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Importance of intracellular Angiotensin II in vascular smooth muscle cell apoptosis: Inhibition by the Angiotensin AT_1 receptor antagonist irbesartan

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Abstract

The intracellular uptake of Angiotensin II has been described, although its physiological role is not yet understood. We aimed to study the role of Angiotensin II internalization in Angiotensin II-induced apoptosis. Vascular smooth muscle cells were cultured from male Wistar–Kyoto rats and treated with Angiotensin II (1 μM, 48 h). Apoptosis was assessed by DNA fragmentation, cell cytometry and caspase-3 activity. The Angiotensin AT_1 receptor antagonist irbesartan (0.1–10 μM) and the inhibitors of Angiotensin II internalization phenylarsine oxide (PAO, 20 μM), but not the AT_2 receptor antagonist PD123319 (*S*-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate) salt), decreased Angiotensin II-mediated apoptosis. Pre-treatment with irbesartan, but not with PD123319, blocked Angiotensin II internalization. We found a strong correlation between intracellular Angiotensin II staining and Angiotensin II-induced apoptosis for all compared groups. We therefore conclude that internalization of Angiotensin II is involved in apoptosis of vascular smooth muscle cells induced by this peptide.

Keywords: Vascular smooth muscle cell; Angiotensin; Apoptosis; Internalization; Cell trafficking

1. Introduction

The octapeptide Angiotensin II is considered as the main effector of the renin–angiotensin system. This peptide plays an important role in normal vascular physiology as well as in cardiovascular disease, mostly through the Angiotensin II type 1 receptor (AT_1) and partially through the Angiotensin II type 2 receptor (AT_2) (Andresen et al., 2004). Angiotensin II acts as a potent vasoconstrictor (Vanhoutte et al., 2005), comitogen (Xiao et al., 2004) and it also induces apoptosis (Bascands et al., 2001). On the other hand, in atherosclerosis and hypertensive vascular remodelling, Angiotensin II is increasingly regarded as an accelerator of vascular cell damage, not only by means of shear stress but also through direct tissue damage at the vessel wall level (Landmesser et al., 2002). Interestingly, this effect seems to be mediated by the Angiotensin AT_1 receptor (Daugherty et al., 2001).

Selective non-peptidic Angiotensin AT_1 receptor antagonists have been developed for the clinical treatment of hypertension and there is intense research of its putative antiatherosclerotic or plaque stabilizing effects. Some studies have revealed that Angiotensin AT_1 receptor antagonists, such as losartan, prevented apoptotic cell death induced by Angiotensin II in vascular smooth muscle cells in several *in vitro* assays (Bascands et al., 2001; Siegert et al., 1999). At the same time, Angiotensin AT_1 receptor antagonists have been described to decrease hypertensive oxidative damage in *in vivo* studies (Daugherty et al., 2001; Landmesser et al., 2002), as well as decrease plaque vulnerability in clinical series (Cipollone et al., 2004).

The classical pathway for Angiotensin II signaling by induction of second messengers has been complemented by the mechanisms which lead to Angiotensin II internalization (Cook et al., 2001). It is generally accepted that Angiotensin II binding to the Angiotensin AT_1 receptor causes rapid internalization of the

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AT_1–Angiotensin II complex (Anderson et al., 1993). The uptake of Angiotensin II into vascular smooth muscle cells *in vivo* was demonstrated more than 30 years ago (Robertson et al., 1971). The mechanism of Angiotensin II internalization is not fully understood. It is thought to be mediated, in part, by β-arrestins. β-arrestins have been shown to link receptors to the cytoplasmic clathrin-binding protein (Kule et al., 2004). Clathrin forms a cage-like structure that forms coated vesicles, which invaginate from the cell surface (Trowbridge et al., 1993). Disruption of the structure of the clathrin coat by treatment with phenylarsine oxide (PAO) markedly inhibits Angiotensin II internalization in vascular smooth muscle cells (Ullian et al., 1989).

The potential importance for intracellular Angiotensin II for cardiovascular medicine is highlighted by the finding of an association between intracellular Angiotensin II and oxidative damage in pathological specimens from diabetic hearts (Frustaci et al., 2000). It has been postulated that the ability of AT_1 antagonists to inhibit intracellular functions of Angiotensin II may depend on their liposolubility (Cook et al., 2001). Since irbesartan is a highly liposoluble molecule, we aimed to study the physiological role of Angiotensin II internalization on the apoptosis induced by Angiotensin II and the preventive role of irbesartan concerning this phenomenon.

2. Methods

2.1. Cell cultures

Male Wistar–Kyoto rats (230±20 g) were used. The investigation followed the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Cultures of rat vascular smooth muscle cells were obtained from enzymatically dissociated rat thoracic aorta. Cells showed the typical hill-and-valley phenotype and stained positive for α-actin. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (Gibco®, Madrid, Spain), supplemented with 100 IU/ml penicillin G (sodium salt) and 100 μg/ml streptomycin (Gibco, Madrid, Spain). Experiments were conducted with cells at passages 3–5. The cells showed a spindle-like morphology.

We set the cell culture conditions for Angiotensin II-induced apoptosis as a low density seed (20,000 cells/cm^2, which yields 70% confluence) and low-serum cell culture medium (0.4% foetal calf serum). These conditions are similar to the ones reported by other groups (Qin et al., 2004).

2.2. Measurement of apoptosis by DNA fragmentation

The cells (5000 cells/well) were plated on 96-well plates and allowed to attach for 24 h. Angiotensin II (1 μM) was added for 48 h (0.4% foetal calf serum). To establish the role of the Angiotensin AT_1 receptor in the apoptosis induced by Angiotensin II, the cells were preincubated with the Angiotensin AT_1 receptor antagonist irbesartan (0.1–10 μM) or with the AT_2 receptor antagonist PD123319 (*S*-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate) salt) at 1–10 μM for 30 min prior to Angiotensin II treatment. We also blocked Angiotensin II internalization with phenylarsine oxide (PAO, 20 μM, 30 min preincubation) which disrupts clathrin-coated pits. Cellular DNA fragmentation was measured with a commercially available cellular DNA fragmentation Enzyme-linked Immunosorbent Assay (ELISA) kit from Roche-Boerhinger®, Spain. After treatment, the cells were washed with phosphate buffer saline (PBS) and incubated with the kit lysis buffer composed by bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and Tween®20 for 30 min at room temperature. Soluble Bromodeoxyuridine (BrdU)-labelled DNA fragments present in the buffer were quantified using the ELISA kit. DNA fragmentation was expressed as fold increase of the control values.

2.3. Western blotting

Protein expression induced by cell treatments was assessed by Western blotting, according to a method previously described (Redondo et al., 2005). Cells (80,000 cells/well) were plated onto 6-well plates and allowed to attach for 24 h. For Bcl-x_s detection, the cells were treated with Angiotensin II 1 μM for 3–18 h in 0.4% foetal calf serum containing medium. The cells were washed with ice-cold PBS, and lysed on ice with 200 μl lysis buffer (10% glycerol, 2.3% sodium dodecyl sulphate, 62.5 mM Trizma base–HCl, pH 6.8; 150 mM NaCl, 10 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml chymostatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride) and boiled for 5 min. Equal amounts of protein were run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Amersham®, Madrid, Spain), and blocked overnight at 4 °C in blocking solution (5% bovine serum albumin in TBS-T: 25 mM Trizma base, 75 mM NaCl, pH=7.4, 0.1% v/v Tween®20). For analysis of Bcl-x_s, blots were incubated with mouse monoclonal anti Bcl-x_s (1:500) (Transduction Labs®, Madrid, Spain). After washing in TBS-T solution, the blots were further incubated for 1 h at room temperature with the horseradish peroxidase conjugated anti-mouse antibodies diluted at 1:10,000 (Santa Cruz Biotechnology®, CA, USA) in the blocking solution. The blots were then washed 5 times in TBS-T, and antibody-bound protein was visualized with Enhanced Chemiluminescence's (ECL) kit (Amersham Biosciences®, Barcelona, Spain). Smooth muscle α-actin was used as a housekeeping protein, and was determined following the same procedure as mentioned above, using a specific anti-α-actin mouse monoclonal antibody (Sigma-Aldrich®, Madrid, Spain), at 1:1000 in TBS-T.

2.4. Measurement of apoptosis by flow cytometry

Cellular DNA content was measured by fluorescence-assessed flow cytometry (FACS). The cells (10^5 cells/flask) were plated and allowed to attach for 24 h and then treated with Angiotensin II 1 μM for 24 or 48 h in 0.4% foetal calf serum containing medium. The cells were then harvested by