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Angiotensin II induces formation of the early growth response gene-1 protein in rat vascular smooth muscle cells

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The effect of angiotensin II (Ang II) on the early growth response gene-1 (Egr-1) mRNA, on the Egr-1 protein and on the phosphoinositide PI turnover signalling system was investigated in the presence and absence of EXP3174, a potent non-peptide Ang II receptor antagonist. Ang II induced an accumulation of 3.4 kb Egr-1 mRNA and the 80 kDa Egr-1 protein, with a maximum at 30 min and 60 min, respectively. EXP3174 blocked the Ang II-induced increase of inositolphosphates, Egr-1 mRNA and the Egr-1 protein, suggesting the involvement of the PI signalling system by the expression of the Egr-1 gene.

Early growth response gene-1 (Egr-1); Angiotensin II; Vascular smooth muscle cell

1. INTRODUCTION

Recently, the early growth response gene-1 (Egr-1) has been identified as a transcription factor belonging to a class of immediate-early genes like *c-fos* expressed upon growth and/or differentiation signals in a large variety of cells and species [1–3]. Using a rabbit antiserum to Egr-1 product the Egr-1 protein has been identified in serum-stimulated fibroblasts as a zinc finger protein with a molecular weight of 80 kDa [4]. It is well established that the *c-fos* protein is a transcriptional factor, belong to the zinc finger protein type [5]. C-fos protein is often coregulated with the Egr-1 protein [2–4]. It is well known that angiotensin II (Ang II) induces *c-fos* expression and vascular smooth muscle cell (VSMC) growth [6]. In the present study, the effect of Ang II on the expression of the Egr-1 mRNA, Egr-1 protein and on the PI-turnover signalling system in rat VSMC was investigated.

2. MATERIALS AND METHODS

2.1. *Materials*

Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Eggenstein, Germany). Angiotensin II (Ang II) was obtained from Sigma Chemical (Deisenhofen, Germany). A 2.1 kb fragment (OC68 insert) of Egr-1 including three zinc-finger domains was used as DNA probe [3]. The rabbit antiserum (R5232-T) to the Egr-1 gene product was obtained as previously described [4]. A 0.77 kb cDNA for β-actin (Dianova/Oncor Science, Hamburg, Germany) was used as DNA probes for β-actin. Hybond N$^+$ membranes, [^{32}P]deoxycytidine triphosphate ([^{32}P]CTP), and ECL Western blotting detection system were obtained from Amersham, Little Chalfont, England Kodak X-Omat, 8 × 10 inch, films were obtained from Kodak, Rochester, USA. Molecular weight standard RNA was obtained from Boehringer Mannheim, Germany PVDF membranes were obtained from Millipore, Bedford, USA 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid was a gift from MSD Research Laboratories, West Point, USA.

2.2. *Cultured of vascular smooth muscle cells*

VSMCs were isolated from rat aorta (strain, female Wistar-Kyoto, 6–10 weeks old, Charles River Wiga GmbH, Sulzfeld, Germany) by the media explantat method and cultured over several passages according to Ross [7].

2.3. *RNA extraction and analysis*

The expression of Egr-1 mRNA was studied after preincubation of the cells in 75 cm^2 culture flasks for 24 h in serum-free quiescent medium, consisting of a mixture of DMEM and Ham's F-10 medium (1:1) The quiescent cells were stimulated with Ang II for different time periods. Total RNA was extracted from VSMC by the guanidinium isothiocyanate/CsCl procedure [8]. 10 μg of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel Northern blotting was performed as previously described [9]. A 2.1 kb fragment (OC68 insert) of Egr-1 including three zinc-finger domains was used as probe. The DNA probes were labelled with [^{32}P]CTP by random oligonucleotide priming. Blots were standardised using a 0.77 kb cDNA probe for β-actin. Densitometric analysis was performed with Hoefer GS-300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA)

2.4 *Analysis of the Egr-1 protein by the enhanced chemiluminescence Western analysis*

VSMCs were seeded in culture dishes (diameter: 33 mm) and culti-

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Abbreviations: VSMCs, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagles medium; PBS, Dulbecco's phosphate-buffered saline, Ang II, Angiotensin II; Egr-1, early growth response gene-1.

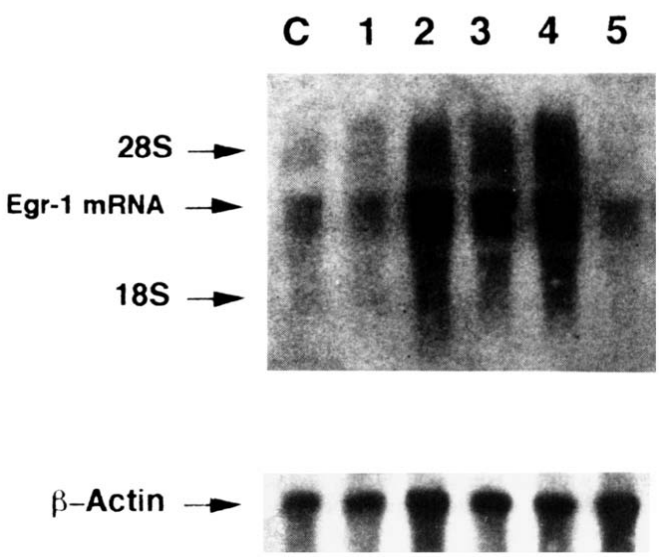

Fig. 1. Effect of angiotensin II (Ang II) on the Egr-1 mRNA induction in vascular smooth muscle cells (VSMC). (Upper) Quiescent cells were stimulated with 10^{-7} Ang II for the times showed. Northern blotting was performed with a ^{32}P-labelled 2.1 kb fragment of Egr-1. C, control; 1, Ang II, 5 min; 2, Ang II, 30 min; 3, Ang II, 45 min; 4, Ang II, 60 min; 5, Ang II, 120 min. (Lower) The same blot previously hybridized with 2.1 kb fragment of Egr-1 was rehybridized with 0.77 kb cDNA probe for β-actin.

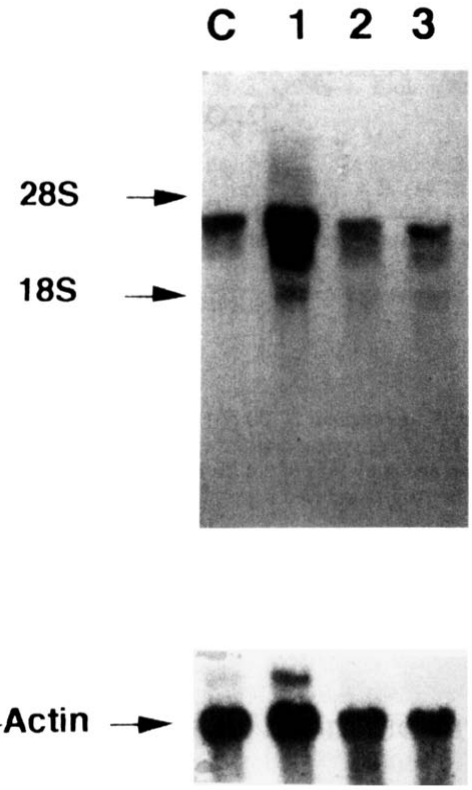

Fig. 2. Effect of angiotensin II (Ang II) on the Egr-1 mRNA induction in the presence of EXP3174. (Upper) Quiescent VSMC were stimulated for 30 min with 10^{-7} M Ang II in the presence (30 min) and absence of EXP3174. Northern blotting analysis was performed with a ^{32}P-labelled 2.1 kb fragment of Egr-1. C, control; 1, 10^{-7} M Ang II; 2, 10^{-6} M EXP3174; 3, 10^{-6} M EXP3174 + 10^{-7} M Ang. (Lower) The same blot previously hybridized with 2.1 kb fragment of Egr-1 was rehybridized with 0.77 kb cDNA probe for β-actin.

vated in culture medium until confluent. Then the medium was replaced by serum-free (quiescent) medium consisting of a mixture of DMEM and Ham's medium (1:1). Following another 24 h cultivation in quiescent medium, cells were stimulated for different time periods. Cells were washed with PBS twice and then incubated with PBS supplemented with 1 mM PMSF, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin and harvested by scraping. Cells were spined down (1500 rpm) at 4°C and then supernatant was discarded. Cells were resuspended in 100 μl 0.25 M Tris, pH 7.8, containing 1 mM PMSF, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin and were lysed by two freeze–thaw cycles (frozen in dry ice/ethanol and warmed briefly at 37°C). Then debris were discarded and the supernatant was kept as cell lysate. Aliquots were used for protein determinations using the Bio-Rad protein assay according to the method of Bradford [9]. 30 μg of protein were separated in a 7.5% polyacrylamide-SDS gel. Proteins were transferred to a PVDF membrane overnight by 100 mA with a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. The protein transfer was checked using Ponceau S. Then membrane was washed using PBS containing 0.1% Tween-20 and 1% non-fat dry milk (standard incubation buffer). Non-specific binding blockade was performed in PBS containing 0.1% Tween-20 and 5% non-fat dry milk for 2 h. Following this the mixture was quickly rinsed with standard incubation buffer, and the membrane was incubated for 1 h in a standard solution containing anti-Egr-1 antisera R5232-T (1:4,000 dilution). After that membrane was incubated for 1 h in standard buffer containing HRP-labelled donkey anti-rabbit Ig (1:5,000 dilution). Membrane was washed 3 times for 5 min with the standard incubation buffer. The detection of the Egr-1 protein was performed with the ECL chemiluminescence Western blotting detection system from Amersham.

2.5. *Determination of inositolphosphates*

The determination of inositolphosphates, inositol 1-phosphate (InsP1), inositol 1,4-bisphosphate (InsP2), and inositol 1,4,5-trisphosphate (InsP3), was performed as previously described [10]. Cells were seeded in petri dishes (60 mm diameter) and grown in the presence of myo-[2-^{3}H]inositol (4 μCi/ml) for 3 days and were treated as previously described. Cells were stimulated with 10^{-7} mol/l Ang II for 3 min in the presence and absence of EXP3174 (30 min) 10^{-6} mol/l Ang II for 3 min. The reaction was terminated and radiolabelled inositolphosphates were analysed by standardised anion-exchange high pressure liquid chromatography as previously described [10].

3. RESULTS

As shown in Fig. 1, Ang II induced a rapid accumulation of 3.4 kb Egr-1 mRNA with maximum between 30 min and 1 h. The Egr-1 mRNA declined to basal levels at 2 h. In addition, the Ang-induced maximal Egr-1 mRNA induction is totally blocked by the specific non-peptide Ang II receptor antagonist EXP3174 (Fig. 2). As illustrated in Fig. 3, stimulation of VSMCs with FCS induced an increase of the 80 kDa Egr-1 protein, in comparison to unstimulated cells. In addition, stimulation of the cells with 10^{-7} M Ang II induced an accumulation of the 80 kDa Egr-1 protein, first detectable at 30 min. Maximum of the Egr-1 protein formation occurred within 60 min, and declined by 3 h to control Egr-1 protein levels. The maximal Ang II-induced Egr-1 protein was totally blocked by pretreatment of the cells with EXP3174, that is a non-peptide Ang II-receptor

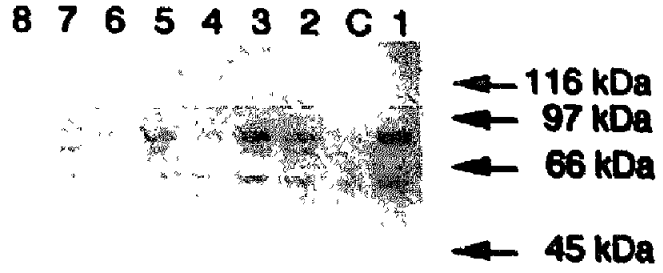

Fig. 3. Immunoblot analysis of the time course of Egr-1 protein induction by angiotensin II (Ang II). VSMCs were incubated for 24 h in serum- free quiescent medium. Cells were stimulated with 10^{-7} M Ang II for different time periods in the presence (30 min) and absence of EXP3174. Cells were harvested and lysed as described in section 2. 30 μg of protein were separated by a 7.5% polyacrylamide-SDS gel and Western blotting analysis was performed with the R5232-T antiserum (1:4,000 dilution). C, control; 1, 10% FCS, 1 h; 2, Ang II, 30 min, 3, Ang II, 1 h; 4, EXP3174, 30 min + Ang II, 1 h; 5, Ang II, 1.5 h; 6, Ang II, 2 h, 7, Ang II, 3 h, 8, Ang II, 4 h.

antagonist. These results show that Ang II induced the formation of the Egr-1 protein in VSMC. As shown in Table I, Ang II (10^{-7} mol/l) induced 70%, 1,200% and 170% increase in InsP1, InsP2, and InsP3 above basal values. After pretreatment of the cells with 10^{-6} M EXP3174 the Ang II-induced elevation of inositolphosphates was completely blocked.

4. DISCUSSION

Using Northern blotting and Western immunoblotting, the Egr-1 mRNA and the Egr-1 protein in rat VSMC has been identified. We found that the Ang II-induced Egr-1 protein (80 kDa) starts to accumulate within 30 min, reaches a peak level of expression between 1 and 2 h, and reached basal levels within 3 h. The time course of protein expression is similar to that seen for the Egr-1 mRNA levels, except that the maximum of mRNA levels occurred between 30 and 60 min and reached basal levels at 2 h. The present kinetic data for the Ang II-induced Egr-1 mRNA and Egr-1 protein are very similar to those obtained by Cao et al. in mouse serum-stimulated fibroblasts [4].

It is well established that Ang II stimulates the phosphoinositide (PI)-turnover signalling system following binding to its specific cell surface receptor leading to an elevation in $[Ca^{2+}]_i$ and most probably to the expression of transcription factors such as c-fos [6,12].

EXP3174, a specific non-peptide Ang II receptor antagonist [13], completely abolished the Ang II-induced intracellular increase in InsP1, InsP2 and InsP3. Consequently, complete blockade of the Ang II-induced Egr-1 mRNA and Egr-1 protein was observed. As previously described, Egr-1 is an early growth response gene that can be induced by ligand–receptor interactions of mitogenic and/or hypertrophic agents in several mammalian cell types. In this context it has been shown that Egr-1 was expressed in fibroblasts (stimulated by epidermal growth factor and serum), kidney epithelial (stimulated by adenosine diphosphate) and mesangial cells (stimulated by vasopressin), hepatocytes (stimulated by insulin) and cardiac myocytes (stimulated by α-agonists) [2–5].

The present study shows that Egr-1 mRNA also can be induced by Ang II in rat VSMC. In addition, the Egr-1 protein with a molecular weight of 80 kDa was identified in these cells. EXP3174, a potent non-peptide Ang II receptor antagonist totally blocked the Ang II-induced stimulation of the PI signalling system, the Egr-1 mRNA and protein induction. From these results, it may be concluded that the Ang II-induced Egr-1 expression in VSMC is mediated by the PI signalling system.

Table I

Effect of EXP3174 on the angiotensin II-induced inositolphosphates formation in vascular smooth muscle cells

	InsP1 (cpm well)	InsP2 (cpm well)	InsP3 (cpm well)
Control	1,568 ± 156	63 ± 6	42 ± 2
EXP3174	1,469 ± 158	58 ± 3	43 ± 3
Ang II	2,689 ± 182	815 ± 139	115 ± 15
EXP3174 + AII	1,453 ± 108	58 ± 3	41 ± 2

Values are means ± S E M , $n = 3$ InsP1, inositol 1-phosphate, InsP2, inositol 1,4-bisphosphate, InsP3, inositol 1,4,5-trisphosphate, Ang II, angiotensin II

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