows the typical cell morphology after incubation of two thiazolidinedione analogues for 48 h. Apparent morphological changes were not observed in either the control cells (Fig. 2A) or the pioglitazone-treated cells (Fig. 2B). In contrast, in the troglitazone-treated cells, many of the cells floated, shrank, and became rounded in shape (Fig. 2C). Further, the cell membranes were blebbed, and the cytoplasm was condensed. This morphological appearance of the troglitazone-treated cells strongly indicates cell death, probably apoptosis. To prove this, we further analyzed the effects of a Caspase-3/7 inhibitor, Ac-DEVD-CHO, on cell death induced by troglitazone. This reagent is well known to act as a potent common executor of apoptosis.

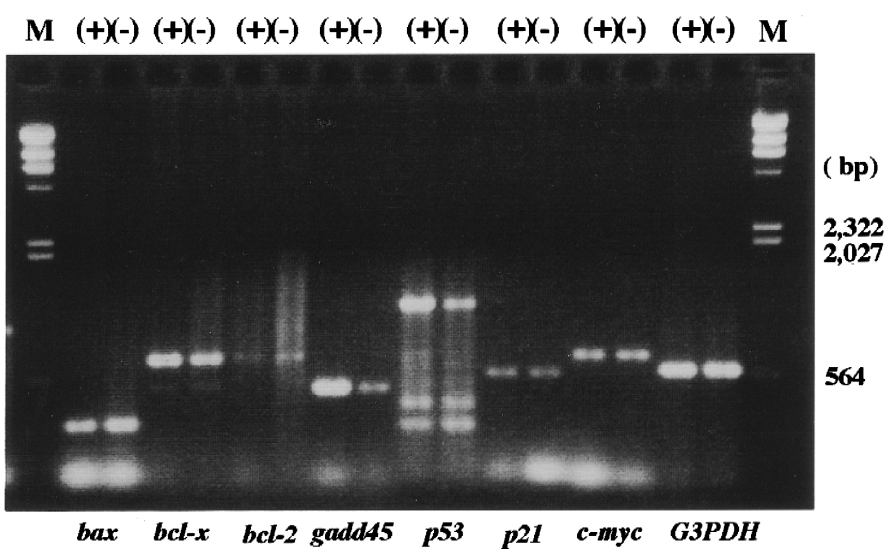

Fig. 4. Apoptosis-related gene expression in vascular smooth muscle cells treated with or without troglitazone. RT-PCR products for *bax*, *bcl-x*, *bcl-2*, *gadd45*, *p53*, *p21*, *c-myc*, and *G3PDH* were analyzed by electrophoresis on a 1.5% agarose gel. (−) indicates the respective mRNA in control vascular smooth muscle cells, and (+) in troglitazone-treated vascular smooth muscle cells. Size markers are shown at both ends of the gel.

There was no significant difference in cell viability between control cells and the Caspase-3/7 inhibitor alone treated cells, indicating Caspase3/7 inhibitor did not influence the cell growth and the cell cytotoxicity (Fig. 1B). The reduction in cell viability by troglitazone was significantly suppressed by the addition of Caspase-3/7 inhibitor (Fig. 2B).

3.2. Vascular smooth muscle cell apoptosis induced by troglitazone

To further confirm the induction of vascular smooth muscle cell apoptosis by troglitazone, both genomic DNA electrophoresis and in situ DNA labeling were carried out (Fig. 3). As shown in Fig. 3A, almost all of the genomic DNA population prepared from the control (lane 1) or pioglitazone-treated cells (lane 2) showed a high molecular DNA band. In contrast, internucleosomal DNA (about 180 bp laddering) was observed in the vascular smooth muscle cells treated with troglitazone (lane 3). In situ DNA labeling also revealed that the troglitazone-treated cells, especially rounded cells, showed condensed and fragmented nuclei that were brown in color (Fig. 3B).

3.3. Apoptosis-related gene expression in troglitazone-treated cells

To characterize the apoptosis-related genes induced in the troglitazone-treated vascular smooth muscle cells, the mRNA levels of *c-myc*, *p21*, *p53*, *gadd45*, *bax*, *bcl-2*, and *bcl-x*, were evaluated by RT-PCR (Fig. 4). The levels of *p53* (expected size 1176 bp) and *gadd45* (555 bp) mRNA were drastically increased by the treatment with troglitazone, whereas those of *c-myc* (697 bp), *p21* (567bp), *bax* (310 bp), *bcl-2* (711bp), and *bcl-x* (721bp) mRNA were not altered when compared with the corresponding control cells. In addition, Northern blotting was performed for *p53* and *gadd45* mRNA from vascular smooth muscle cells treated with 30-μM troglitazone for 6, 12, and 24 h (Fig. 5). While some *p53* mRNA was expressed before treatment, troglitazone increased *p53* mRNA expression during first 12 h and then reduced at a time of 24 h after treatment (upper panel). Baseline levels of *gadd45* mRNA were very low or almost negligible, but the levels markedly increased in response to troglitazone treatment in a time-dependent manner during a 24-h incubation (mid panel).

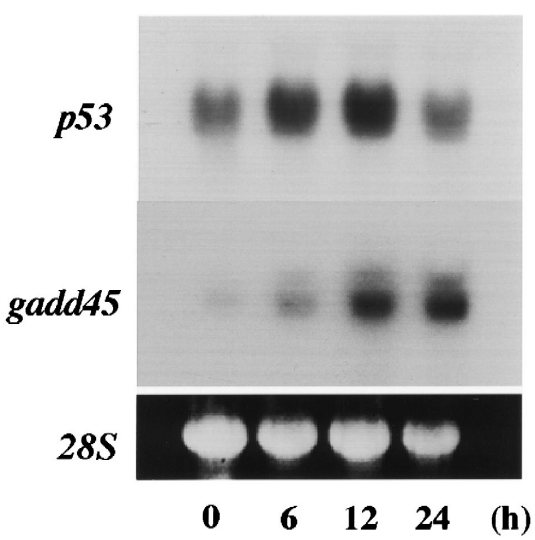

Fig. 5. Time-course of the mRNA induction of *p53* and *gadd45* by troglitazone. Vascular smooth muscle cells were treated with troglitazone (30 μM), and total cellular RNA was extracted from the cells after 0–24 h as indicated. Total RNA (20 μg) was analyzed by Northern blotting for *p53* and *gadd45* mRNA.

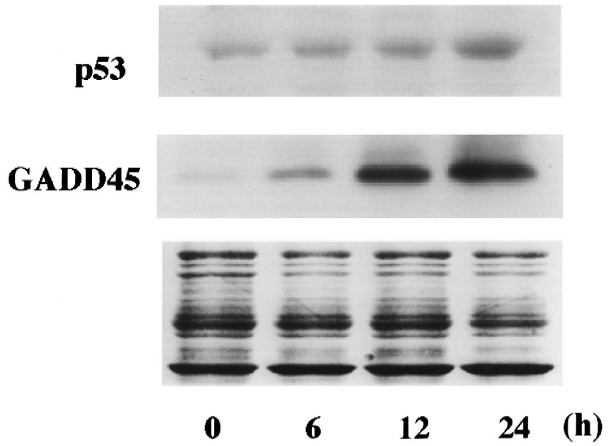

Fig. 6. Induction of p53 and Gadd45 protein levels by troglitazone. Total cell lysates (50 μg) obtained from vascular smooth muscle cells treated with troglitazone (30 μM) were directly subjected to Western blotting for p53 and Gadd45.

3.4. Protein levels of p53 and Gadd45 in troglitazone-treated cells

To further confirm the results of the RT-PCR or Northern blotting, protein levels of p53 and Gadd45 were assessed by Western blotting (Fig 6). Baseline levels of p53 protein were detectable, whereas the levels of Gadd 45 protein were almost negligible. After treatment with troglitazone, both the p53 and Gadd45 protein levels were increased in a time-dependent manner during a 24-h incubation (Fig. 6).

4. Discussion

Both troglitazone and pioglitazone are active antidiabetic thiazolidinediones that increase tissue sensitivity to the actions of insulin though the activation of PPARγ (Lee et al., 1994; Iwamoto et al., 1991; Nolan et al., 1994). Recent studies have also shown that PPARγ activation induces apoptosis in endothelial cells (Bishop-Bailey and

Hla, 1999), monocyte-derived macrophages (Chinetti et al., 1998), JEG3 choriocarcinoma cells (Keelan et al., 1999), and gastric cancer cells (Takahashi et al., 1999). In this study, we demonstrated that troglitazone, but not pioglitazone, reduces vascular smooth muscle cell viability and induces vascular smooth muscle cell apoptosis at a concentration of 5–30 μM. Pioglitazone has been reported to be a more potent and specific antidiabetic insulin-sensitizing drug than troglitazone (Ikeda and Watanabe, 1998). The effective doses used for diabetes in Zucker fatty rats have been reported to be 0.3–10 mg/kg for pioglitazone (Ikeda et al., 1990) and 100–150 mg/kg for troglitazone (Fujiwara et al., 1988). For troglitazone, another pharmacological action has recently been reported in addition to anti-diabetic insulin-sensitization. Troglitazone has an inhibitory effect on platelet aggregation and cholesterol biosynthesis (Keelan et al., 1999; Hattori et al., 1999). In addition, troglitazone has a significant effect on excitability, action potential configuration and membrane currents of ventricular myocytes (Ikeda and Watanabe, 1998). These actions, which appear to be specific to troglitazone, have been thought to be caused by another pathway rather than PPARγ activation. In the present study, we have demonstrated that only troglitazone, but not pioglitazone induce vascular smooth muscle cell apoptosis, even if the same doses (5–30 μM) are used for each drug. Furthermore, apoptosis-related genes, *p53* and *Gadd45*, expressions were increased with the induction of vascular smooth muscle cell apoptosis by the treatment with troglitazone (Fig. 4) but not pioglitazone (data not shown). These results strongly suggest that the apoptotic effects of troglitazone are not due to PPARγ activation.

Clinically, the maximum plasma concentrations after treatment with therapeutic doses of troglitazone have been reported to be 0.4–2.4 mg/l (0.9–5.5 μM) (Spencer and Markham, 1997). In the present study, we have shown that troglitazone reduces cell viability and induces apoptosis at a concentration of 5 to 30 μM. The concentrations of troglitazone used here were slightly higher than in plasma or vascular tissues under therapeutic conditions in humans. However, clinically, troglitazone is administered for a long term. We need to clarify the chronic effect of troglitazone on vascular smooth muscle cell viability. If troglitazone may induce vascular smooth muscle cell apoptosis in vivo, the potential clinical importance of this phenomenon is controversial. Vascular smooth muscle cell apoptosis may contribute to an attenuation of the neointimal thickening seen in early atherosclerosis (Libby and Ross, 1996). On the other hand, increased apoptosis may contribute to the instability of plaque in atherosclerotic lesion (Ross and Fuster, 1996).

Although the precise mechanisms of troglitazone's induction of apoptosis remain unclear, our results indicate that the p53 tumor-suppressor gene plays an important role in this apoptotic pathway. p53 induces cell-cycle arrest or apoptosis in many types of cells, including vascular smooth muscle cells (Vogelstein and Kinzler, 1992; Speir et al., 1994, Johnson et al., 1996; Yonemitsu et al., 1998), and activates the transcription of selected target genes including *p21* (Waga et al., 1994), *bax* (Miyashita and Reed, 1995), *bcl-2* (Miyashita et al., 1994) and *gadd45* (Kastan et al., 1992; Hollander et al., 1993). Further, we were able to show the up-regulation of *gadd45* expression, but not *p21*, *bax*, and *bcl-2* expression in apoptotic cells after treatment with troglitazone. These results suggest that *gadd45* is activated by an intracellular pathway through p53 induction, and that the subsequent cell-death pathway is induced by troglitazone. Gadd45 is up-regulated in response to growth-arrest conditions as well as to a variety of DNA-damaging agents (Hollander et al., 1993; Yoshida et al., 1996). Gadd45 mediates the growth arrest caused by inhibition of the cell cycle through the interaction of two cell-cycle components, p21(Kearsey et al., 1995) and cell nuclear antigen (Smith et al., 1994). As another growth-arrest or apoptotic pathway for Gadd45, Takekawa and Saito (1988) have recently reported that Gadd45 mediates cell-growth inhibition or apoptosis through the activation of p38/JNK, via MTK1/MEKK4, in response to environmental stresses. However, the pathway down stream from Gadd45 observed in troglitazone-induced vascular smooth muscle cell apoptosis remains unknown.

Recently, Hattori et al. (1999) have shown that troglitazone up-regulates the nitric oxide (NO) synthesis stimulated by cytokines such as interleukin-1, tumor necrosis factor-α, interferon-γ, and lipopolysaccharides in vascular smooth muscle cells through a PPARγ-independent pathway. NO can act either as a proapoptotic or antiapoptotic factor depending on the cell-type specificity (Nicotera et al., 1997). In vascular smooth muscle cells, NO commonly acts as an inducer of vascular smooth muscle cell apoptosis and plays an important role in regulating the vascular smooth muscle cells number in atherosclerotic or restenotic lesions after balloon injury (Pollman et al., 1996; Bochaton-Piallat et al., 1995)). Iwashina et al. (1999) have reported that NO-induced apoptosis is seen in vascular smooth muscle cells accompanied by the induction of p53 expression. Although we do not yet have any direct evidence for the effect of troglitazone on NO synthesis in vascular smooth muscle cells, NO may contribute to vascular smooth muscle cell apoptosis in response to troglitazone treatment through a pathway involving p53 accumulation.

In conclusion, we have shown the direct effects of thiazolidinediones on vascular smooth muscle cell proliferation, demonstrating that only troglitazone induces vascular smooth muscle cell apoptosis by the pathway of p53 and Gadd45 induction, but not by PPARγ activation.

Acknowledgements

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Sci-

ence, Sports, and Culture of Japan (No. 11838012), a Japan Heart Foundation Grant for research on Hypertension and Vascular Metabolism, and the Takeda Medical Foundation.

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