FEBS 17977 FEBS Letters 400 (1997) 119–121

Troglitazone inhibits angiotensin II-induced DNA synthesis and migration in vascular smooth muscle cells

Kristof Graf[a,b,*], Xiao-Ping Xi[a], Willa A. Hsueh[a], Ronald E. Law[a]

[a] *University of Southern California, School of Medicine, Department of Medicine, Division of Endocrinology, Diabetes and Hypertension, Los Angeles, CA 90033, USA*

[b] *Department of Internal Medicine/Cardiology, Virchow Klinikum HU Berlin and German Heart Institute, Berlin, Germany*

Received 11 November 1996

Abstract Angiotensin II (AII) plays a crucial role in controlling the proliferation and migration of vascular smooth muscle cells (VSMCs). The present study was undertaken to determine if troglitazone (Tro) has an effect on the G-protein coupled signaling through AII type I (AT-1) receptors in cultured rat aortic VSMCs. AII-induced MAP kinase activation was inhibited 67.9% by Tro. AII-induced DNA synthesis and migration was completely inhibited by Tro or by the AT-1 receptor blocker irbesartan. The present study demonstrates that troglitazone inhibits AII-induced DNA synthesis, migration and MAP kinase activation in VSMCs which are important molecular events for the development of neointimal hyperplasia and atherosclerosis.

Key words: Angiotensin II; DNA synthesis; MAP kinase; Migration; Smooth muscle cell; Troglitazone

1. Introduction

Intimal accumulation of vascular smooth muscle cells (VSMCs) is an important event in the development of both restenosis and atherosclerosis. The accumulation of VSMC in the intima results from both cell proliferation and directed migration of VSMCs from the media into the intima [1,2]. These processes are regulated by several growth factors which may be released by cells within the injured vessel or from circulating cells. We have recently shown that troglitazone (Tro), a member of the thiazolidinedione class of oral antidiabetic drugs, prevents VSMC proliferation and migration in the vitro and substantially attenuates neointimal hyperplasia in the injured rat aorta [3]. The mechanism of this effect appears to be through the inhibition of the MAP kinase pathway which is activated in response to, and integrates signals from both, the basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) tyrosine kinase receptors. More specifically, we determined that Tro inhibited the nuclear actions of MAP kinase at a point downstream of MAP kinase activation by MAP kinase kinase (MEK) [3].

Angiotensin II is a peptide growth factor, generated in the vascular wall [4] which is involved in regulation of VSMC proliferation and migration, and, when elevated in the circulation, significantly enhances neointimal hyperplasia [5,6]. In VSMC, most of these effects of AII are mediated via the AT-1 receptor, which is a G-protein coupled receptor [7]. Activation of the AT-1 receptor leads to increased Ca^{2+} mobilization, activation of protein kinase C, increased inositol triphosphate formation, and activation of the RAS-RAF-MEK-MAP kinase pathway [8]. We have recently reported that AII and PDGF-induced VSMC migration requires MAP kinase activation [9]. In the present study, we investigated the effect of troglitazone on AII mediated MAP kinase activation, migration and DNA synthesis in cultured rat aortic smooth muscle cells.

2. Materials and methods

2.1. Cell culture

Rat aortic smooth muscle cells were prepared from thoracic aorta of 2–3-month-old Sprague-Dawley rats using the explant technique [10]. The cells were cultured in DMEM containing 10% FBS, 150 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 mmol/l glutamine (Sigma, St. Louis). The purity and identity of the smooth muscle cell cultures were verified by using a monoclonal antibody against smooth muscle α-actin (Sigma). For all experiments, early passaged (4 or less) rat VSMCs were grown to 60–70% confluency and made quiescent by serum starvation for at least 16 h, when MAP kinase activity was assayed. For all data shown, each individual experiment represented in the *n* value was performed using an independent preparation of VSMCs.

2.2. MAP in-gel kinase assay

MAP kinase activity was measured by the in gel kinase assay as described [3]. Troglitazone was given 30 min before inducing MAP kinase activation with AII for 10 min. For the data shown, each individual experiment represented in the *n* value was performed using an independent preparation of VSMCs. Densitometric analysis was performed using NIH Image 1.60 software on a Macintosh PC.

2.3. VSMC DNA synthesis

Incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), was measured to determine the effect of AII on DNA synthesis as described previously [11]. VSMC were plated out at 3.0×10^4 cells on 24-well plates (Falcon primaria) in DMEM with 10% FBS for 48 h. After serum starvation for 36 h in DMEM/0.2% FBS, incubation with AII (1 μM, Bachem, Torrance, CA), irbesartan (100 μM), and Tro (1, 10 or 20 μM) was performed for the next 20 h. Troglitazone was a generous gift from Parke-Davis, Ann Arbor. The AT-1 receptor blocker irbesartan was kindly provided by Bristol Myers Squibb (Princeton, NJ). Then 15 μM BrdU (Sigma) was added, and the incubation was continued for another 4 h. After several washes with PBS, cells were fixed, permeabilized with 1 N HCl and stained with an anti-BrdU monoclonal antibody (Zymed, San Francisco, CA) using the ABC method. A fast red substrate system (Dako Corp., Capinteria, CA) was used as a chromagen; counterstaining was performed with hematoxylin. Cell nuclei with BrdU incorporation appeared red and were counted in 4–6 different high power fields/well and related to total cell number/high power field.

2.4. Migration

Chemotaxis experiments were performed as described in [3]. VSMC migration was examined in transwell cell culture chambers (Costar,

*Corresponding author. USC Medical Center, 1333 San Pablo, Bishop Building Room B-11, Los Angeles, CA 90033, USA. Fax: 1 (213) 342-3528. E-mail: graf@hsc.usc.edu

PII S0014-5793(96)01371-3

Cambridge, MA) using a gelatin-coated polycarbonate membrane with 8 μm pores. The number of VSMCs per 320× high power field (HPF) that migrated to the lower surface of the filters was determined microscopically. 4–6 randomly chosen HPFs (magnification ×320) were counted per filter. Experiments were performed in duplicate or triplicate, and were repeated at least three times.

2.5. Statistical analysis

Analysis of variance, paired or unpaired *t*-test, was performed for statistical analysis, as appropriate. *P* values less than 0.05% were considered to be statistically significant. Data are expressed as mean ± S.E.M.

3. Results

3.1. Troglitazone inhibits MAP kinase activation by AII

Rat aortic VSMCs were made quiescent by 16 h treatment in serum-free culture medium. Quiescent VSMCs exhibited low MAP kinase activity, as demonstrated by the faint signals produced by the phosphorylation of myelin basic protein by p44 (ERK1) and p42 (ERK2) in the in-gel kinase assay (Fig. 1A, lane 1). Strong induction of ERK1 and ERK2 (≈5-fold) activity was observed after 10 min treatment with AII (Fig. 1a,b). AII-induced MAP kinase activation was inhibited in a concentration-dependent manner by Tro (Fig. 1a). At 20 μM Tro inhibited the AII-induced activation of ERK1 and ERK2 by 67.9% in VSMCs ($n=4$, $p<0.01$ vs. AII alone).

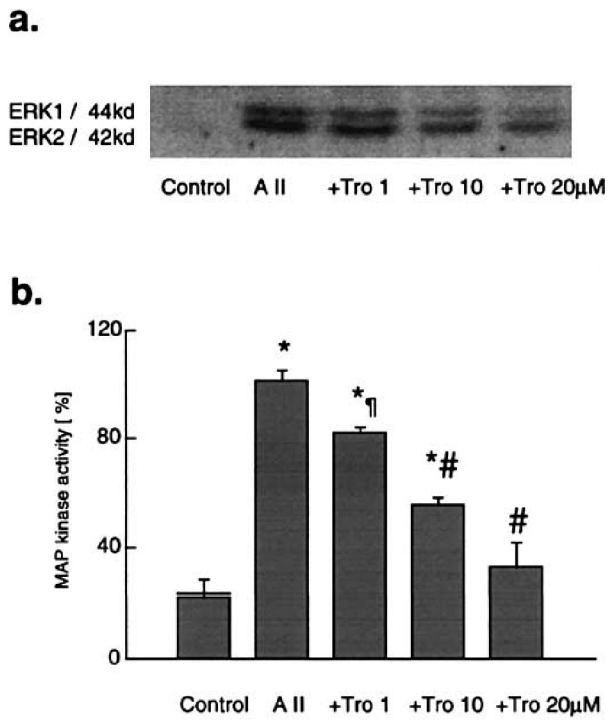

Fig. 1. AII (1 μM) induces phosphorylation of ERK1 and ERK2 in quiescent rat VSMC, which is inhibited by increasing concentrations of Tro (1–20 μM). Cells were starved for at least 16 h in serum-free DMEM and stimulated with AII for 10 min. MAP kinase activity was measured by in-gel kinase assay, which demonstrates phosphorylation at 42 and 44 kDa size, bands specific for ERK1 and ERK2. (a) Representative autoradiography of MAP kinase activity induced by AII in the absence and presence of Tro (1–20 μM). (b) Results of the densitometric analysis of 4 different experiments. MAP kinase activity induced by AII is set as 100% (*$p<0.01$ vs. control, ¶$p<0.05$ vs. AII alone, $p<0.01$ vs AII alone; $n=4$, mean ± S.E.M.).

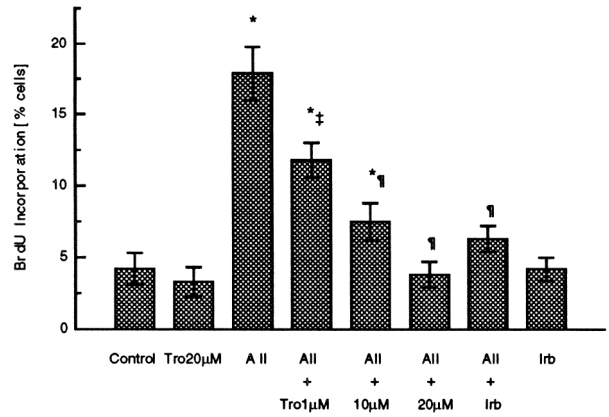

Fig. 2. DNA synthesis was measured by incorporation of bromodeoxyuridine (BrdU) and visual quantification of 4–6 high power fields (HPF)/well. Experiments were performed in duplicates. Quiescent VSMCs were stimulated with AII in the presence or absence of troglitazone (Tro 1–20 μM) or the AT-1 receptor blocker irbesartan (Irb 100 μM) for 20 h before addition of BrdU. AII induced DNA synthesis was inhibited by Tro in a concentration-dependent fashion and was also prevented by losartan. Tro (20 μM) or Irb alone did not affect DNA synthesis compared to unstimulated cells (*$p<0.01$ vs. control, ‡$p<0.04$ vs AII, ¶$p<0.01$ vs. AII alone, $n=12$).

3.2. Troglitazone inhibits AII mediated DNA synthesis in rat VSMC

DNA synthesis was determined by incorporation of bromodeoxyuridine (BrdU). AII (1 μM) induced a 4-fold increase in DNA synthesis (from 4.4 ± 1.1 control to 18.2 ± 2.2% positive cells, $p<0.01$, $n=8$), which was completely inhibited by the AT-1 receptor blocker irbesartan (100 μM, $p<0.01$ vs. AII alone). Tro (1–20 μM) demonstrated a concentration-dependent inhibition of AII-induced DNA synthesis (Fig. 2). The maximal concentration of Tro (20 μM) completely inhibited the effect of AII on DNA synthesis ($p<0.01$ vs. AII alone).

3.3. Troglitazone inhibits AII mediated migration in rat VSMC

VSMC migration induced by AII was increased more than 3-fold ($n=8$, $p<0.01$) as compared to unstimulated cells. Pretreatment with various concentrations of Tro resulted in a significant inhibition at 1 μM Tro (79%, $p<0.01$) of the AII-induced effect with complete inhibition observed at 10 and 20 μM Tro ($p<0.01$, Fig. 3). The AT-1 receptor blocker irbesartan significantly inhibited the AII-induced migration ($p<0.01$), whereas the AT-2 receptor blocker PD 123319 (100 μM, Parke-Davis, Ann Arbor, MI) did not interfere with the migration towards AII (Fig. 3).

4. Discussion

We have recently demonstrated that Tro attenuates neointimal hyperplasia in the balloon injured rat aorta by inhibiting PDGF and bFGF actions on VSMC [3]. In in vitro experiments Tro inhibited PDGF-mediated migration and bFGF-induced VSMC proliferation. These effects may have resulted from Tro's ability to block the MAP kinase pathway at a point downstream of MAP kinase activation by MEK resulting in inhibition of two nuclear endpoints: (1) induction of c-fos and (2) transactivation of serum response elements by the MAP kinase regulated transcription factor ELK1, which also activates c-fos [3]. In the present study, Tro also prevented

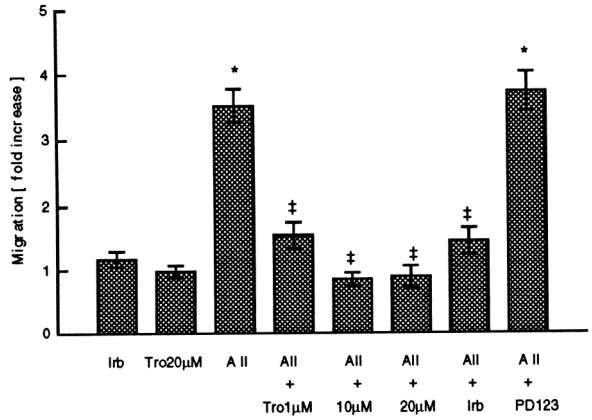

Fig. 3. Migration of rat aortic VSMC towards AII was inhibited by troglitazone (Tro) in a concentration-dependent manner. AII (1 μM) induced a significant increase in migrated cells, which was inhibited by Tro and by Irb (100 μM). Treatment with Tro or Irb alone in unstimulated cells did not affect baseline values. The AT-2 receptor antagonist PD123319 (PD123, 100 μM) did not inhibit the AII-induced migration ($n = 8$–16, mean ± S.E.M., $^{*}p < 0.01$ vs. baseline, $^{\ddagger}p < 0.01$ vs AII alone).

AII-induced migration and DNA synthesis. In contrast to its action on the PDGF and bFGF signalling pathway, the AII-induced signaling was inhibited at a point upstream of MAP kinase activation, since we consistently observed a concentration-dependent inhibition of AII-mediated MAP kinase activation by Tro. This result is somewhat surprising, since it suggests that Tro targets two different intracellular sites, which both ultimately modulate the RAS-RAF-MEK-MAP kinase pathway. We and others have shown that mitogenic signalling induced by growth factors like AII, PDGF, bFGF and insulin involves this pathway and is inhibited by a synthetic inhibitor of MEK PD 98059 and antisense oligodinucleotides against MAP-kinase [3,12–14]. Both agents inhibited AII- and PDGF-induced migration [9] (Graf and Law, unpublished data). Thus, the MAP kinase pathway is important for both VSMC growth and chemotaxis.

The present study demonstrates that Tro inhibits AII signalling upstream of MAP kinase activation in VSMC, which mediates the inhibition of AII-induced VSMC migration and proliferation. This potent effect of troglitazone on AII-induced signalling might contribute to the observed in vivo effects of troglitazone to prevent neointimal hyperplasia in the injured vessel and to lower blood pressure in some animal models of hypertension. Tro and related thiazolidinediones have been shown to attenuate hypertension an a variety of animal models [15,16]. As insulin sensitizers these agents could lower blood pressure indirectly by improving insulin resistance [17]. Thiazolidinediones have also been shown to have direct effects on the vasculature [18] and VSMCs [3,19]. It is possible, therefore, that troglitazone and other thiazolidinediones may regulate blood pressure more directly by interfering with the action of growth factors, such as AII, involved in the development of hypertension. Taken together, troglitazone has now been shown to inhibit the signal transduction through MAP kinase that is initiated by either tyrosine kinase growth factor receptors [3] or a G-protein coupled, seven transmembrane domain receptor. Activation of MAP kinase, in turn, is required for both VSMC growth and migration. These results underscore the potential clinical efficacy of troglitazone for prevention of both human restenosis and atherosclerosis. Troglitazone has already proved beneficial in improving insulin resistance and reducing hyperinsulinemia in initial clinical trials involving obese subjects and patients with non-insulin dependent diabetes mellitus [17,20]. Future investigation will likely reveal whether troglitazone is also beneficial for vascular diseases.

Acknowledgements: We thank Denise Edwards and Janie Teran for secretarial contributions. This study was supported by a Specialized Center for Research Grant in Hypertension from the National Heart Lung and Blood Institute (1P50-HL4404) to W.A.H. and a Research Grant from the American Diabetes Association (ADA 96) to R.E.L. We thank Randy Whitcomb, Dean Lockwood and Thomas Valiquett for their generosity in providing troglitazone. This work was also supported in part by a grant from Parke-Davis (Ann Arbor, MI).

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