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Short communication

Carbocyclic thromboxane A_2 enhances the angiotensin II-induced DNA synthesis in smooth muscle cells

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Abstract

The present study describes the influence of carbocyclic thromboxane A_2 on the proliferative effects of angiotensin II on vascular smooth muscle cells. Angiotensin II (10^{-7} M) and carbocyclic thromboxane A_2 (10^{-6} M) per se caused an increase in [^{3}H]thymidine incorporation and cell number. The exposure of cells to both agonists resulted in a 2.5-fold elevation of the angiotensin II dependent effect on DNA synthesis and a 1.6-fold increase in cell number. 2-Ethoxy-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid (CV-11974), the active metabolite of the specific non-peptide angiotensin AT_1 receptor antagonist (±)-1-(cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate (TCV-116, Candesartan) suppressed the effect of angiotensin II on cell growth as well as reduced the synergistic effect of carbocyclic thromboxane A_2. Simultaneous cell stimulation with carbocyclic thromboxane A_2 and angiotensin II for 30 min resulted in a 26 ± 9% elevation of the angiotensin II-induced increase of c-fos mRNA (100%).

Keywords: Angiotensin II; Thromboxane A_2; c-fos; Smooth muscle cell

1. Introduction

Abnormal vascular smooth muscle cell growth may participate in the development of hypertension and atherosclerosis (Schwartz and Reidy, 1987; Schwartz et al., 1986). Thromboxane A_2 is a lipid metabolite of arachidonic acid which is synthesized from activated platelets and vessel wall tissues. It has been established that thromboxane A_2 is a potent vasoconstrictor for vascular smooth muscle cells acting through a pharmacologically defined binding site, the thromboxane A_2/prostaglandin H_2 receptor (Morinelli et al., 1989; Fitzgerald et al., 1986). Several thromboxane analogues have been synthesised which are stable in aqueous solutions. It is well known that following binding to G_q protein-coupled AT_1 receptor, angiotensin II activates the phosphoinositide signalling system leading to expression of transcription factors, such as *c-fos* (Taubman et al., 1989). *C-fos* belongs to a class of immediate-early genes expressed upon growth and/or differentiation signals in a large variety of cells and species (Verma and Sassone-Corsi, 1987) and thus it is implicated in the regulation of vascular smooth muscle cell growth. We investigated the influence of carbocyclic thromboxane A_2 (Smith et al., 1981) on the growth promoting effects of angiotensin II on vascular smooth muscle cells. Furthermore, the influence of CV-11974, the active metabolite of the specific non-peptide angiotensin AT_1 receptor antagonist TCV-116 (Candesartan), on the growth promoting effects of angiotensin II in the presence and absence of carbocyclic A_2 was investigated. In order to elucidate possible intracellular pathways for the transmission of growth signals by thromboxane A_2 in combination with angiotensin II, the effect of carbocyclic thromboxane A_2 on the angiotensin II-induced expression of c-fos was investigated.

2. Materials and methods

2.1. Materials

Carbocyclic thromboxane A_2 was obtained from Calbiochem (Bad Soden/TS, Germany). The 0.77 kb cDNA

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probe for β-actin was obtained from Dianova/Oncor Science (Hamburg, Germany). Angiotensin II was obtained from Sigma Chemical (Deisenhofen, Germany). 2-Ethoxy-1-[[2'-(1*H*-tetrazol-5-yl)bipheny!-4-yi]methyl]-1*H*-benzimidazole-7-carboxylic acid (CV-11974) was obtained from Takeda Chemical Industries (Osaka, Japan; via Takeda Euro R&D Centre, Frankfurt/Main, Germany).

2.2. Methods

Rat aortic smooth muscle cells were isolated using thoracic aortas from Wistar-Kyoto rats (6–8 weeks old, Charles River Wiga, Sulzfeld, Germany) by enzymatic dispersion according to the method of Chamley (Chamley et al., 1979) as previously described (Sachinidis et al., 1995).

The expression of c-fos mRNA was studied after pre-incubation of the cells for 24 h in serum-free medium in 75 cm^2 culture flasks. Then quiescent cells were stimulated with carbocyclic thromboxane A_2 or angiotensin II. Total RNA was extracted from vascular smooth muscle cells by the guanidinium isothiocyanate/CsCl procedure (Chirgwin et al., 1979). 10 μg of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel. The expression of c-fos mRNA was analysed by Northern blot analysis as previously described (Sachinidis et al., 1995). Blots were standardised using a 0.77 kb cDNA probe for β-actin.

The effect of angiotensin II and carbocyclic thromboxane A_2 on DNA synthesis was measured as described previously (Sachinidis et al., 1995). Cells were seeded in 24-well culture plates and cultivated in culture medium until confluent. Then the medium was replaced by serum-free medium consisting of a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-10 medium (1 : 1). Following another 24 h cultivation in serum-free medium stimulators were added to the cells. Then cultures were exposed to the stimulating agents in the presence and absence of CV-11974 for 20 h before 3 μCi/ml [^{3}H]thymidine were added to the serum-free medium. 4 h later experiments were terminated.

For cell counting, cells were seeded in 24-well culture plates (5×10^4 cells/well, well diameter 12 mm) and cultured until cells reached a confluence of approximately 70%. The medium was then replaced by serum-free medium consisting of DMEM and Ham's F-10 (1 : 1, v/v) and cells were stimulated with carbocyclic thromboxane A_2 and angiotensin II in the presence and absence of CV-11974. After 24 h, cells were trypsinized and resuspended in DMEM plus trypan blue. Cell counting was performed with a Neubauer cell box by light microscopy.

2.3. Statistical analysis

Statistical analysis of the data was performed using the Mann-Whitney *U*-test.

3. Results

Data from three individual experiments each performed with triplicate wells were normalised by calculating the means ± S.E. of the individual experiment and expressed as % increase above the basal value of the unstimulated cells (= 100%). As shown in Fig. 1, the exposure of cells to both carbocyclic thromboxane A_2 and angiotensin II caused a 2.5-fold elevation of the angiotensin II dependent effect on [^{3}H]thymidine incorporation. CV-11974 (10^{-9} M) suppressed the effect of angiotensin II down to basal values as well as reduced the synergistic effect of carbocyclic thromboxane A_2 down to carbocyclic thromboxane A_2 values. CV-11974 at a concentration of 10^{-9} M per se had no effect on basal and on the carbocyclic A_2-induced DNA synthesis. Angiotensin II (10^{-7} M) and carbocyclic thromboxane A_2 (10^{-6} M) per se induced a 57 ± 9 and 29 ± 5% increase in cell number over the basal value (basal value = 100%), respectively. Carbocyclic thromboxane A_2 in combination with angiotensin II enhanced the angiotensin II dependent increase in cell number to 94 ± 11% (mean ± S.E., $P < 0.05$ for angiotensin II effect vs. angiotensin II + carbocyclic thromboxane A_2 effect). Again, CV-11974 suppressed the effect of angiotensin II down to basal values as well as reduced the synergistic effect of carbocyclic thromboxane A_2 down to carbocyclic thromboxane A_2 values.

Maximal induction of c-fos mRNA in vascular smooth muscle cells by angiotensin II (Flesch et al., 1995) and

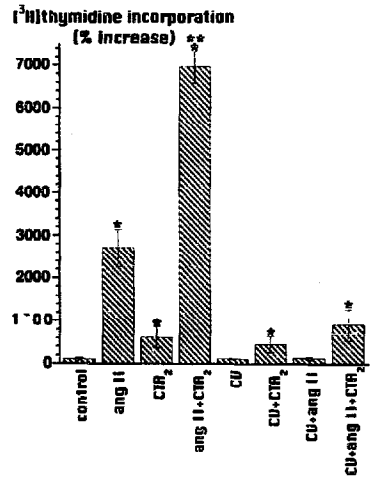

Fig. 1. Effect of angiotensin II (ang II) and carbocyclic thromboxane A_2 (CTA_2) on DNA synthesis in vascular smooth muscle cells. Confluent cells (24-well plates) were precultured for 24 h in the serum-free medium. Carbocyclic A_2 (10^{-6} M) was added 5 min before stimulation with angiotensin II (10^{-7} M). Following another 20 h incubation, cells were exposed to 3 μCi/ml [^{3}H]thymidine. 4 h later the reaction was terminated and cell protein and [^{3}H]thymidine incorporation into cell DNA were quantified. Data from three individual experiments each performed with triplicate wells were normalised by calculating the means ± S.E. of the individual experiment and expressed as % increase above the basal value of the unstimulated cells (= 100%) (* $P < 0.05$ for angiotensin II or carbocyclic thromboxane A_2 vs. control). ** $P < 0.05$ for angiotensin II + carbocyclic thromboxane A_2 vs. the angiotensin II effect.

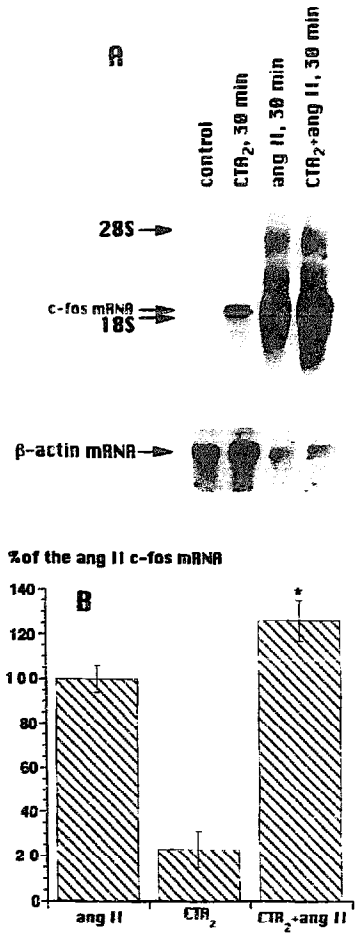

Fig. 2. Effects of carbocyclic thromboxane A_2 (CTA_2) on the angiotensin II (ang II)-induced expression of c-fos in vascular smooth muscle cells. A: Cells were stimulated with angiotensin II (10^{-7} M) in the presence or absence of carbocyclic thromboxane A_2 (10^{-6} M) for 30 min. 10 μg of total RNA were separated on a formaldehyde-agarose gel, blotted on Hybond N^+ membranes and probed with a ^{32}P-labeled 1.0 kb v-fos cDNA that hybridized to the 2.2 kb mRNA of c-fos. The same membranes were rehybridized with a 0.77 kb cDNA probe for β-actin. Arrows indicate the 28 S (4.6 kb) and 18 S (1.8 kb) ribosomal RNA. B: Densitometric analysis from three separate experiments (mean ± S.D., * $P < 0.05$ for the carbocyclic A_2 + angiotensin II effect vs. the angiotensin II effect.

carbocyclic thromboxane A_2 (Sachinidis et al., 1995) occurs at 30 min. Fig. 2A shows a representative experiment showing the effect of angiotensin II and carbocyclic thromboxane A_2 per se or in combination on the 2.2 kb c-fos mRNA expression at 30 min. Data from the densitometric analysis from three independent experiments are summarised in Fig. 2B. Blots were standardised using a 0.77 kb cDNA probe for β-actin. The carbocyclic thromboxane A_2-induced amount of the c-fos mRNA was estimated to be $23 \pm 8\%$ of the angiotensin II-induced c-fos mRNA expression in vascular smooth muscle cells (= 100%). Simultaneous cell stimulation with carbocyclic thromboxane A_2 and angiotensin II for 30 min caused a $26 \pm 9\%$ elevation of the angiotensin II-induced increase of c-fos mRNA.

The proliferation of vascular smooth muscle cells in response to various growth factors differs considerably and is dependent on several factors, such as isolation procedure, strain and age of the animal, cultivating conditions, cell seeding density and the culture time (Campbell and Campbell, 1993).

Thus, we investigated the effect of carbocyclic thromboxane A_2 and angiotensin II or their combination on the [^{3}H]thymidine incorporation in cells derived from another cell line as the cells used in previous experiments. Angiotensin II (10^{-7} M) and carbocyclic thromboxane A_2 (10^{-6} M) per se induced an increase in [^{3}H]thymidine incorporation in these cells from 421 ± 44 to 945 ± 45 and 565 ± 67 cpm/μg protein (mean ± S.E. of triplicate wells), respectively. Again, the exposure of cells to both enhanced the angiotensin II-induced [^{3}H]thymidine incorporation from 945 ± 45 to 2159 ± 68 cpm/μg protein. Carbocyclic thromboxane A_2 caused a 2.3-fold elevation of the angiotensin II dependent effect on [^{3}H]thymidine incorporation in these cells.

4. Discussion

The present study shows that the thromboxane A_2/prostaglandin H_2 mimetic carbocyclic thromboxane A_2 enhances the growth promoting effects of angiotensin II in vascular smooth muscle cells as well as the angiotensin II-induced expression of c-fos mRNA. Although carbocyclic thromboxane A_2 enhanced the angiotensin II dependent increase in [^{3}H]thymidine incorporation in a synergistic fashion, its effects on the angiotensin-induced elevation in cell number and c-fos mRNA induction seem to be rather additive than synergistic. Since the relative change in DNA synthesis is greater than the relative increase in cell number, both agonists and/or their combination may induce polyploidy of the vascular smooth muscle cells.

CV-11974, the active metabolite of the specific nonpeptide angiotensin AT_1 receptor antagonist TCV-116 at a concentration of 1 nM completely blocked the growth promoting effects of angiotensin II per se or in combination with carbocyclic thromboxane A_2. CV-11974 (1 nM) inhibits the angiotensin II-induced increase in cytosolic Ca^{2+} concentration and c-fos mRNA (Flesch et al., 1995).

Recently, we demonstrated that the thromboxane A_2/prostaglandin H_2 mimetic carbocyclic thromboxane A_2 stimulates the expression of immediate early growth response genes and promotes the proliferation of vascular smooth muscle cells (Sachinidis et al., 1995). The present study shows that carbocyclic thromboxane A_2 also significantly enhanced the effect of angiotensin II on the c-fos mRNA expression. This finding may explain the synergistic or additive effects of carbocyclic thromboxane A_2 on the angiotensin II effect on vascular smooth muscle cell growth.

Thromboxane A_2 is mainly synthesized from activated

platelets. It may be assumed that after an endothelial injury, activated platelets accumulate at the vessel lesion and release thromboxane A_2 to the subendothelial layer of the vessel wall resulting in an increase of the local concentration of thromboxane A_2. The renin-angiotensin system represents a cascade of biochemical events leading to the generation of angiotensin II. Local generation and autocrine or paracrine actions of angiotensin II have been shown in the vascular system and the heart (Campbell, 1986). Thus, it is conceivable that thromboxane A_2 may significantly enhance the growth promoting effects of angiotensin II on vascular smooth muscle cells and therefore might contribute to the pathogenesis of cardiovascular diseases. Furthermore, CV-11974 may be beneficial in the treatment of cardiovascular diseases as CV-11974 not only prevents the direct proliferative effect of angiotensin II on vascular smooth muscle cells but also abolishes the synergistic effect of thromboxane A_2.

Acknowledgements

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