



# Effects of Angiotensin II on human endothelial cells survival signalling pathways and its angiogenic response

Baijun Kou<sup>a,\*</sup>, Manu Vatish<sup>b</sup>, Donald R.J. Singer<sup>a</sup>

<sup>a</sup> *Clinical Pharmacology, Clinical Science Research Institute, Warwick Medical School, University of Warwick Coventry, CV2 2DX, UK*

<sup>b</sup> *Obstetrics, Clinical Science Research Institute, Warwick Medical School, University of Warwick Coventry, CV2 2DX, UK*

Received 1 March 2007; accepted 1 June 2007

## Abstract

Reduced capillary density (rarefaction) is an early event of cardiovascular disease. The PI-3K–Akt pathway is a key player in anti-endothelial cells (ECs) apoptosis. VEGF is a key growth factor for angiogenesis. We investigated the effect of Angiotensin II (Ang II) on ECs survival signalling and angiogenesis *in vitro*. We found that Ang II had a biphasic effect on Akt phosphorylation by western blotting analysis. Low concentration Ang II caused a dose-dependent increase in Akt phosphorylation, while high concentration of Ang II led to a decrease of Akt phosphorylation. This effect was negative regulated by its type II receptor. Ang II  $10^{-4}$  M induced ECs apoptosis by its type II receptor was completely blocked by VEGF. Cell viability was increased by Ang II  $10^{-6}$  M and decreased by Ang II  $10^{-4}$  M. It was further decreased by pre-treatment with PI-3K/Akt inhibitor LY294002, but unaffected by p38-MAPK inhibitor SB202190. Ang II  $10^{-4}$  M reduced ECs' proliferation and vascular tube length, which were in part regulated by type II receptor. Our findings support a dose-dependent role of Ang II in effect on ECs survival and angiogenesis by PI-3K/Akt pathway. The anti-angiogenic effect of Ang II was mediated by its type II receptor.

© 2007 Elsevier Inc. All rights reserved.

**Keywords:** Ang II; Angiogenesis; VEGF; Cell signalling; Apoptosis

## 1. Introduction

Chronic arterial hypertension, as well as other cardiovascular diseases, is associated with a secondary increase in peripheral microvascular resistance, which causes structural reduction in the lumina of the small distributing vessels (Vicault, 1992). Vascular structural rarefaction is the result of ablation or complete disappearance of capillary vessels. (Noon et al., 1997). The disappearance of vessels requires the removal of endothelial cells (ECs) mainly after cells apoptosis (Gobe et al., 1997). ECs rarefaction is an early event in patients with cardiovascular disease or just with risk factor. Therefore, it is very important to clarify which signal pathway controls ECs survival. Phosphoinositide 3-kinases (PI-3K) and its downstream serine/threonine kinase Akt, also named protein kinase B(PKB), plays a central role in promoting the survival of a wide range of cell types

(Khawaja, 1999). PI-3K constitutes a multifunctional family of enzymes activated by receptor tyrosine kinases such as insulin, platelet-derived growth factor, and vascular endothelial growth factor (VEGF) receptors (Wymann and Pirola, 1998). Ligand-dependent recruitment of PI-3K subunits induces phosphorylation of the inositol ring of phosphatidylinositol lipids. These inositol lipids are implicated in numerous cellular functions (Leevers et al., 1996). PKB/Akt is a critical downstream effector (Shiojima and Walsh, 2002). Upon receptor activation, it is recruited to the plasma membrane and binds to inositol lipids *via* its pleckstrin homology domain. Once in the membrane, Akt is phosphorylated by phosphoinositide-dependent kinases, which enhances its catalytic activity toward a variety of diverse substrates (Downward, 1998). Akt controls the cell survival by several signal pathway including BAD, NF-Kb et al. (Coffer et al., 1998). The survival of endothelial cells is critical for angiogenesis and the maintenance of blood vessel integrity (Risau, 1997). VEGF is an endothelial cell-specific mitogen that induces blood vessel formation during normal development and

\* Corresponding author. Tel.: +44 2476968646; fax: +44 2476535004.

E-mail address: [koubaijun@yahoo.com](mailto:koubaijun@yahoo.com) (B. Kou).

various pathological processes. VEGF biological activity is mediated through its binding to at least two endothelial cell-specific receptors, fms-like tyrosine kinase (flt-1) and fetal liver kinase (flk-1). VEGF activates receptor tyrosine kinases leading to the activation of various signal transducers including PI-3K (Guo et al., 1995; Xia et al., 1996). In addition to its mitogenic effects, VEGF acts to promote endothelial cell survival both *in vitro* and *in vivo* (Alon et al., 1995; Gerber et al., 1998a). Previous studies have indicated that VEGF activates the PI-3K–PKB/Akt signal transduction pathway by its receptor 2 (flk-1/KDR) (Tanimoto et al., 2002).

Mechanisms that participate in endothelial cell rarefaction include oxidative stress and vasocative peptide Ang II. Ang II, the most important effector peptide of the renin–angiotensin system, is implicated in cardiovascular and renal pathology and has been recognized as a growth-promoting and apoptosis-regulating factor contributing to vascular structural alteration (Dzau, 1994; Otani et al., 1998). Ang II initiates its effects by interaction with at least two pharmacologically distinct subtypes of cell-surface receptors, AT<sub>1</sub> and AT<sub>2</sub>. The major functions of Ang II in cardiovascular system are mediated through AT<sub>1</sub>, whereas AT<sub>2</sub> exerts anti-growth and anti-hypertrophic effects (Stoll et al., 1995). In ECs, Ang II has been reported to potentiate induce VEGF-mediated angiogenic activity (Otani et al., 1998).

However, the effect of Ang II on PI-3K/Akt signalling and its type II receptor function in regulating of Ang II signalling to downstream reaction has remained elusive. In addition, little is known about whether Ang II plays an anti- or pro-Akt phosphorylation role in microvascular angiogenesis. On the other hand, the effect of Ang II on ECs' fate may not only be influenced by PI-3K/Akt pathway. Recent studies have indicated that cross-talk between different signal transduction pathways, including p38-MAPK pathway, is involved in ECs survival (Gratton et al., 2001).

Here, we examined the survival signalling Akt phosphorylation of cultured human umbilical vein endothelial cells (HUVEC). We also examined the effect of Ang II and its type II receptor on VEGF-mediated HUVECs survival signalling, as well as its correlation with p38-MAPK pathway and its effect on ECs angiogenic response.

## 2. Materials and methods

### 2.1. Materials

The following reagents were obtained from Sigma-Aldrich: Human Recombinant VEGF, Ang II (acetate human) and its type 2 receptor antagonist PD123319, MTT, Hoechst 33342, propidium iodide, endothelial cell culture medium 199, endothelial cell growth supplement, collagenase and heparin (sodium salt). Cell RIPA lysis buffer was from Upstate. Antibodies against phosphorylated-Akt and caspase-3 or cleaved caspase-3 were purchased from Cell Signalling Technology, with second antibodies (polyclonal goat anti-rabbit immunoglobulins) from DAKO Cytomation. ECL plus Western blotting detection reagents were from Amersham Biosciences.

Fetal bovine serum was from Biowest. Cell harvest reagent trypsin-EDTA (10×) was from Invitrogen Corporation. PI-3K/Akt inhibitor LY294002 and p38-MAPK inhibitor SB202190 were obtained from Calbiochem.

### 2.2. Cell culture

We grew primary cultures of endothelial cells from human umbilical cords (HUVECs) obtained following elective Caesarean Section with written informed consent and local ethics committee approval. Each cord was collected into 4 °C PBS and treated as soon as possible. After digestion with 0.2% collagenase for 15–20 min at 37 °C 5% CO<sub>2</sub>, the resulting supernatant with rich endothelial cells was centrifuged and seeded in a 25 cm flask pre-coated with 1% gelatin in 20% serum M199 and cultured for 18 h. After replacing the medium with 10% serum, 75 µg/ml endothelial cell growth supplement, cells were cultured for another 5–7 days reaching 70% confluence.

For each study, the cells were cultured in a serum-free medium for 24 h before study agents were added. To assess receptor specificity of Ang II effect on cell signalling, cells were pre-treated for 30 min with Ang II type II receptor's antagonist PD123319 10<sup>-7</sup> M.

### 2.3. Western blotting analysis of PKB/Akt phosphorylation and apoptosis signalling

At the end of each treatment, cells were rapidly lysed in 1× modified RIPA buffer (1% Triton X-100, 158 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl, pH 7.2, 100 mM sodium vanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM PMSF) on ice. Cell lysates were then sonicated for 15 s, boiled at 100 °C for 5 min then cooled on ice and centrifuged at 2000 rpm for 10 min at 4 °C. Protein concentration was assayed with a Bradford protein assay kit (Bio-Rad). Cell lysates (20 µg per well) were resolved on a 10%(p-Akt)-14% (caspase-3/cleaved caspase-3) SDS-PAGE gel, and the separated proteins were then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with the specific antibody against phosphorylated Akt or caspase-3 (cleaved-caspase-3) (1:1000) at 4 °C overnight. The antibody against β-actin (1:100) was used as a positive house-keeping control. The membrane was then washed and incubated with second antibody (1:2000) at room temperature for 1 h. The immunoreactive protein was detected by enhanced chemiluminescence (ECL) plus and developed with KODAK film in a dark room. The blotting film was quantitatively analyzed with Syngene Genetools (Bio Imaging systems) software.

### 2.4. Apoptosis assay

To assess apoptotic activity, HUVECs were seeded on to 6-well plates at 5 × 10<sup>4</sup> cells/well. Morphological changes in the nuclear chromatin of apoptotic cells were determined by staining with bis-benzimide (Hoechst 33342) and propidium iodide. A minimum of 500 cells was scored for apoptotic chromatin changes. The effects of VEGF 50 ng/ml were studied after 24 h incubation. Stress was induced by 24 h incubation with Ang II. Cell survival signalling

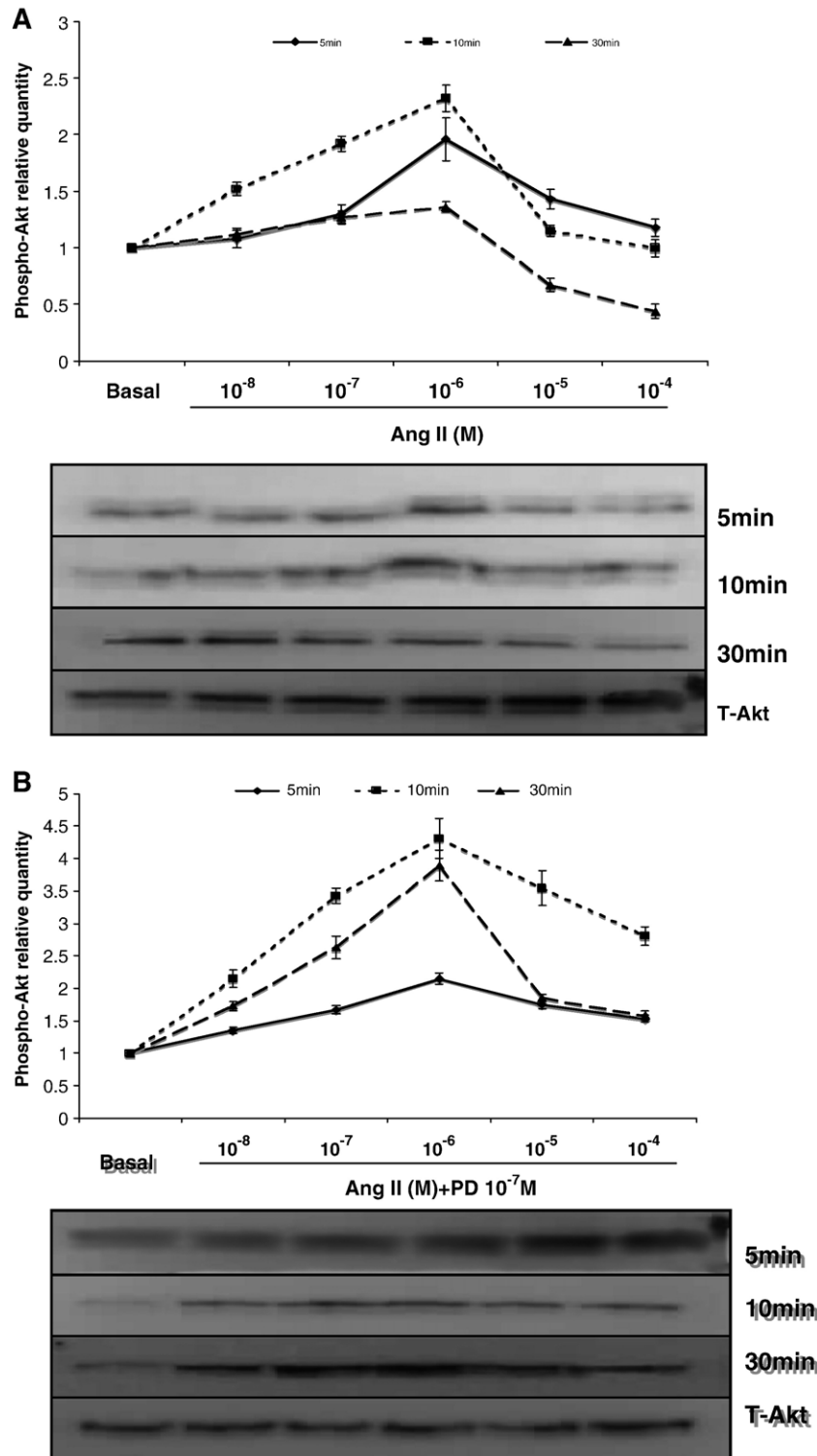


Fig. 1. The effect of Ang II and its type 2 receptor on Akt signalling in HUVECs. A: Ang II dose and time dependent effect on Akt phosphorylation in HUVECs (ANOVA,  $p < 0.0001$ ). In the range  $10^{-8}$  M to  $10^{-6}$  M, Ang II caused a dose-dependent increase in phospho-Akt level, starting at 5 min after stimulation, maximal  $2.3 \pm 0.1$  fold basal after 10 min  $10^{-6}$  M Ang II stimulation ( $p < 0.001$  vs. basal). Thereafter, phospho-Akt signalling decreased rapidly. In the range of  $10^{-5}$ – $10^{-4}$  M, Ang II caused a dose-dependent decrease in phospho-Akt level. B: Ang II plus PD123319  $10^{-7}$  M dose and time dependent effect on Akt phosphorylation in HUVECs (ANOVA,  $p < 0.001$ ). HUVECs were pretreated with Ang II type 2 receptor antagonist PD123319 ( $10^{-7}$  M) for 30 min before adding Ang II. PD123319 in  $10^{-7}$  M generally increased Akt phosphorylation compared with Ang II alone in parallel time and dose. 10 min exposure to  $10^{-6}$  M Ang II increased Akt phosphorylation maximal up to  $4.3 \pm 0.3$  fold basal ( $p < 0.0001$  vs. basal) and the inhibitory response to higher concentration Ang II ( $10^{-5}$ – $10^{-4}$  M) was abolished —  $10^{-5}$ – $10^{-4}$  M Ang II now enhancing Akt-phosphorylation.

was tested by 2 h pre-treated with the PI-3K/Akt inhibitor LY294002 (25  $\mu$ M) or the p38-MAPK inhibitor SB202190 (10  $\mu$ M). Data for the cell viability ratio (CVR) are expressed as percentage of apoptotic cells in total counted cells.

### 2.5. Cells proliferation assay

The proliferation of endothelial cells was assessed by MTT assay. Following an overnight treatment of cultured cells, MTT (2 mg/ml in PBS) was added to each well of 96-well plates and the cells were incubated for a further 4 h. The media containing

MTT was then removed, 200  $\mu$ l of DMSO was added to each well and absorbance was read at 570 nm. Each treated sample was compared with an untreated control, each experiment was repeated three times.

### 2.6. 3-D cultures of HUVECs and vascular tube formation

The day before experiment, Matrigel was stored at 4  $^{\circ}$ C with related materials. Thawed Matrigel (200  $\mu$ l) was added to 24-well plates and then incubated at 37  $^{\circ}$ C for 30 min to form a gel layer. HUVECs at passage 2 (about  $10^4$  cells/well) being treated

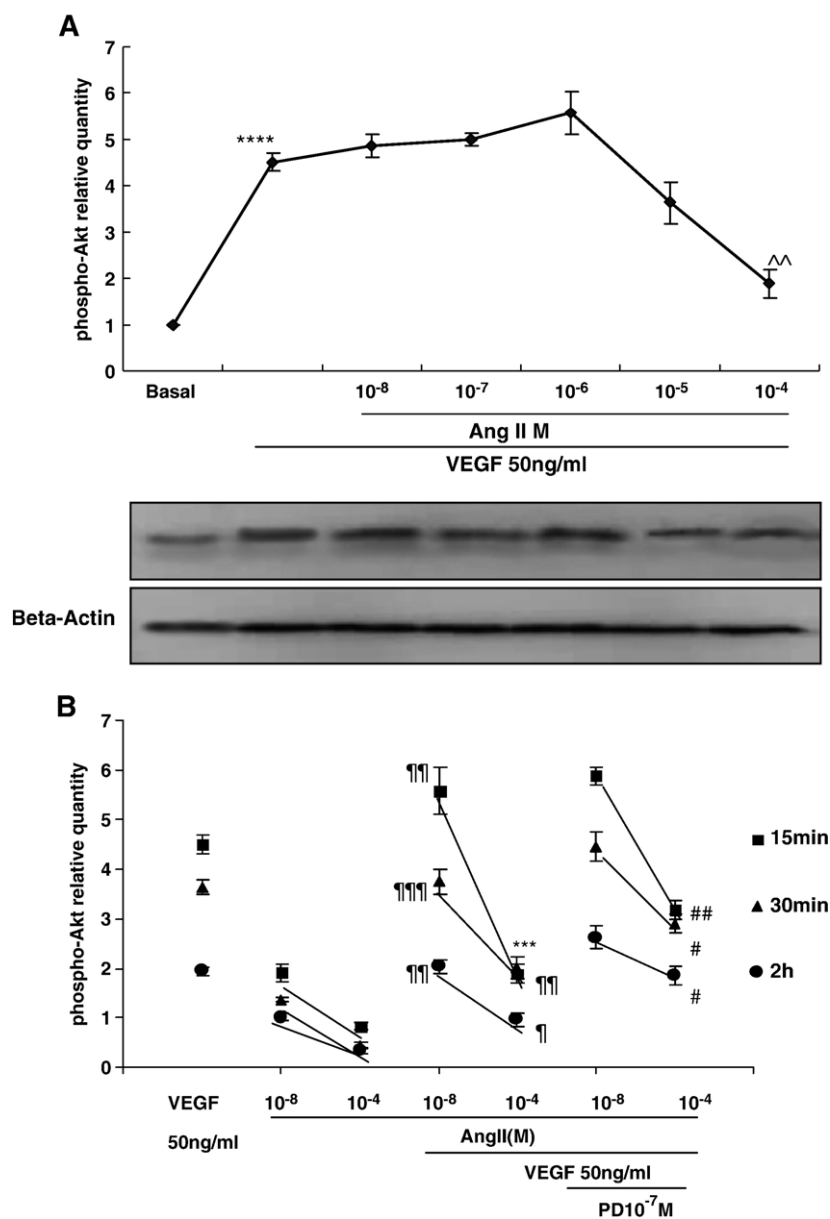


Fig. 2. Dual effect of Ang II on VEGF-stimulated Akt signalling in HUVECs. A: VEGF 50 ng/ml significantly increased Akt phosphorylation (\*\*\*\* $p < 0.0001$ ). Ang II  $10^{-8}$ – $10^{-6}$  M slightly enhanced VEGF-stimulated Akt phosphorylation ( $p > 0.05$  vs. VEGF alone). Ang II  $10^{-5}$ – $10^{-4}$  M inhibited VEGF-stimulated Akt phosphorylation ( $p < 0.01$ , Ang II  $10^{-4}$  M+V vs. V). B: After 15 min of stimulation, the Akt phosphorylation was maximal either with Ang II alone, or VEGF stimulation, or plus PD 123319. Afterward, the phospho-Akt level went down with time dependent. VEGF increased Akt phosphorylation both in low and high concentrations of Ang II compared with Ang II alone ( $p < 0.05$ ) with time dependent, this action was enhanced when adding PD 123319 (\*\*\* $p < 0.001$ , Ang II+V vs. V alone; ¶ $p < 0.05$ , ¶¶ $p < 0.01$ , ¶¶¶ $p < 0.001$ , Ang II vs. Ang II+V; # $p < 0.05$ , ## $p < 0.01$ , Ang II+V+PD vs. Ang II+V).

overnight were seeded to the Matrigel-coated 24-well plate with 10% FBS medium and incubated for 4 h. Tube formation was observed using phase contrast microscopy. Images were captured using a Scion digital camera and analyzed using Imaging J software.

## 2.7. Statistical analysis

Western blotting results for basal data were normalised to 1. Each experiment was repeated three times. Data are expressed as mean  $\pm$  SEM. Differences for repeated measures among

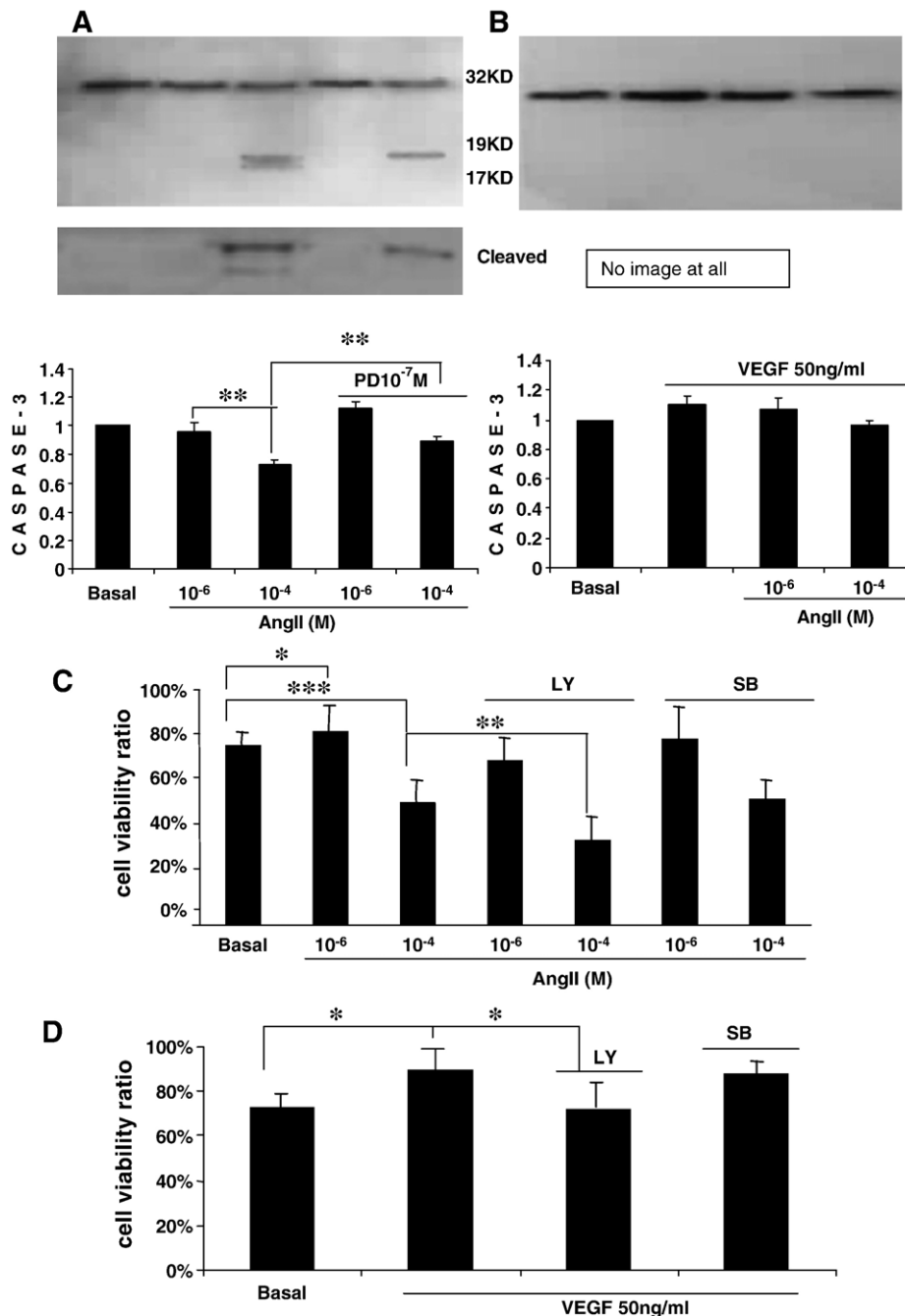


Fig. 3. HUVECs' apoptosis assessment by antibodies against caspase-3 and cleaved caspase-3 western blotting and HUVECs' viability assessment by Hoechst33342 and PI staining. A. Ang II  $10^{-4}$  M induced cleaved caspase-3 expression with no effect of Ang II  $10^{-6}$  M. The Ang II type 2 receptor antagonist PD123319  $10^{-7}$  M reduced cleavage of caspase-3 expression by high concentration Ang II ( $10^{-4}$  M). (\*\* $p$  < 0.01). B. VEGF 50 ng/ml completely blocked the cleavage of caspase-3 expression induced by high concentration Ang II ( $10^{-4}$  M) ( $p$  < 0.01 vs. Ang II  $10^{-4}$  M alone). C. Ang II  $10^{-6}$  M increased CVR to  $82 \pm 11\%$  (\* $p$  < 0.05 vs. basal). Ang II  $10^{-4}$  M decreased CVR to  $52 \pm 7\%$  (\*\* $p$  < 0.001 vs. basal); CVR was further decreased by pre-treated with PI-3K/Akt inhibitor LY294002 (25  $\mu$ M) (\*\* $p$  < 0.01 vs. Ang II  $10^{-4}$  M alone) but unaffected by p38-MAPK inhibitor SB202190 (10  $\mu$ M). D. VEGF 50 ng/ml increased CVR to  $89 \pm 9\%$  (\* $p$  < 0.05 vs. basal). LY294002 completely blocked VEGF-induced increase in CVR, with no effect of SB202190. (Note: PD123319 was pretreated for 30 min before Ang II was added to the cells and assessment was carried out after 24 h incubation. PI-3K/Akt inhibitor LY294002 (25  $\mu$ M) or p-38 MAPK inhibitor SB202190 (10  $\mu$ M) was pretreated for 2 h before Ang II/VEGF were added.)

experimental groups were evaluated by ANOVA, with *post-hoc* testing for pairs of data sets. Student's two-tailed *t*-test was used for comparing two treatment groups in paired experiments.

### 3. Results

#### 3.1. Biphasic effect of Ang II on Akt phosphorylation and role of Ang II type 2 receptor

To study the effect of Ang II on Akt phosphorylation signalling in HUVECs, we incubated serum-deprived HUVECs with and without  $10^{-8}$ – $10^{-4}$  M Ang II for 5 min–30 min. In the range  $10^{-8}$  M to  $10^{-6}$  M, Ang II caused a dose-dependent increase in phospho-Akt level, maximal  $2.3 \pm 0.1$  folds basal after 10 min  $10^{-6}$  M Ang II stimulation ( $p < 0.001$  vs. basal). Thereafter, phospho-Akt signalling decreased rapidly. However, within the range of  $10^{-5}$ – $10^{-4}$  M, Ang II caused a dose-dependent decrease

in phospho-Akt level.  $10^{-6}$  M Ang II significantly increased Akt phosphorylation compared with other concentration of Ang II and basal (ANOVA,  $p < 0.0001$ ) (Fig. 1A).

Next, we pre-treated serum-deprived HUVECs with Ang II type II receptor antagonist PD123319 ( $10^{-7}$  M) for 30 min before adding Ang II. Under these conditions, 10 min exposure to  $10^{-6}$  M Ang II increased Akt phosphorylation up to  $4.3 \pm 0.3$  folds basal ( $p < 0.0001$  vs. basal) and the inhibitory response to higher concentration Ang II was abolished with  $10^{-5}$ – $10^{-4}$  M Ang II now enhancing Akt-phosphorylation (Fig. 1B).

#### 3.2. Effects of Angiotensin II on VEGF-stimulated Akt phosphorylation in HUVECs

To investigate effect of Ang II on VEGF-stimulated phospho-Akt signalling, we incubated HUVECs with 50 ng/ml VEGF with and without  $10^{-8}$ – $10^{-4}$  M Ang II for 15 min. At lower

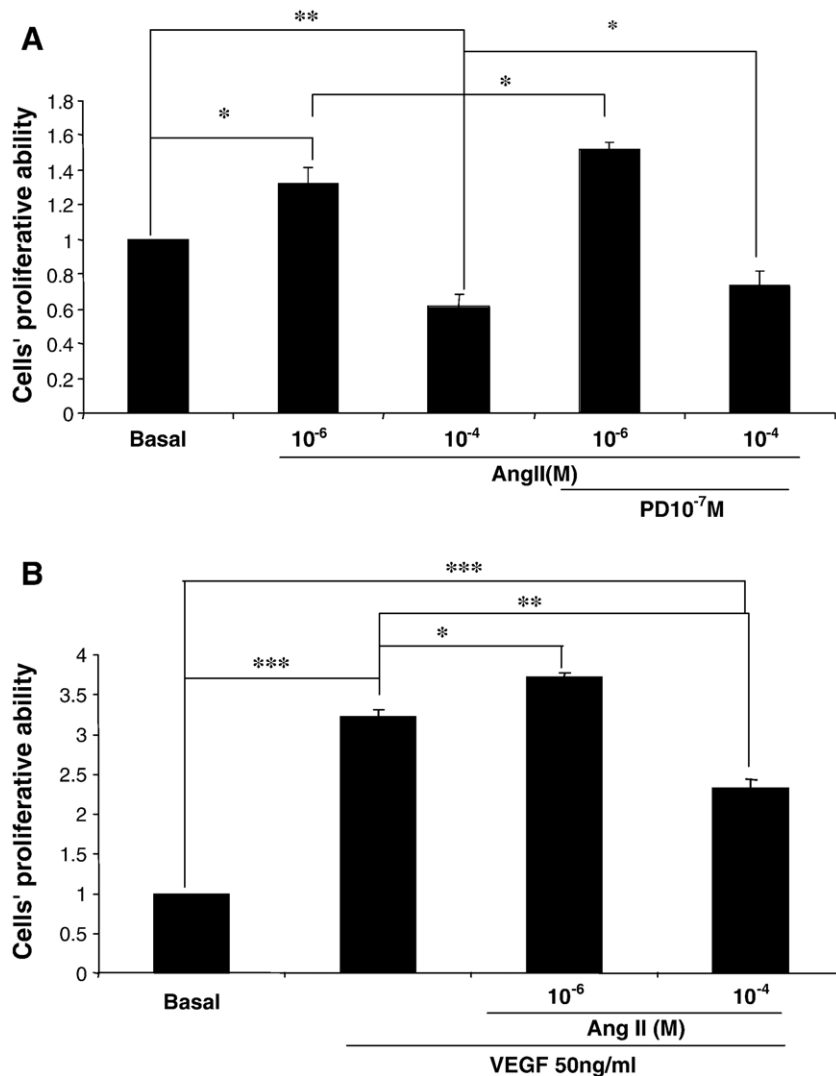


Fig. 4. HUVECs' proliferation assessment by MTT assay. A. Cell's proliferation assessment under Ang II/PD123319  $10^{-7}$  M. After 24 h incubation, Ang II  $10^{-6}$  M increased the cells' proliferation ( $*p < 0.05$  vs. basal), but Ang II  $10^{-4}$  M decreased the cells' proliferation ( $**p < 0.01$  vs. basal). PD123319  $10^{-7}$  M slightly increased cells' proliferation compared with Ang II alone ( $*p < 0.05$  vs. Ang II alone). (PD123319  $10^{-7}$  M was pretreated for 30 min before Ang II was added). B. Cell's proliferation assessment under VEGF/Ang II. VEGF 50n g/ml significantly increased cells' proliferation compared with baseline ( $***p < 0.001$ ) with Ang II  $10^{-6}$  M increased VEGF stimulated cells' proliferation. ( $*p < 0.05$  vs. VEGF alone). Ang II  $10^{-4}$  M inhibited VEGF-stimulated cells' proliferation ( $**p < 0.01$ , vs. VEGF alone); however, the cells' proliferation was still much higher than basal ( $***p < 0.001$ ).

concentrations, Ang II slightly enhanced VEGF-stimulated Akt phosphorylation ( $p > 0.05$  vs. VEGF alone). Higher concentrations of Ang II inhibited VEGF-stimulated Akt phosphorylation to a minimum of 58% below the response to VEGF alone on incubation with  $10^{-4}$  M Ang II for 15 min ( $p < 0.01$  vs. VEGF alone) (Fig. 2A). We also compared phospho-Akt signalling at different time points in the presence of lower or higher concentration of Ang II, with and without Ang II type II

receptor antagonist PD123319  $10^{-7}$  M. Akt phosphorylation was maximal after 15 min stimulation with VEGF 50 ng/ml, or in the presence of low or high concentration Ang II. VEGF increased Akt phosphorylation in the presence of low and high concentrations of Ang II ( $p < 0.01$  vs. Ang II alone). VEGF stimulated Akt phosphorylation under Ang II  $10^{-4}$  M was enhanced on PD123319 involved ( $p < 0.05$  vs. Ang II+V) (Fig. 2B).

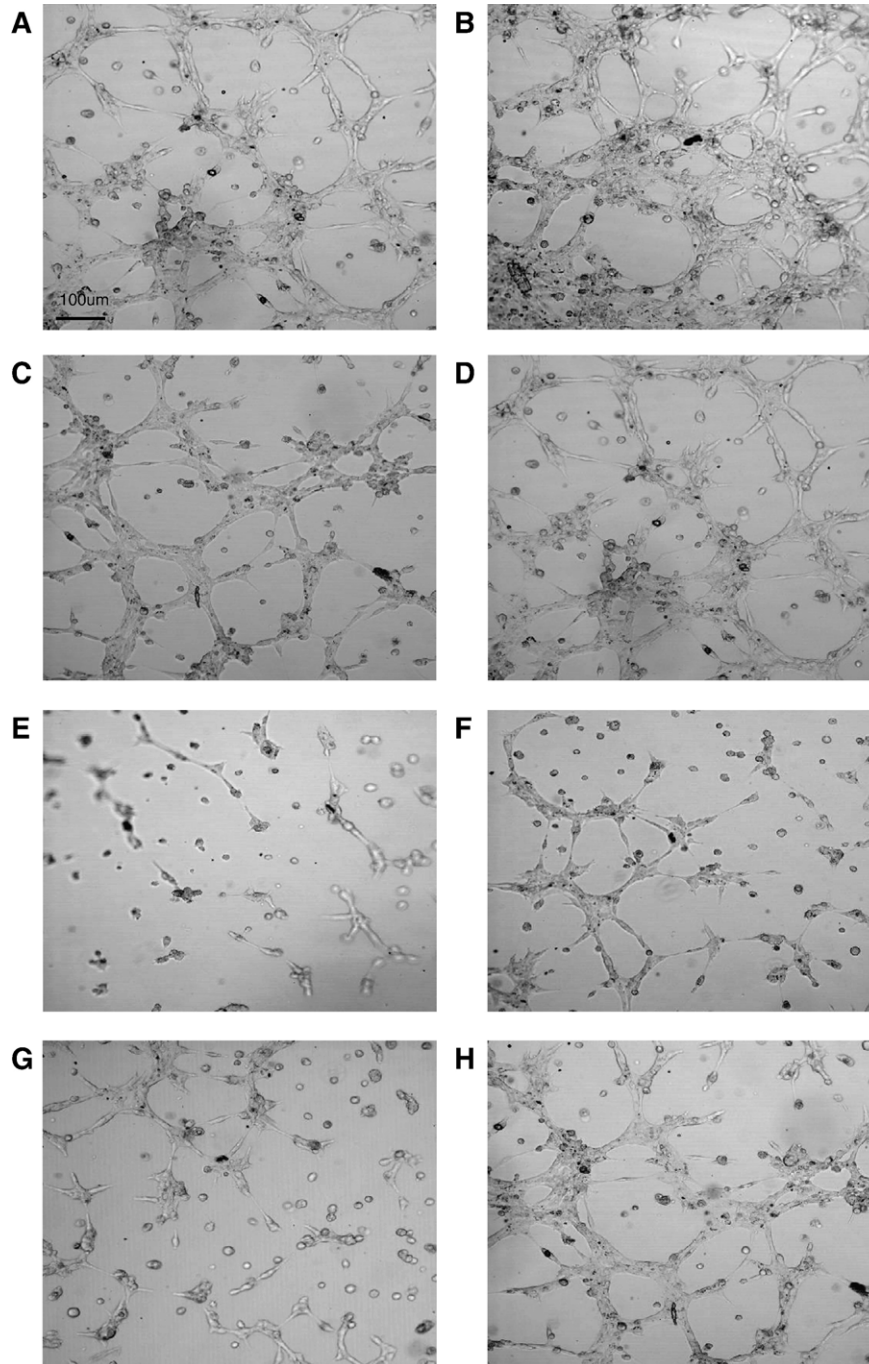


Fig. 5. Effects of Ang II on HUVECs vascular tube formation in 3-D Matrigel culture. Photographs were taken 4 h after initial seeding of cells. A. control, B. VEGF 50 ng/ml, C. Ang II  $10^{-6}$  M, D. VEGF+Ang II  $10^{-6}$  M, E. Ang II  $10^{-4}$  M, F. VEGF+Ang II  $10^{-4}$  M, G. Ang II  $10^{-4}$  M+PD123319  $10^{-7}$  M, H. Ang II  $10^{-4}$  M+PD123319  $10^{-7}$  M+VEGF 50 ng/ml. ( $2 \times 10^{-4}$  HUVECs cells were seeded each well of 24-well plate pre-coated with matrigel. The cells were cultured with serum-free medium for overnight and Ang II/VEGF were added for 24 h before seeding to matrigel coated well. PD123319 was pretreated for 30 min before Ang II was added in relative studies).

### 3.3. Effect of Angiotensin II on expression of cleaved caspase-3

24 h incubation with  $10^{-4}$  M Ang II induced expression of cleaved caspase-3 (cleaved: uncleaved caspase-3  $2.8 \pm 0.3/1.5 \pm 0.4$ ), however caspase-3 cleavage in lower concentration  $10^{-6}$  M Ang II was undetectable. Co-treatment with Ang II type II receptor antagonist PD123319  $10^{-7}$  M reduced expression of cleaved caspase-3 induced by high concentration Ang II (cleaved: uncleaved caspase-3  $2.6 \pm 0.4/3.2 \pm 0.3$ ;  $p < 0.01$  vs.  $10^{-4}$  M Ang II alone). VEGF 50 ng/ml completely blocked cleavage of caspase-3 expression induced by high concentration of Ang II (Fig. 3A and B).

### 3.4. Role of PI-3K/Akt and p-38 MAPK pathways on Ang II's effect of ECs viability

$10^{-6}$  M Ang II increased the cell viability ratio (CVR) to  $82 \pm 11\%$  ( $p < 0.05$  vs. basal); whereas,  $10^{-4}$  M Ang II decreased the CVR to  $52 \pm 7\%$  ( $p < 0.001$  vs. basal); the CVR was further decreased by pre-treated with 25  $\mu$ M LY294002 ( $p < 0.01$ ; LY +  $10^{-4}$  M Ang II vs.  $10^{-4}$  M Ang II alone) but unaffected by SB202190 ( $p > 0.05$ ). VEGF 50 ng/ml increased the CVR to  $89 \pm 9\%$  ( $p < 0.05$  vs. basal). LY294002 completely blocked the VEGF-induced increase of CVR in HUVECs ( $p < 0.05$  vs. VEGF alone), with no effect of SB202190 ( $p > 0.05$ ) (Fig. 3C and D).

### 3.5. Effect of Ang II/VEGF on HUVECs proliferation

The MTT assay was used to assess cells proliferation.  $10^{-6}$  M Ang II increased cell proliferation compared with baseline ( $p < 0.05$ ; Fig. 4A); however,  $10^{-4}$  M Ang II decreased cell proliferation ( $p < 0.01$  vs. basal Fig. 4A).  $10^{-7}$  M PD123319 increased the level of cell proliferation in response to low or high concentration Ang II ( $p < 0.05$ ; Fig. 4A). VEGF 50 ng/ml significantly increased cell proliferation to  $3.1 \pm 0.1$  folds ( $p < 0.001$  vs. basal) and completely blocked the inhibitory effect of high concentration Ang II ( $10^{-4}$  M) on cell proliferation (Fig. 4B).

### 3.6. Effects of Ang II on HUVECs vascular tube formation

Incubation with VEGF 50 ng/ml significantly increased HUVECs angiogenic activity vs. control (Total length: VEGF  $5365 \pm 165$   $\mu$ m vs. control  $4247 \pm 306$   $\mu$ m;  $p = 0.03$ ) in 3-D culture coated with Matrigel. There was little effect of  $10^{-6}$  M Ang II on tube formation (total length  $4391 \pm 169$   $\mu$ m). However,  $10^{-4}$  M Ang II reduced endothelial tube length markedly ( $2458 \pm 245$   $\mu$ m;  $p = 0.008$  vs. control) and inhibited the angiogenic effects of VEGF ( $10^{-4}$  M Ang II + VEGF  $3523 \pm 385$   $\mu$ m;  $p = 0.012$  vs. VEGF alone). The anti-angiogenic effects of  $10^{-4}$  M Ang II were partly prevented by its type II receptor antagonist PD123319 ( $10^{-4}$  M Ang II +  $10^{-7}$  M PD123319:  $3696 \pm 295$   $\mu$ m;  $p = 0.032$  vs.  $10^{-4}$  M Ang II alone) (Fig. 5, Table 1).

## 4. Discussion

Endothelial cell survival and sprouting are critical steps in vascular formation and maintenance. A growing body of evidence indicates that the survival signal mediated by various growth factors and cytokines is dependent on PI-3K–PKB/Akt signal pathway (Yao and Cooper, 1995; Zachary and Gliki, 2001). It is well established that VEGF promotes endothelial cell survival via Flk-1 receptor-mediated Akt activation (Gerber et al., 1998b). VEGF is known to stimulate Akt-dependent phosphorylation of eNOS  $Ca^{2+}$ -independently via its receptor Flk-1/KDR. This results in an activation of eNOS and an increased NO production of endothelial cells. Hence, VEGF-induced phosphorylation of Akt plays a key role in VEGF-stimulated angiogenesis. Our study (data not shown in this paper) indicated that VEGF significantly stimulated Akt activity and by this pathway to protect ECs from apoptosis. Although growth factors mediate cell survival through other signalling pathways, such as MAPK, PKA and PKC; blocking MAPK/ERK activity using a specific inhibitor PD98059 does not affect endothelial cell survival (Fujikawa et al., 1999); which supports the theory that Akt is the main mediator of ECs survival. Although Akt signalling plays a pivotal role in mediating endothelial cell survival, Akt can be regulated by growth factors acting through receptor protein tyrosine kinases mediated by PKC pathway. This indicates that PKC mediates Akt activity in VEGF-stimulated cell survival and angiogenesis (Gliki et al., 2002).

The role of Ang II in angiogenesis has been extensively studied by applying different *in vivo* models, and there is consensus that Ang II promotes angiogenesis via the AT<sub>1</sub> receptor (Munzenmaier and Greene, 1996). Recent evidence further indicated that the angiogenic properties of Ang II might be due to an AT<sub>1</sub> receptor-mediated increase in VEGF protein expression and an up-regulation of VEGF receptor (KDR) expression (Otani et al., 1998). Moreover, AT<sub>2</sub> receptor-mediated anti-angiogenic actions in microvascular growth have been described (Munzenmaier and Greene, 1996; Benndorf et al., 2003), but with contradicting reports. Our study indicated that Ang II had a biphasic effect on PKB/Akt phosphorylation and its type II receptor induced a negative regulation on Akt signalling. The concentration of Ang II determines its effect on Akt phosphorylation and therefore

Table 1  
Effect of Ang II on VEGF induced HUVECs tube formation

Group	Number of tube mean $\pm$ SEM	Total length ( $\mu$ m) mean $\pm$ SEM
Control	7 $\pm$ 1	4247 $\pm$ 306
VEGF 50 ng/ml	13 $\pm$ 1 <sup>a</sup>	5367 $\pm$ 165 <sup>b</sup>
Ang II $10^{-4}$ M	3 $\pm$ 1 <sup>c</sup>	2458 $\pm$ 245 <sup>d</sup>
VEGF + Ang II $10^{-4}$ M	4 $\pm$ 1	3523 $\pm$ 385 <sup>e</sup>
Ang II $10^{-6}$ M	8 $\pm$ 1	4391 $\pm$ 169
Ang II $10^{-6}$ M + VEGF	10 $\pm$ 1	4768 $\pm$ 393
Ang II $10^{-4}$ M + PD $10^{-7}$ M	5 $\pm$ 1	3696 $\pm$ 295 <sup>f</sup>
Ang II $10^{-4}$ M + PD $10^{-7}$ M + VEGF	6 $\pm$ 1	4110 $\pm$ 200

<sup>a</sup>  $p = 0.005$  vs. control.

<sup>b</sup>  $p = 0.03$  vs. control.

<sup>c</sup>  $p = 0.01$  vs. control.

<sup>d</sup>  $p = 0.008$  vs. control.

<sup>e</sup>  $p = 0.012$  vs. VEGF alone.

<sup>f</sup>  $p = 0.032$  vs. Ang II  $10^{-4}$  M alone.

clarifies one of the controversies over why Ang II induces vascular rarefaction as well as promotes angiogenesis. Furthermore, our data is in agreement with other reports that AT<sub>2</sub> receptor inhibition counteracts the VEGF-induced endothelial cell Akt activity both in low and high concentrations of Ang II. It has recently been reported that AT<sub>2</sub> receptor-mediated deactivation of Akt may exert pro-apoptotic actions (Cui et al., 2002). Thus, another explanation for the inhibition of ECs survival might be the induction of endothelial cell apoptosis by AT<sub>2</sub> receptor (Lehtonen et al., 1999) and we might expect that the induction of apoptosis by AT<sub>2</sub> receptors become relevant after prolonged incubation, and is probably responsible for the inhibitory effect of Ang II on VEGF-induced endothelial cell survival. In the present study, low concentration Ang II induced endothelial cell Akt phosphorylation, which was probably mediated through AT<sub>1</sub> receptor signalling. The AT<sub>2</sub> receptor antagonist PD123319 significantly increased Akt phosphorylation both in low and high concentrations, which strongly suggests anti-angiogenic and AT<sub>1</sub> receptor-antagonistic actions were mediated by the AT<sub>2</sub> receptor. Ang II has recently been found to cause rapid phosphorylation of the Akt mediated by PI-3K pathway (Das et al., 2004) and this process is blocked by a PI-3K inhibitor Wortmannin or by the over-expression of catalase, indicating that Ang II potentiates Akt phosphorylation in a redox-regulate pathway. In fact, evidence is rapidly accumulating to support the theory that Ang II stimulate NAD(P) H oxidase-dependent superoxide(O<sub>2</sub><sup>-</sup>) generation in ECs, and that the major source of ROS in ECs is a NAD(P) H oxidase (Ushio-Fukai, 2006). Through this pathway, it seems possible to explain why low concentration of Ang II enhances transient Akt phosphorylation, but higher concentration of Ang II inhibits Akt phosphorylation.

Further, a growing body of evidence indicates that endothelial cells rarefaction usually expresses as endothelial cells apoptosis. Caspases are a family of proteases that mediate cell death and are important to the process of apoptosis. Caspase-3 is one of the critical enzymes of apoptosis with its cleavage leading to cell death including chromatin condensation and DNA fragmentation. Ohashi et al.'s study indicated that low concentration of Ang II induced inhibition of apoptosis in ECs by PI-3K/Akt pathway (Ohashi et al., 2004). Additional, our results showed that there was strongly cleaved caspase-3 expression when endothelial cells were applied with high concentration of Ang II, whereas, stimulation with VEGF attenuated the cleaved caspase-3 expression.

However, we need to identify whether ECs apoptosis induced by high concentration of Ang II is mediated by the PKB/Akt pathway, and whether cross-talk exists with the PKB/Akt pathway causing ECs apoptosis. Accumulating studies indicate that endothelial dysfunction is associated with an increase of p38-MAPK activity in endothelial cells, and that this influences the cellular decision regarding migration and proliferation. Prolonged activation of p38-MAPK may result in an anti-angiogenic phenotype that contributes to endothelial dysfunction, however activation of p38-MAPK is not sufficient to induce apoptosis (McMullen et al., 2005). Another report indicated that stress induced apoptosis mediated through the

p38-MAPK pathway and inhibition of PI-3K/Akt and MAPK pathways markedly up-regulated stress-mediated p38-MAPK activation resulting in enhanced endothelial cell apoptosis, which indicated that local balance between pro- and anti-apoptotic stimuli as well as different related signal transduction pathways will determine the fate of each individual cell (Gratton et al., 2001). In contrast, VEGF-treated endothelial cells are protected by stress induced apoptosis through the PI-3K/Akt pathway. Our study once again demonstrated the VEGF stimulated ECs apoptosis signalling is mediated by PI-3K/Akt, but not by p38-MAPK. Study showed that p38-MAPK activation inhibited endothelial cells proliferation, differentiation and tube formation through the stimulation of FGF-2 but not through VEGF (Matsumoto et al., 2002).

On the other hand, although the presence of high concentration Ang II significantly reduces HUVECs cell proliferation, it can still be stimulated by VEGF. However, even if Ang II type II receptor antagonist PD123319 was added to the media before application of stress for 30 min, the tube formation numbers were more than the group of stress alone but less than the control.

In conclusion, our research described in this paper indicated that ECs survival signal pathway-Akt activity is regulated by many kinds of factors, with VEGF playing a significant role in stimulating Akt phosphorylation. Ang II, initiated by NAD(P)H, activates its two types of receptors to produce a biphasic effect on Akt phosphorylation. High concentration of Ang II induces cleaved caspase-3 expression and cell death. Cell viability is regulated by the PI-3K/Akt pathway with no effect of p38-MAPK pathway under Ang II/VEGF application. On the other hand, the regulatory action is not balanced, with VEGF able to protect HUVECs proliferation but providing only partial protection for tube formation from exogenous stress.

### Acknowledgement

This study was funded by University of Warwick. We thank the labour ward of Walsgrave Hospital to provide the human umbilical cords.

### References

- Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J., Keshet, E., 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med.* 1, 1024–1028.
- Benndorf, R., Bogerm, R.H., Ergun, S., Steenpass, A., Wieland, T., 2003. Angiotensin II type 2 receptor inhibits vascular endothelial growth factor-induced migration and in vitro tube formation of human endothelial cells. *Circ. Res.* 93 (5), 438–447.
- Coffer, P.J., Jin, J., Woodgett, J.R., 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* 335, 1–13.
- Cui, T.X., Nakagami, H., Nahmias, C., Shiuchi, T., Takeda-Matsubara, Y., Li, J.M., Wu, L., Iwai, M., Horiuchi, M., 2002. Angiotensin II subtype 2 receptor activation inhibits insulin-induced phosphoinositide 3-kinase and Akt and induces apoptosis in PC12W cells. *Mol. Endocrinol.* 16, 2113–2123.
- Das, D.K., Maulik, N., Engelman, R.M., 2004. Redox regulation of angiotensin II signalling in the heart. *J. Cell. Mol. Med.* 8 (1), 144–152.
- Downward, J., 1998. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.* 10, 262–267.

- Dzau, V.J., 1994. Cell biology and genetics of angiotensin in cardiovascular disease. *J. Hypertens. Suppl.* 12, S3–S10.
- Fujikawa, K., de Aoa Scherpenseel, I., Jain, S.K., Presman, E., Christensen, R.A., Varticovski, L., 1999. Role of PI 3-kinase in angiotensin-1-mediated migration and attachment-dependent survival of endothelial cells. *Exp. Cell Res.* 253, 663–672.
- Gerber, H.-P., Dixit, V., Ferrara, N., 1998a. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J. Biol. Chem.* 273, 13313–13316.
- Gerber, H.P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V., Ferrara, N., 1998b. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* 273 (46), 30336–30343.
- Gliki, G., Wheeler-Jones, C., Zachary, I., 2002. Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3'-kinase-mediated PKC delta phosphorylation: role of PKC in angiogenesis. *Cell Biol. Int.* 26 (9), 751–759.
- Gobe, G., Browning, J., Howard, T., Hogg, N., Winterford, C., Cross, R., 1997. Apoptosis occurs in endothelial cells during hypertension-induced microvascular rarefaction. *J. Struct. Biol.* 118 (1), 63–72.
- Gratton, J.P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., Sessa, W.C., 2001. Akt down-regulation of p38 signalling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J. Biol. Chem.* 276, 30359–30365.
- Guo, D., Jia, Q., Song, H.-Y., Warren, R.S., Donner, D.B., 1995. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. *J. Biol. Chem.* 270, 6729–6733.
- Khwaja, A., 1999. Akt is more than just a bad kinase. *Nature* 401, 33–34.
- Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., Waterfield, M.D., 1996. The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J.* 15, 6584–6594.
- Lehtonen, Y.A., Horiuchi, M., Daviet, L., Akishita, M., Dzau, V., 1999. AT<sub>2</sub> receptor induces apoptosis via sphingolipid biosynthesis. *J. Biol. Chem.* 274, 16901–16906.
- Matsumoto, T., Turesson, I., Book, M., Gerwins, P., Claesson-Welsh, L., 2002. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J. Cell Biol.* 156 (1), 149–160 Jan 7.
- McMullen, M.E., Bryant, P.W., Glembotski, C.C., Vincent, P.A., Pumiglia, K.M., 2005. Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. *J. Biol. Chem.* 280 (22), 20995–21003.
- Munzenmaier, D.H., Greene, A.S., 1996. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension* 27, 760–765.
- Noon, J.P., Walker, B.R., Webb, D.J., Shore, A.C., Holton, D.W., Edwards, H.V., Watt, G.C., 1997. Impaired microvascular dilatation and capillary rarefaction in young adults with a predisposition to high blood pressure. *J. Clin. Invest.* 99 (8), 1873–1879.
- Ohashi, H., Takagi, H., Oh, H., Suzuma, K., Suzuma, I., Miyamoto, N., Uemura, A., Watanabe, D., Murakami, T., Sugaya, T., Fukamizu, A., Honda, Y., 2004. Phosphatidylinositol 3-kinase/Akt regulates angiotensin II-induced inhibition of apoptosis in microvascular endothelial cells by governing surviving expression and suppression of caspase-3 activity. *Circ. Res.* 94 (6), 785–793 Apr 2.
- Otani, A., Takagi, H., Suzuma, K., Honda, Y., 1998. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ. Res.* 82, 619–628.
- Risau, W., 1997. Mechanisms of angiogenesis. *Nature* 386 (6626), 671–674.
- Shiojima, I., Walsh, K., 2002. Role of Akt signalling in vascular homeostasis and angiogenesis. *Circ. Res.* 90 (12), 1243–1250.
- Stoll, M., Steckelings, U.M., Paul, M., Bottari, S.P., Metzger, R., Unger, T., 1995. The angiotensin AT<sub>2</sub>-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.* 95, 651–657.
- Tanimoto, T., Jin, Z.G., Berk, B.C., 2002. Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). *J. Biol. Chem.* 277 (45), 42997–43001.
- Ushio-Fukai, M., 2006. Redox signalling in angiogenesis: role of NADPH oxidase. *Cardiovasc. Res.* 71 (2), 226–235.
- Vicault, E., 1992. Hypertension and the microcirculation: a brief overview of experimental studies. *J. Hypertens.* 10, S59–S68.
- Wymann, M.P., Pirola, L., 1998. Structure and function of phosphoinositide kinases. *Biochim. Biophys. Acta* 1436, 127–150.
- Xia, P., Aiello, L.P., Ishii, H., Jiang, Z.Y., Park, D.J., Robinson, G.S., Takagi, H., Newsome, W.P., Jirousek, M.R., King, G.L., 1996. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J. Clin. Invest.* 98, 2018–2026.
- Yao, R., Cooper, G.M., 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 267 (5206), 2003–2006.
- Zachary, I., Gliki, G., 2001. Signalling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc. Res.* 49, 568–581.