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Troglitazone inhibits α_1-adrenoceptor-induced DNA synthesis in vascular smooth muscle cells

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Abstract

While vascular smooth muscle cell proliferation is important in hypertension, relatively little is known about the contribution of catecholamines. Novel insulin sensitizing agents, thiazolidinediones, have been demonstrated to inhibit angiotensin II-, basic fibroblast growth factor (FGF)-induced growth of vascular smooth muscle cells. We hypothesize that these agents might also inhibit the effect of the stimulation of α_1-adrenoreceptors on the proliferation of vascular smooth muscle cells. Troglitazone (1–20 μM), a member of the thiazolidinediones, significantly inhibited the stimulation of α_1-adrenoreceptor-induced DNA synthesis, c-*fos* induction and mitogen-activated protein (MAP)-kinase activation. This effect was associated with inhibition by troglitazone of the transactivation of the serum response element (SRE), which regulates c-*fos* expression. Inhibition of c-*fos* induction by troglitazone appeared to occur via blockade of the upstream of MAP kinase activation in vascular smooth muscle cells. At this dose, troglitazone inhibited the ternary complex factor (TCF)-dependent activation, which is regulated by MAP kinase activation, but did not inhibit the TCF-independent SRE activation. Besides, the degree of the inhibitory effect of troglitazone on MAP kinase activation, DNA synthesis, c-*fos* expression differs. This may show that troglitazone work on multiple sites. These results suggest that troglitazone is a potent inhibitor of vascular smooth muscle cells proliferation through the downregulation of c-*fos* expression and may be a useful agent for prevention of atherosclerosis which is a result of hypertension. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: α_1-Adrenoceptor; Smooth muscle cell vascular; c-*fos*; Troglitazone; SRE (serum response element); SRF (serum response factor); TCF (ternary complex factor)

1. Introduction

Enhancement of the hyperplasia of vascular smooth muscle cells is thought to be one of the main events implicated in the pathogenesis of atherosclerosis. Several stimuli, for example the stimulation of α_1-adrenoceptors and injury, are reported to induce the hyperplasia of vascular smooth muscle cells. Therefore, it is important to inhibit the hyperplasia of vascular smooth muscle cells by several stimuli to prevent atherosclerosis.

Stimulation of α_1-adrenoceptors by endogenous catecholamines regulates a host of physiological responses in different cell types (Chambers et al., 1993; Anofossi and Trovati, 1996). With respect to vascular smooth muscle cells, it has been demonstrated that, apart from any acute effects on vascular smooth muscle cells tone (Barman, 1995), activation of α_1-adrenoceptors regulate gene expression (Okazaki et al., 1994), eicosanoid metabolism (Nishio et al., 1996), and proliferation of cultured vascular smooth muscle cells (Noveral and Grunstein, 1994). Indeed, in this context, it has been suggested that α_1-adrenoceptor-mediated regulation of cell growth may play an important role in the development of vascular smooth muscle cells hyperplasia, with the resultant increase in atherosclerosis and restenosis.

Thiazolidinediones are novel insulin-sensitizing agents that have been shown to decrease insulin resistance and hyperglycemia in patients with non-insulin dependent diabetes mellitus (Kumar et al., 1996). Recent studies demonstrated that troglitazone, a member of the thiazolidinedione class of oral antidiabetic drugs, prevents vascular smooth muscle cells proliferation and migration in vitro and substantially attenuates neointimal hyperplasia in the injured rat aorta (Law et al., 1996). But the precise mechanism of this effect is unknown.

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In the present study, we investigated the effect of troglitazone on phenylephrine-induced proliferation in cultured rat vascular smooth muscle cells as well as its mechanism.

2. Materials and methods

2.1. Cell Culture

Vascular smooth muscle cells were obtained from thoracic aorta of rat by the method described (Nishio and Watanabe, 1997a). The cells (1×105) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were used between the third and sixth passage. Cells were grown to confluence, at which time they were rendered quiescent by the DMEM medium containing 0.5% (V/V) fetal bovine serum and maintained for 72 h before experimentation. The number of viable cells was determined in duplicate by hematocytometry with trypan blue exclusion. Viable cells is beyond 95% in spite of treatment.

2.2. Assay for DNA synthesis by vascular smooth muscle cells

The assay was performed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials (Nishio and Watanabe, 1997b). Vascular smooth muscle cells were seeded at a density of 1×10^5 cells/dish and grown to confluence. Then the medium was replaced by DMEM containing 0.5% (V/V) fetal bovine serum. Following another 3-day cultivation, the cells were exposed for 24 h to DMEM containing phenylephrine in the presence of [^{3}H]thymidine (5 μCi/ml). Acid-insoluble [^{3}H]thymidine was extracted in 0.5 M NaOH and quantified by use of a liquid scintillation counter.

2.3. Oligonucleotide synthesis

Synthesis and purification of phosphorothioate oligonucleotides for culture experiments were performed as described previously (Monia et al., 1996). c-*fos* antisense (5′-GAA-CAT-CAT-GGT-CGT-3′) oligonucleotides was designed to span the region around the start codon of the rat c-*fos* mRNA. Also the corresponding sense (5′-ACG-ACC-ATG-ATG-TTC-3′) and scrambled (5′-GTA-CCA-ATC-GGG-ATT-3′) oligonucleotides were synthesized.

2.4. Western blotting

Cell lysates were separated by using 12.5% SDS-polyacrylamide gel. Separated proteins were transferred to polyvinylidene difluoride nylon membrane and subjected to Western blotting as described elsewhere using polyclonal anti c-*fos*, polyclonal anti-active MAP kinase or polyclonal anti-ERK-1(C-16) (Nishio and Watanabe, 1996).

2.5. Treatment of cells with oligonucleotides

The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of DMEM containing 10% fetal bovine serum for 24 h. Thereafter, cells were incubated with oligonucleotides at a concentration of 15–45 μg/ml in DMEM supplemented with *N*-[1-(2, 3-dioleoyloxy) propyl]-*N*, *N*-trimethylammonium methylsulfate (DOTAP) solution (lipofectin) at a concentration of 0.25 μg/10 mM oligonucleotide. After 18 h, the medium was removed and replaced with DMEM only for 24 h. Thereafter, the cells were stimulated with DMEM containing phenylephrine (Ciffi et al., 1997).

2.6. Reporter gene expression

The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of DMEM containing 10% fetal bovine serum for 24 h. Thereafter, cells were incubated with reporter plasmid and control plasmid in DMEM supplemented with DOTAP solution (lipofectin). After 4 h, the medium was removed and replaced with DMEM only for 24 h. Thereafter, the cells were stimulated with DMEM containing phenylephrine (Ciffi et al., 1997). Serum response element (SRE)-dependent gene expression was measured in cotransfection assays with the reporter plasmid pSRE-Luc, which contains two copies of the c-*fos* SRE cloned upstream of a minimal promoter element and the firefly luciferase gene or pSRE.L-Luc, which contains three copies of the c-*fos* SRE.L (Hill et al., 1993) cloned upstream of a minimal promoter element. Transfection efficiency was monitored by using a control plasmid expressing β-galactosidase. The luciferase and β-galactosidase activities were measured as described previously (Seth et al., 1992). The data are presented as the ratio of luciferase activity to β-galactosidase activity.

2.7. Inositol triphosphate (IP_3) assay

The cells (1×10^6) were seeded into 35-mm diameter dishes and maintained in 2 ml of DMEM containing 10% fetal bovine serum for 24 h. Thereafter, cells grown to confluence were left for 4 h in serum-free DMEM. After pretreatment of troglitazone for 30 min, phenylephrine stimulated vascular smooth muscle cells for the indicated times. The medium was then removed rapidly and 1 ml of ice-cold 10% solution of trichloroacetic acid was added to the cells, which were then scraped off and placed on ice for 30 min. Samples were centrifuged to remove precipitated proteins, and the supernatant fractions were washed with 1 ml of water-saturated diethyl ether three times and neutralized by the addition of 1 M sodium bicarbonate.

Aliquots of these supernatants were then analyzed for IP_3 quantification, using IP_3 binding kits (Amersham, Amersham, UK).

2.8. Materials

[^{3}H]thymidine was obtained from Amersham. Anti-c-*fos* polyclonal antibodies were from Oncogene science. Polyclonal anti-active MAP kinase was from Promega. Anti-ERK-1 (C-16) was from Santa Cruz Biotechnology. Lipofectin was from Gibco BRL. All cell culture materials were from Life Technologies. Troglitazone was generously supplied by Sankyo.

2.9. Statistics

Values are expressed as the arithmetic mean ± S.D. Statistical analysis of the data was performed by the one factor Anova–Scheffe *F*-test (StatView 512$^+$, version 1.0, Apple Computer). Duplicate wells were analyzed for each experiment and each experiment was performed independently at least three times. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Troglitazone inhibits phenylephrine-induced vascular smooth muscle cells proliferation

Previously, we reported phenylephrine induced vascular smooth muscle cells proliferation in a concentration-dependent manner (Nishio and Watanabe, 1997b). Furthermore, we confirmed that this response of phenylephrine is highest at 24 h (data not shown). To determine whether troglitazone inhibits phenylephrine-induced proliferation in vascular smooth muscle cells, growth-arrested vascular smooth muscle cells were treated for 24 h with phenylephrine (10 μM) in the presence and absence of troglitazone, and DNA synthesis was measured by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable materials. Phenylephrine stimulated vascular smooth muscle cells DNA synthesis 4-fold compared to untreated cells, and this response was significantly blunted by troglitazone (1–20 μM). Troglitazone inhibited phenylephrine-induced vascular smooth muscle cells DNA synthesis in a dose-dependent manner with 50 and 95% inhibition at 10 and 20 μM concentration, respectively. Prazosin (100 μM), a selective α_1-adrenoceptor antagonist, also significantly inhibited phenylephrine-induced vascular smooth muscle cells DNA synthesis (Fig. 1). Yohimbine (100 μM), a selective α_2-adrenoceptor antagonist, did not significantly inhibited phenylephrine-induced vascular smooth muscle cells DNA synthesis (Nishio and Watanabe, 1997b). When growth-arrested cells were treated with 10 (data not shown) and 20 μM troglitazone for 24 h in the absence of phenylephrine, no significant difference was observed as compared to control, suggesting that troglitazone was not toxic to the cells (Fig. 1).

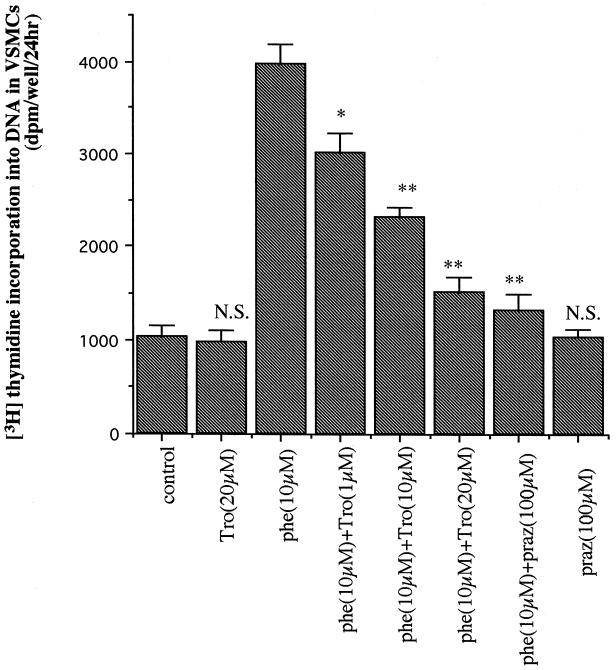

Fig. 1. Troglitazone inhibits phenylephrine-induced vascular smooth muscle cell DNA synthesis. Growth-arrested vascular smooth muscle cells were treated with and without phenylephrine (phe, 10 μM) in the presence and absence of troglitazone (Tro, 1–20 μM) for 24 h, and DNA synthesis was measured by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material. Values are the means ± S.D. of three independent experiments performed in duplicate each time. ($^*P < 0.05$, $^{**}P < 0.01$ vs. phenylephrine only, N.S. vs. control).

3.2. Troglitazone inhibits the induction of c-fos expression by phenylephrine

A transient induction of c-*fos* mRNA expression accompanies the stimulation of growth-arrested cells to proliferate by a variety of growth factors (Campan et al., 1992). Therefore, we determined whether troglitazone inhibits phenylephrine-induced c-*fos* expression. Troglitazone concentrations as low as 20 μM totally blocked phenylephrine-induced c-*fos* expression. Significant inhibition of both phenylephrine (10 μM) simulated-DNA synthesis and c-*fos* induction occurs by troglitazone (1 μM) (Figs. 1 and 2), suggesting that its antiproliferative effect in vascular smooth muscle cells may result from its ability to inhibit the induction of the important early response gene c-*fos*, which occurs when cells undergo transition from quiescent to the proliferative stage.

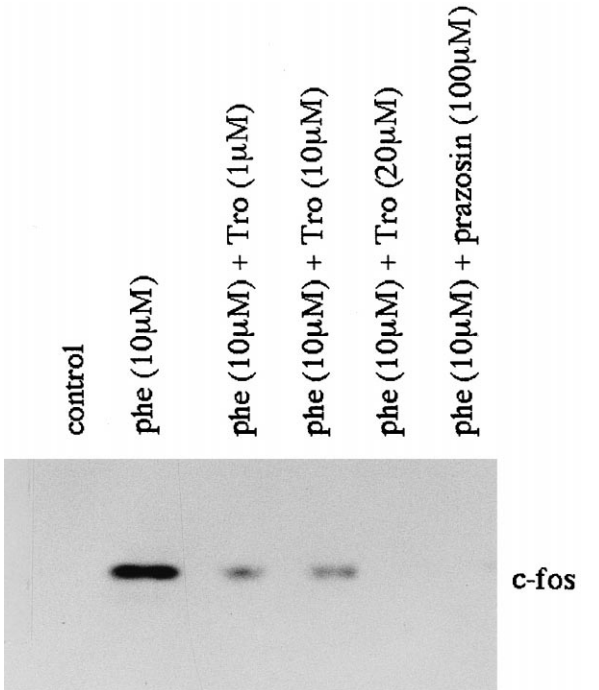

Fig. 2. Troglitazone inhibits phenylephrine-induced c-*fos* expression. Growth-arrested vascular smooth muscle cells were stimulated for 3 h with phenylephrine (phe, 10 μM) in the presence and absence of troglitazone (Tro, 1–20 μM). The c-*fos* expressions were analyzed by Western blotting. Results shown here are representative of one experiment and were reproduced in three separate experiments.

3.3. Effect of c-fos antisense oligonucleotides on phenylephrine-induced vascular smooth muscle cell proliferation

Figs. 1 and 2 demonstrates that troglitazone inhibits phenylephrine (10 μM)-induced vascular smooth muscle cells proliferation accompanying c-*fos* downregulation. In order to elucidate the cause-and-effect relationship between the phenylephrine-induced vascular smooth muscle cells proliferation and c-*fos* expression, we examined the effect of c-*fos* antisense oligonucleotides on phenylephrine-induced vascular smooth muscle cells proliferation. A phosphorothioate oligonucleotide was synthesized originating at the initiation site and proceeding toward the 3′ end of the c-*fos* cDNA. Cells were incubated for 18 h with the antisense oligonucleotide in order to provide enough time to maximally effect c-*fos* protein (data not shown). After this time, vascular smooth muscle cells were incubated for 24 h with DMEM only. Thereafter, phenylephrine was added, and the cells were incubated for an additional 24 h. Cells were monitored throughout the experiment, and no greater cytotoxicity was observed compared with the control. Furthermore, we assessed another early response gene expression, c-*myc*, to verify the specificity of the c-*fos* antisense oligonucleotide. However, there was no decrease in induction of c-*myc* expression by phenylephrine that may be required for DNA synthesis (Bennett et al., 1994) (data not shown). The concentration-dependent effect of the oligonucleotide on phenylephrine-induced vascular smooth muscle cells proliferation was examined using [^{3}H]thymidine incorporation assay. Fig. 3 demonstrates that antisense c-*fos* oligonucleotides reduce cell level of c-*fos* expression induced by phenylephrine and Fig. 4 demonstrates that antisense c-*fos* partially inhibits phenylephrine-induced vascular smooth muscle cells proliferation dependent on oligodeoxynucleotide concentrations. Antisense c-*fos* oligodeoxynucleotides decreased phenylephrine-induced vascular smooth muscle cells proliferation to 74, 63, and 32% of control at 15, 30, 45 (μg/ml), respectively. In contrast, corresponding sense c-*fos* and scramble c-*fos* did not suppress c-*fos* expression nor inhibit phenylephrine-induced vascular smooth muscle cells proliferation. These studies confirm that c-*fos* expression is required for vascular smooth muscle cells proliferation and c-*fos* downregulation caused the inhibition of phenylephrine-stimulated proliferation. Furthermore, in spite of complete inhibition of c-*fos* expression by antisense oligodeoxynucleotide (15 μg/ml), phenylephrine-induced vascular smooth muscle cells proliferation was partially inhibited. This result may suggest that phenylephrine-induced vascular smooth muscle cells proliferation is partially independent of c-*fos* expression.

3.4. Troglitazone blocks transactivation of the SRE

The SRE mediators increase immediate early gene expression concluding c-*fos* in cells. To determine whether

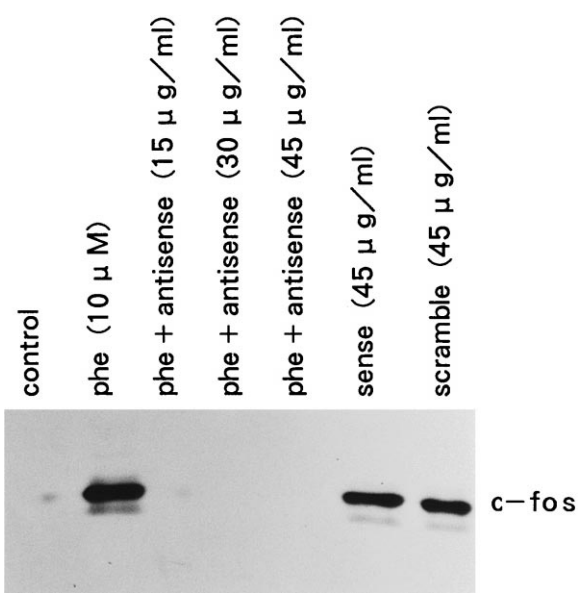

Fig. 3. Antisense c-*fos* oligonucleotide inhibited c-*fos* expression. Vascular smooth muscle cells were incubated for 24 h with DMEM only, following incubation with oligonucleotides for 18 h. Thereafter, cell lysates were prepared from vascular smooth muscle cells stimulated by phenylephrine (phe, 10 μM) for 3 h. The c-*fos* expressions were analyzed by Western blotting. Results shown here are representative of one experiment and were reproduced in three separate experiments.

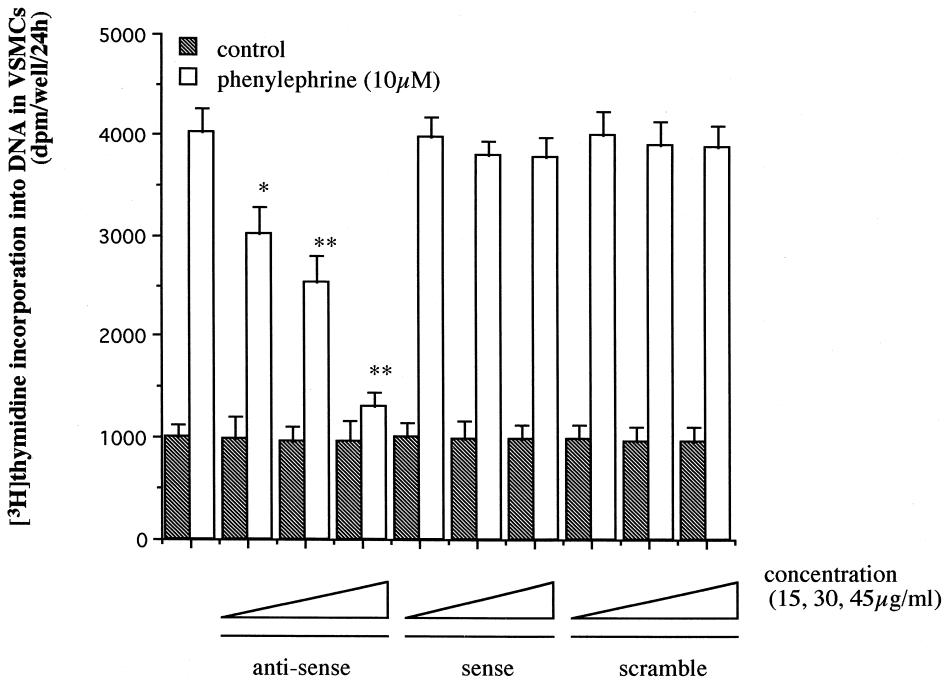

Fig. 4. Antisense c-*fos* oligodeoxynucleotide inhibited phenylephrine-induced vascular smooth muscle cell DNA synthesis. Vascular smooth muscle cells were incubated for 24 h with DMEM only, following incubation with oligonucleotides for 18 h. Thereafter, vascular smooth muscle cells were stimulated with phenylephrine (phe, 10 μM) in the presence of [^{3}H]thymidine for 24 h and DNA synthesis was measured by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material. Values are the means ± S.D. of three independent experiments performed in duplicate each time. (*$P < 0.05$, **$P < 0.01$ vs. phenylephrine only).

the inhibition of phenylephrine-induced c-*fos* expression by troglitazone reflected diminished transcriptional activation of c-*fos* through the SRE, 2 × SRE-luciferase reporter plasmids were transiently transfected into vascular smooth muscle cells. In the presence of phenylephrine, SRE-directed luciferase activity was 3-fold greater than that in lysates from transfected cells in the absence of phenylephrine. Troglitazone inhibited phenylephrine-inducible SRE-luciferase activity in a dose-dependent fashion (Fig. 5). In the presence of 1 μM troglitazone, phenylephrine-stimulated SRE-luciferase activity was reduced to 65 ± 4% of expression in cells treated with phenylephrine alone. Troglitazone at 10 and 20 μM further inhibited SRE-luciferase activity to 43.8 ± 5.6 and 21 ± 6.5% of the phenylephrine-induced effects, respectively.

3.5. Troglitazone inhibits mitogen-activated protein (MAP) kinase activation by phenylephrine

Transcriptional activation of the c-*fos* gene by phenylephrine is mediated, at least in part, through the interaction of transcriptional factor with SRE located in the c-*fos* 5′-flanking DNA. The transcriptional factor, ternary complex factor (TCF) protein, belongs to a subfamily of the ETS-domain family (Jankenecht and Nordheim, 1993). Phosphorylation of TCF by MAP kinase is associated with activated, SRE-dependent gene expression. Therefore, we investigated the effect of troglitazone on the MAP kinase activity. Levels of activated MAP kinase were visualized

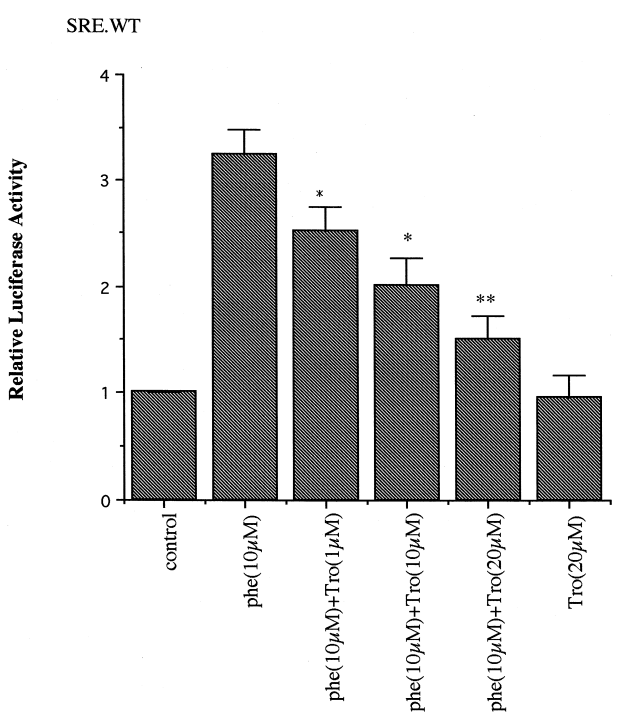

Fig. 5. The effect of troglitazone on the phenylephrine-induced SRE activity. Cells were transfected with thymidine kinase (TK)-luciferase reporter plasmids controlled by the wild-type SRE. Following incubation in serum-free medium for 24 h, the cells were treated with phenylephrine (phe, 10 μM) in the absence or presence of troglitazone (Tro 1–20 μM). The data are presented as relative luciferase activities (mean ± S.D.; $n = 3$) (*$P < 0.05$, **$P < 0.01$ vs. phenylephrine only).

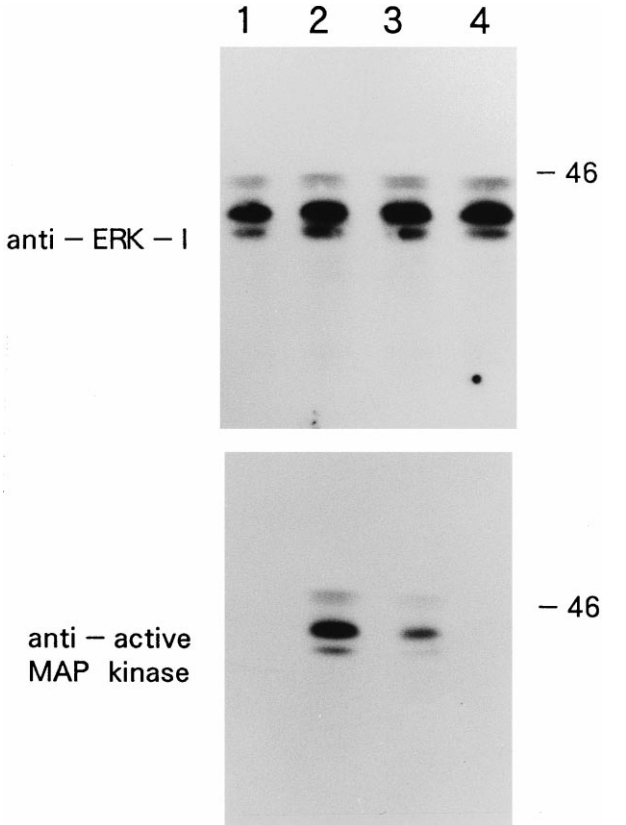

Fig. 6. Troglitazone inhibits the enzymatic activation of ERK. Arrested cells were treated with phenylephrine (phe 10 μM) in the presence or absence of troglitazone (Tro 1–10 μM) for 5 min. Levels of activated cellular MAP kinase in 35 μg of cell extracts were visualized by Western blotting using the anti-active MAP kinase antibody which selectively recognizes the activated phosphorylated forms of ERK1 and ERK2. Blots were stripped and reprobed with anti-MAP kinase that recognizes both active and inactive forms of ERK1 and ERK2. Lanes 1–4 are: 1, control 2, phe (10 μM) 3, phe (10 μM)+Tro(1 μM) 4, phe (10 μM)+Tro(10 μM). Similar results were obtained with three independent experiments.

by Western blotting, using an antibody that selectively recognizes the phosphorylated activated forms of the phenylephrine-regulated MAP kinases extracellular signal-regulated kinase-1 and extracellular signal-regulated kinase-2. Although MAP kinase phosphorylation was detected in lysates from cells which were treated by phenylephrine (10 μM) for 10 min, MAP kinase phosphorylation was not detected in lysates from cells pre-treated with troglitazone (10 μM) for 30 min in spite of the treatment with phenylephrine for 10 min (Fig. 6). This result may suggest that troglitazone suppresses the c-*fos* induction by the stimulation of α_1-adrenoceptor via MAP kinase inactivation. Furthermore, in spite of complete inhibition of MAP kinase activation by troglitazone (10 μM), phenylephrine-induced vascular smooth muscle cells proliferation was partially inhibited (Fig. 1). These results may suggest that phenylephrine-induced vascular smooth muscle cells proliferation is partially independent of MAP-kinase activation.

3.6. Troglitazone has no effect on interaction of serum response factor (SRF) with the SRE

Although c-*fos* promoter mutants that cannot bind TCF are not responsive to the activated MAP kinase pathway, they remain responsive to TCF-independent serum-induced SRE activator. Therefore, we investigated the effects of interaction of SRF and SRE by troglitazone. To determine the effects of troglitazone on the interaction of SRF and SRE, 3× c-*fos* SRE derivative, SRE.L-luciferase, which cannot bind TCF, was transiently transfected into vascular smooth muscle cells. In the presence of phenylephrine, SRE.L-directed luciferase activity was 3-fold greater than that detectable in lysates from transfected cells in the absence of phenylephrine. Troglitazone cannot inhibit phenylephrine-inducible SRE.L-luciferase activity in a dose-dependent fashion. In the presence of 1, 10 and 20 μM troglitazone, phenylephrine-stimulated SRE.L-luciferase activity was almost unchanged compared with expression in cells treated with phenylephrine alone (Fig. 7). The results suggest that troglitazone has no effect on the interaction of SRF with SRE.

3.7. Troglitazone has no effect on α_1-adrenoceptor induced-phospolipase C activation

There is the possibility that the inhibition of MAP kinase activation by troglitazone could be attributed to

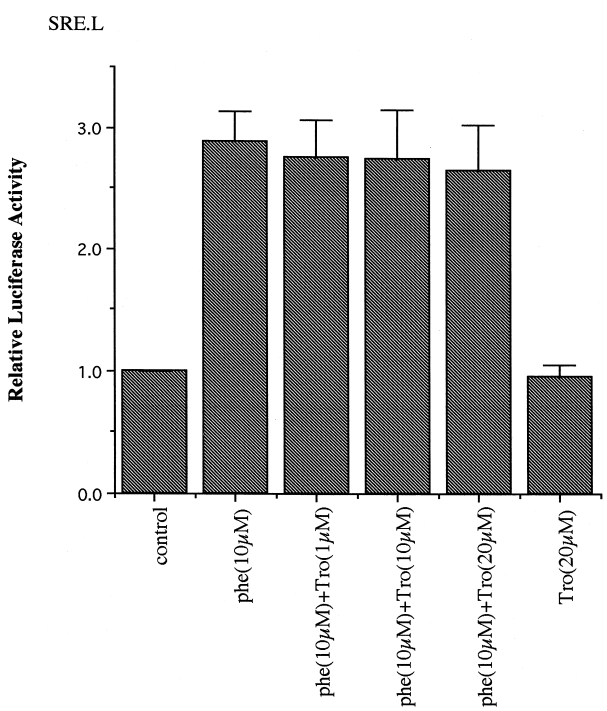

Fig. 7. The effect of troglitazone on the phenylephrine-induced SRE.L activity. Cells were transfected with TK-luciferase reporter plasmids controlled by the SRE.L. Following incubation in serum-free medium for 24 h, the cells were treated with phenylephrine (phe, 10 μM) in the absence or presence of troglitazone (Tro, 1–20 μM). The data are presented as relative luciferase activities (mean ± S.D.; $n = 3$).

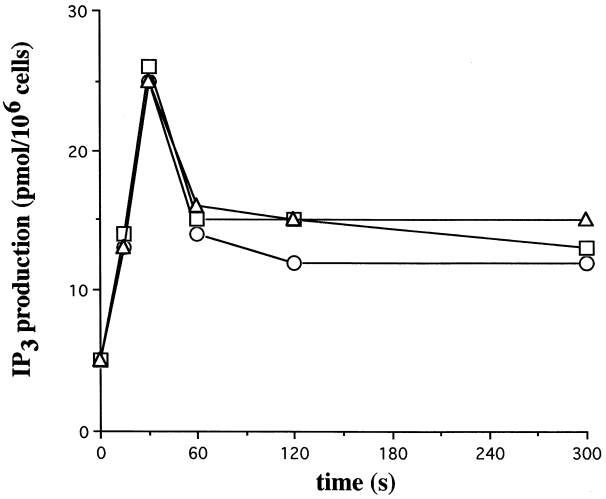

Fig. 8. Time course of troglitazone effect on phenylephrine-induced IP_3 formation in vascular smooth muscle cells. Vascular smooth muscle cell (1×10^6) were seeded and grown to confluence. Thereafter, vascular smooth muscle cells were left for 4 h in serum free-DMEM and vascular smooth muscle cells were stimulated with phenylephrine (10^{-5} M) at indicated times after pretreatment of troglitazone (0 (○), 10 μM (□) or 20 μM (△)). IP_3 determination was carried out as described in Section 2. Data are shown as pmol $IP_3/1\times10^6$ cells and values are means of four independent determinations from two separate experiments.

α_1-adrenoceptor blocking properties. Therefore, we investigated the effect of α_1-adrenoceptor induced phospolipase C activation by troglitazone. In Fig. 8, we showed the time course of the effect of troglitazone on IP_3 release, which follows the phosphatidylinositol (PI) hydrolysis caused by a specific phospolipase C. The pretreatment of troglitazone to the cells cannot inhibit phenylephrine-elicited rapid and transient increase of IP_3. This result suggest that troglitazone does not produce a general, non-specific inhibitor of the response induced by the stimulation of α_1-adrenoceptor.

4. Discussion

The present study examined inhibitory effects of troglitazone on phenylephrine-induced DNA synthesis. This inhibition of phenylephrine-induced DNA synthesis is due to inhibition of phenylephrine-mediated MAP kinase activation and downregulation of c-*fos* expression. This result agrees with other work showing that the inhibition of angiotensin II-induced DNA synthesis by troglitazone is attributed to the inhibition of angiotensin II-mediated MAP kinase activation (Graf et al., 1997). However, in contrast to the action of troglitazone on the phenylephrine or angiotensin II signalling pathway, it was reported that the PDGF and basic FGF signalling pathways were inhibited at a point downstream of MAP kinase activation by troglitazone. Troglitazone cannot inhibit the platelet-derived growth factor (PDGF)- nor basic FGF-induced activation of MAP kinase (Kumar et al., 1996). These results may suggest that troglitazone targets two different intracellular sites.

The present study clearly demonstrated that the stimulation of α_1-adrenoceptor by phenylephrine induces DNA synthesis and c-*fos* expression of growth-arrested rat aortic smooth muscle cells. This result agrees with early work that stimulation of smooth muscle cell proliferation by growth factors is preceded by dramatic upregulation of immediate early genes like as c-*fos*, c-*myc* and c-*jun* (Yu et al., 1996). The proto-oncogene c-*fos* is a major nuclear target for the signal transduction pathway involved in the regulation of cell growth (Coolican et al., 1997). Since it was reasonable to determine the involvement of c-*fos* on the enhancement of DNA synthesis by phenylephrine, we evaluated the effect of c-*fos* antisense oligonucleotide on phenylephrine-induced vascular smooth muscle cells proliferation. The sequence was carefully chosen from a region lacking substantial homology with other sequenced rat genes. The oligonucleotides were phosphorothioate-modified to limit degradation and purified by high performance liquid chromatography prior to use to remove all incomplete synthesis products, thereby limiting non-specific effects. Phenylephrine-stimulated DNA synthesis was inhibited by antisense c-*fos* oligonucleotides in a dose-dependent fashion but was not inhibited by sense oligonucleotides. These observations suggest that the involvement of c-*fos* in the α_1-adrenoceptor-mediated stimulation for DNA synthesis. Troglitazone also inhibits the expression of c-*fos* induced by phenylephrine. Therefore, the inhibition of α_1-adrenoceptor-stimulated DNA synthesis by troglitazone may be due to the downregulation of c-*fos* expression.

The SRE is a regulatory sequence found in many growth factor-regulated promoters (for example, c-*fos*) (Treisman, 1990). The ubiquitous transcription factor, SRF, binds to the SRE along with a TCF (Triesman, 1994). The TCF, which comprises members of a small family of Ets domain proteins, including ELK-1, sphingolipid activator protein (SAP)-1 and SAP-2/ERP/NET. The TCF regulates SRE activity in activation of the Ras–Raf-ERK pathway (Whitmarsh et al., 1995). By itself, the SRF can mediate transcriptional activation induced by serum, lysophosphatidic acid or intracellular activation of heterotrimetric G protein (for example Rho family) (Hill et al., 1995).

Recently, it has been reported that both Rho and Ras are required for $G\alpha_q$ and α_1-adrenoceptor signalling in cardiomyocytes, although Rho and Ras are involved independently of each other in the α_1-adrenergic signalling pathway (Sah et al., 1996). Therefore, it is reasonable to think that the induction of early genes (c-*fos*) by phenylephrine is mediated by the two kinds of signalling, that is, Rho family GTP ase and Ras-MAP signalling. In this study, we have analyzed regulated transcription by SRE in more detail. First to provide direct evidence for the involvement of SRF in phenylephrine-induced transcription,

we used a previously described functional assay (Hill et al., 1993). Smooth muscle cells were transfected with luciferase reporter genes controlled by either two copies of the wild-type c-*fos* SRE, or three copies of its derivative SRE.L, which cannot bind TCF. Both the wild-type c-*fos* SRE and the SRE.L reporter responded to phenylephrine. This result suggests that a part of the activation of the SRE by phenylephrine is dependent on SRF and independent on TCF. Next, we investigated to see whether troglitazone's inhibitory effect on the activation of the SRE by phenylephrine is dependent on SRF or not. Troglitazone did not inhibit phenylephrine-induced SRE.L luciferase activity under the present experimental conditions. The result suggests that troglitazone does not inhibit the potentiation of SRF transcriptional activity by phenylephrine. In contrast, troglitazone inhibits the phenylephrine-induced SRE wild-type luciferase activity. These results established for the first time that troglitazone inhibits the SRE transcriptional activity through the mechanism which is dependent on TCF and is independent on SRF. Furthermore, we demonstrate that the inhibitory effect of troglitazone on TCF-dependent SRE transcriptional activity is mediated through the inhibition of MAP-kinase activity.

Lastly, in this study, the mechanism of blockade of α_1-adrenoceptor-induced MAP kinase activation by troglitazone is unclear. The time course analysis of IP_3 production showed that there is no effect of troglitazone on α_1-adrenoceptor-induced phospolipase C activation. Further study is required to elucidate the mechanism for blockade of MAP kinase activation. It is possible that troglitazone and all other thiazolideniones may regulate vascular function directly, in addition to improving insulin resistance and reducing hyperglycemia. Further investigation will likely reveal whether troglitazone is also beneficial for vascular proliferative disease such as atherosclerosis.

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