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24 h pre-incubation of EA.hy926 cells with angiotensin II regulates insulin-dependent activation of eNOS in a concentration-dependent manner

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The rennin-angiotensin II system (RAS) and the insulin-PI3kinase signalling pathways cross-interact with important physiological and pathophysiological consequences for cells and the whole organism. Here, the effect of 24 h pre-incubation of EA.hy926 with two different concentrations of angiotensin II, on insulin-mediated activation of the PI3kinase-AKT-eNOS signalling was investigated. Quiescent EA.hy926 cells were treated with insulin (100 nM, 30 min) following 24 h pre-treatment with or without either 0.1 or 1 µM of angiotensin II. Cell lysates were immunoblotted for phospho AKT Ser-473, phospho eNOS Ser-1177 and normalized with β-actin. Homogenates of EA.hy926 treated with insulin in the presence or absence of 1 µM angiotensin II, were also subjected to nitric oxide synthase (NOS) activity assay using titrated arginine as substrate. To exclude cytotoxicity of the 1 µM angiotensin II concentration, Trypan blue cell viability assay as well as the microscopic examination of unstained and DAPI/Phalloidin stained EA.hy926 cells were undertaken. Insulin resulted in about 2 fold increase in phospho-eNOS Ser-1177 and 8 fold increase in phospho-AKT Ser-473 levels in treated compared to untreated cells. A 2 fold increase was observed in the NOS activity of insulin-treated and untreated cells preincubated with Ang II. 24 h pre-treatment with 0.1 µM Ang II did not significantly interfere with the responses to insulin but the 1 µM Ang II pre-treated EA.hy926 showed significant attenuated insulin induced phosphorylation of eNOS Ser-1177 and AKT Ser-473, alongside impaired NOS activity. The Ang II-treated cells showed normal nuclei and cytoskeletal architecture. Angiotensin II concentration-dependently regulated basal and insulin-mediated PI3Kinase-AKT-eNOS signalling in cultured endothelial cells.

Key words: 24 h pre-incubation, angiotensin II, regulates, insulin-PI3Kinase-AKT-eNOS signalling, concentration-dependently.

INTRODUCTION

Just like skeletal muscle cells, adipocytes and hepatocytes, endothelial cells are important targets for insulin action

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(Kubota et al., 2011). Endothelial cells increase their nitric oxide (NO) output in response to insulin through the calcium-independent PI3kinase mediated enhancement of endothelial NO synthase (NOS III) activity (Muniyappa et al., 2011). The released NO dissolves in the aqueous phase of the circulating blood and diffuses to reach vascular smooth muscle cells resulting in smooth muscle relaxation and vasodilation (Zhao et al., 2015). Insulin binds to and activate its receptor, to recruit and activate the insulin receptor substrate (IRS)-PI3-Kinase-AKT signalling upstream of eNOS (Lizcano et al., 2002). Endothelial dependent vasodilation is vital to the regulation of vascular tone and blood pressure (Coretti et al., 2002; Yunn et al., 2016). NO also possesses anti-atherosclerotic, anti-inflammatory and anti-coagulative functions as well as being vital for vasculogenesis (Brasier et al., 2004; Ahiuwalia et al., 2004). In chronic disorders such as type II diabetes mellitus, obesity, dyslipidemias, dysmetabolic syndrome, cardiac failure and coronary heart diseases, insulin-target cells are less responsive to insulin (Greenfield and Campbell, 2004; Nolan et al., 2011).

The earliest manifestation of endothelial resistance to insulin constitute endothelial dysfunction, characterised mainly by impaired activation of the Insulin-PI3kinase-AKT-eNOS signalling in a setting of either normo or hyperinsulinaemia (Wheatcroft et al., 2003; Calles-Escandon and Cipolia, 2001; Cefalu, 2001). The consequential impaired NO-dependent vasodilation leads to heightened risk of cardiovascular morbidities and mortalities (Mombouli and Vanhoutte, 1999; Tangvarasittichai, 2015; Abbasi et al., 2016; Haffner et al., 2000). The blood concentration of Ang II is elevated in insulin-resistant states as a result of the activation of the rennin aniotensin aldosterone system (RAAS) (Surapongchai et al., 2017; Mehta and Griendling, 2007). Inhibition of Ang II action with either Ang II type-1 receptor antagonists or angiotensin converting enzyme inhibitors (ACEI) could restore insulin-sensitivity in hypertensives and type II diabetic patients (Pavo et al., 2003; Shiuchi et al., 2004). Apart from Ang II, other pro-inflammatory cytokines including TNF-alpha and IL-6 are elevated and implicated in the pathogenesis of insulin resistance (Bastard et al., 2006; Shoeison et al., 2006). These peptide hormones and cytokines provide useful tools for the *in vitro* induction of insulin resistance in cultured target cells (Bastard et al., 2006; Shoeison et al., 2006; Andreozzi et al., 2004).

Pre-treatment of skeletal muscle cells with Ang II induced insulin-resistance manifested as impaired insulin-mediated glucose transporter 4 (GLUT 4) translocation from intracellular storage sites to the plasma membrane and intracellular glucose uptake (Wei et al., 2008). Acute pre-treatment of cultured endothelial cells (ECs) with Ang II impaired insulin-PI3Kinase-AKT-eNOS signalling via the induction of serine phosphorylation of IRS resulting in impeded tyrosine Phosphorylation needed for IRS dependent PI3K activation (Andreozzi et al., 2004). Here, the effect of 24 h pre-incubation of confluent monolayer of EA.hy926 cells with two different concentrations of Ang II to determine any concentration-dependent regulation of the insulin-PI3K-eNOS signalling was investigated.

METHODOLOGY

Materials

EA.hy926 cells (ATCC, USA), monoclonal rabbit phospho AKT-Ser 473, phospho eNOS Ser-1177 antibodies, polyclonal secondary goat anti-rabbit, and anti-mouse antibodies were from cell signalling technology. Dubelcco's Modified Eagles Medium (DMEM), fetal bovine serum, penicillin, streptomycin, angiotensin II, polyclonal mouse β-Actin primary antibody, sodium dodecyl sulfate (SDS), polyacrylamide, Trypan blue dye, diamidino-2-phenylindole dihydrochloride (DAPI), rhodamine-phalloidin, insulin, methanol, sodium orthnovanidate, betaglycerophosphate, protease inhibitor cocktail, ammonium persulfate TEMED, nicotinamide adenine dinucleotide phosphate (NADPH), scintillation fluid, bovine serum albumin, Tween-20, polyvinylidene difluoride (PVDF) membrane, glycine, sodium chloride, sodium EDTA, Trypan blue dye, PBS, glycine, tertrahydrobiopterine (BH4) and Triton X-100 were from Sigma Aldrich. ECL solution A and B, NOS activity assay kit (Caymans), and titrated arginine were from Cambridge Bioscience. Tris HCl and sodium deoxycholate were from Fishers Scientific. Bradford ultra protein assay kit was from Expedeon protein solutions.

Cell culture

Cells were cultured and maintained in DMEM containing 5 mM glucose supplemented with 10% foetal bovine serum, 1 µM BH4, 100 U/ml of penicillin and 100 µg/ml of streptomycin. They were used for experiments when they became 80 to 100% confluent in either 10 c or 6 cm corning cell culture dishes. They first had their culture media replaced with serum free DMEM conditioned with or without either 0.1 or 1 µM Ang II for 24 h in the incubator (air 95%, O_2 5% and humidified). At the end of the Ang II pre-incubation period, the cells were treated with or without insulin (100 nM, 30 min). Phenol red free DMEM was used as starvation and treatment media in the NOS activity experiments.

Western blot

The cells were rinsed with ice cold wash buffer (1X PBS, Na_2VO_3, EDTA) and lysed in 200 µl of RIPA lysis buffer (50 mM Tris HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% w/v SDS, 200 µM Na_2VO_4, 1 mM lytic cocktail) on ice for 10 min, harvested with the aid of a plastic cell scrapper and transferred into 0.5 ml Eppendorf. The lysate was clarified by micro centrifugation (10000 g, 10 min) before the proteins in 100 µl were resolved by SDS polyacrylamide gel electrophoresis (4% stacking gel, pH 6.8; 8.5% resolving gel, pH 8.0), in a running buffer (190 mm glycine, 25 mM Tris, 0.1% SDS). Resolved gel proteins were transferred to PVDF membrane by passing a constant current of 100 mA for 4 h through the transfer buffer (190 mM glycine, 25 mM Tris, 20% methanol). The protein blots were subsequently incubated with polyclonal primary antibodies to β-actin (1:10000), phospho AKT Ser-473 (1:1000) and phospho eNOS Ser-1177 (1:1000) for 1 h each before they were washed and probed with polyclonal goat anti-mouse (1:5000) and anti-rabbit (1:1000) for 1 h

each. Following repeat final washes, the immunoblots were incubated with ECL chemilumniscence solution A and B. Detection of protein bands was with the aid of Chemidoc (Biorad). Protein quantification was with Imagelab software (Biorad).

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NOS activity assay

Treated and untreated confluent cells in 10 cm corning cell culture dishes were rinsed with ice cold wash buffer (1X PBS, 1 mM EDTA) and then harvested with a cell scrapper in 1 ml of same buffer. They were subsequently pelleted by centrifugation (10000 g, 5 min) and the wash buffer discarded before homogenizing in 50 µl homogenization buffer by sonicating for 20 min on ice. Splashed homogenate on the sides of the microfuge tubes were recovered by repeating the spin for 1 min before pipetting up and down to disperse, and the protein content of the supernatant was determined (Bradford Ultra Protein Assay). The protein concentrations of all samples were standardized to 5 mg/ml or more. The NOS activities of the homogenates were measured by the rate of conversion of titrated arginine to titrated citrulline according to the assay kit manufacturer's instruction.

Cell viability assay

Confluent EA.hy926 cells were serum starved for 24 h in DMEM with or without 1 µM Ang II conditioning before they were detached by trypsinization and re-suspended in 1 ml of PBS. 50 µl of each cell suspension was incubated with equal volume of 4% Trypan blue on ice for 5 min (Strober, 2001; Altman et al., 1993). Thereafter, 20 µl of the dispersed cell-dye mixture was counted with a haemocytometer and the number of dead cells (dark-stained from dye retention) and total number of cells were counted for 10 squares. The percentage of cell viability was subsequently calculated from:

$$\text{Cell viability (\%)} = \frac{\text{Total number of live cells}}{\text{Total number of cells}} \times 100$$

Microscopy

Normal and Ang II induced insulin-resistant cultured EA.hy926 cells were first examined under the light microscope (x40 magnification). Thereafter, EA.hy926 cells grown over glass cover slips were fixed in 4% paraformaldehyde and stained with DAPI (1:20000 dilution of 2 mg/ml stock) and Rhodamine conjugated Phalloidin (1:100 methanol dilution of 0.5 mg/µl DMSO stock), to view their actin cytoskeleton using the Snapshot Widefield fluorescence microscope. Image capturing was with the Coolsnap camera at x20 magnification.

Statistical analyses

Results are expressed as mean ± standard error mean (SEM) of 3 or more independent experiments and statistical analyses was with graph pad prism 7.0 using either the student's t test or one way analysis of variance (ANOVA) with Tukey's multiple comparison tests. Level of statistical significance was set at P values less than 0.05.

RESULTS

Initial assessment of EA.hy926 cell responses to insulin treatment (Figure 1A to D) showed that the level of phosphorylated AKT Ser-473 was 780.20 ± 134.00% of untreated cells (n=11) while phosphorylated eNOS Ser-1177 level was 205.90 ± 33.15% of untreated cells (n=14). The NOS activity in insulin-treated cells was 363.30 ± 60.68% of untreated cells (n=5). The level of phosphorylated eNOS Ser-1177 in insulin-treated EA.hy926 following their pre-incubation with either 0.1 or 1 µM Ang II for 24 h were 151.54 ± 12.91% and 90.58 ± 20.23% of their respective treatment-matched control, compared to 166.99 ± 23.07% obtained in insulin-treated normal cells (Figure 2A). Insulin-induced NOS activity in Ang II pre-treated cells was 66.47 ± 7.82% of control compared to 181.90 ± 11.25% in non-Ang II pre-treated cells (Figure 2B). The basal level of phosphorylated eNOS Ser-1177 in EA.hy926 cells pre-treated with Ang II (0.1 or 1 µM Ang II, 24 h) were 120.36 ± 6.30 and 171.38 ± 21.44% of untreated cells, respectively (Figure 3A). Basal NOS activity was also increased following 24 h 1 µM Ang II pre-treatment (220.70 ± 26.73% of untreated cells (Figure 3B). Microscopic examination of 1 µM Ang II treated cells and untreated cells showed normal histology (Figure 4A and B) and actin cytoskeleton (Figure 4C and D). The percentages of cell viability assessed using the

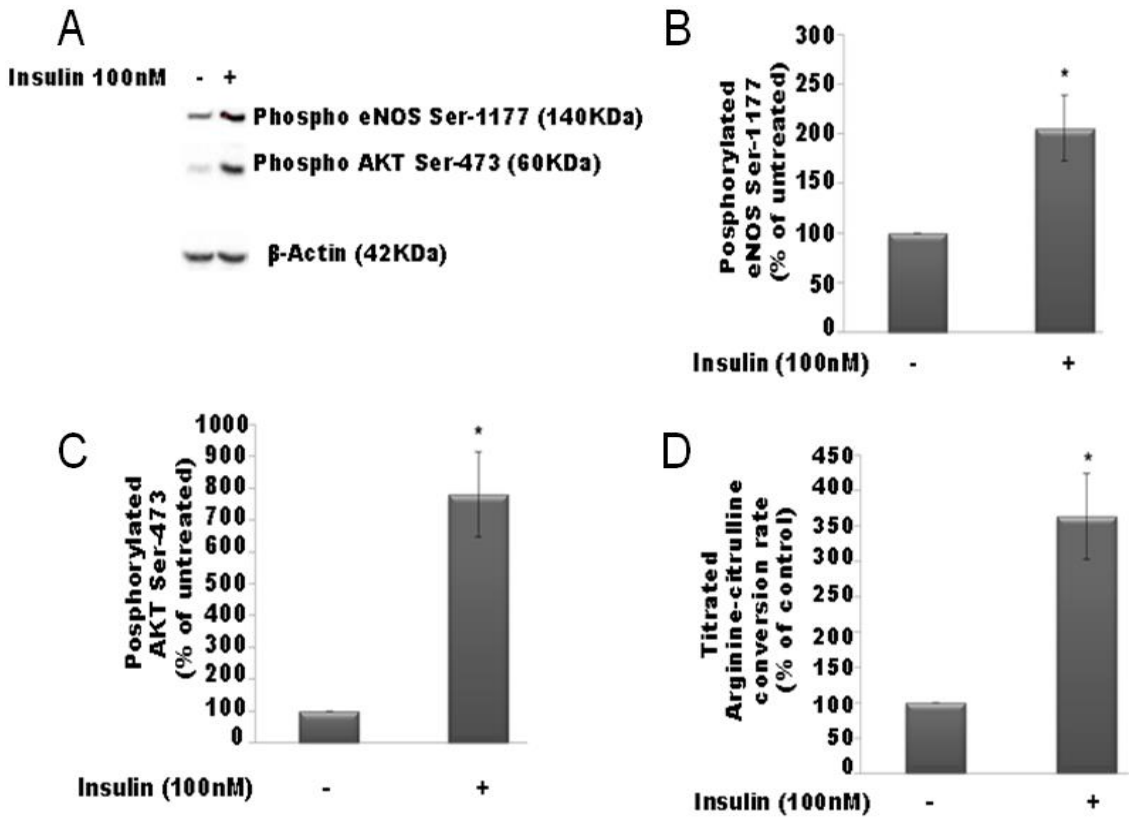

Figure 1. EA.hy926 cell responses to insulin. The effect of treating quiescent EA.hy926 cells with insulin (100 nM, 30 min). (**A**) Representative immunoblot of phosphorylated eNOS Ser-1177, phosphorylated AKT Ser-473 and β-actin of insulin treated and untreated EA.hy926 cells. (**B**) Densitometry of phospho eNOS Ser-1177 shows that insulin resulted in 2 fold increase in the mean level of phosphorylation of eNOS at Ser-1177 (*P< 0.05, Student's t test). Data are mean ± SEM of 14 separate experiments. (**C**) Densitometry of phospho AKT Ser-473. Insulin treatment resulted in 8 fold increase in the mean level of phosphorylation of AKT at Ser-473 relative to untreated controls (*P<0.05, Student's t test). Data are mean ± SEM of 11 different experiments. (**D**) NOS activity of EA.hy926 cell homogenates measured by the rate of conversion of titrated Arginine to citrulline expressed a percentage of untreated control. Insulin resulted in 4 fold increase in the NOS activity of treated EA.hy926 cells compared to untreated controls (*P<0.05, Student's t test). Data represent mean ± SEM of 5 separate experiments.

Trypan blue dye exclusion assay were 97.55 ± 1.22 and 99.11 ± 0.03 in untreated and 1 µM Ang II-treated EA.hy926 cells, respectively (Figure 5).

DISCUSSION

Treatment of serum-starved EA.hy926 cells with insulin (100 nM, 30 min), resulted in significant activation of their PI3K-AKT-eNOS pathway, evident from the increased levels of AKT phosphorylated at Ser-473 and eNOS phosphorylated at Ser-1177 on immunoblots of treated EA.hy926 cell lysates as compared to untreated controls (Figure 1A, B and C). The observations here are in line with previously published data (Montagnani et al., 2001; Repetto et al., 2006; Xiao et al., 2015). The increased level of insulin-mediated eNOS phosphorylation observed in immunoblots was further corroborated by the observation of significantly higher NOS activity of insulin-treated EA.hy926 cells compared to untreated controls (Figure 1D), in line with previously reported data where resveratrol induced eNOS phosphorylation at Ser-1177 correlated with enhanced NOS radioactivity (Notas et al., 2006). Taken together, both results suggest enhanced NO output from EA.hy926 cells following insulin stimulation. *In-vivo*, NO produced by endothelial cells dissolves in the aqueous phase of the blood and diffuses to influence vascular smooth muscle cells (VSMCs) by activating their guanylyl cyclase with consequent activation of myosin light chain dephosphorylase, which dephosphorylates the myosin light chains, resulting in the dissociation of the myosin light chain heads from actin culminating in smooth muscle relaxation (Ling et al., 2015; Sausbier et al., 2000; Somiyo and Somiyo, 2003; Lincoln et al., 2001). Dysregulation of this endothelial NO-dependent vasodilation plays important pathogenic

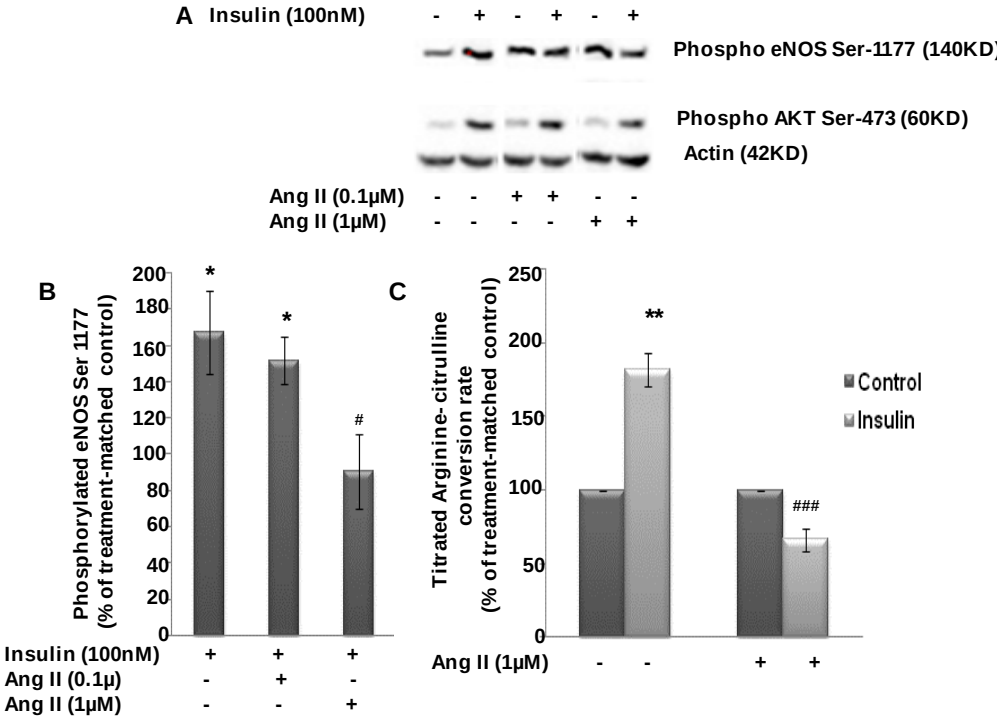

Figure 2. Insulin responses of EA.hy926 cells pre-treated with two different concentrations of Ang II. (A) Representative immunoblot of insulin-treated and untreated EA.hy926 cell lysates showing phosphorylated eNOS Ser-1177, phosphorylated AKT Ser-473 and β- actin. (B) Densitometry of phosphorylated eNOS Ser-1177 expressed as percent of treatment-matched control. Insulin resulted in significant increase in the levels of phosphorylation of eNOS at Ser 1177 in Ang II-untreated and 0.1μM Ang II pre-treated EA.hy926 cells but the response in 1μM Ang II pre-treated EA.hy926 cells were significantly impaired relative to untreated cells. The results represent the mean ± SEM of 3-4 separate experiments. (C) NOS activity of insulin treated and untreated EA.hy926 cells in the presence or absence Ang II pre-treatment (1μM, 24 h). The insulin mediated increase in the NOS activity was significantly impaired in the presence of Ang II pre-treatment (**P<0.01 vs. treatment-matched control, $^{###}$P<0.01 vs. insulin treated in the absence of Ang II, One way ANOVA, Tukey's multiple comparison). Data represent mean ± SEM of 3 separate experiments

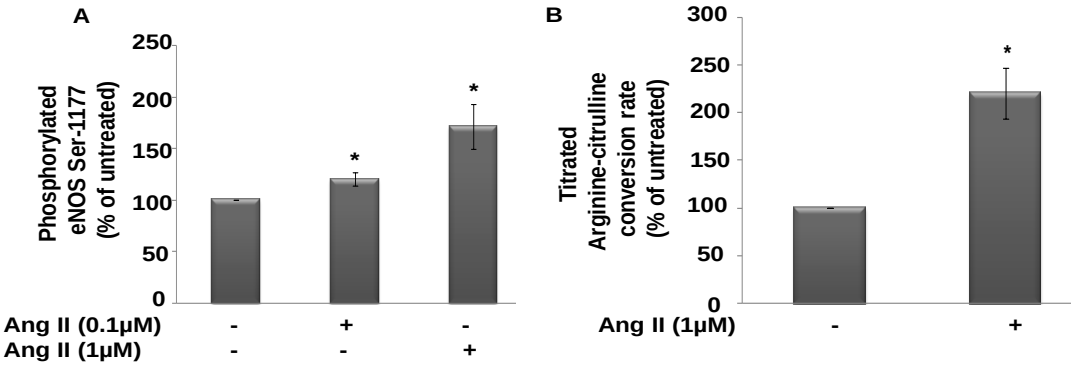

Figure 3. Basal eNOS activation is enhanced following 24 h Ang II pre-treatment of EA.hy926 cells. (**A**) Densitometry of anti-phospho eNOS Ser-1177 immunoblot of EA.hy926 pre-treated with or without Ang II (0.1 μM or 1 μM, 24 h). The level of phosphorylated eNOS Ser-1177 was significantly higher in Ang II pre-treated cells (120 ± 6.0% and 171 ± 21% of untreated for 0.1 and 1 μM respectively) compared to untreated (*P<0.05 versus untreated, Student's t test. Data represents mean ± SEM of 3-4 independent experiments. (**B**) NOS activity of EA.hy926 in the presence or absence of Ang II pre-treatment (1 μM, 24 h). NOS activity was higher in the 1 μM Ang II pre-treated cells (221 ± 26.73% of untreated (*P<0.05, Student's t test). Data represents mean ± SEM of 4 independent experiments.

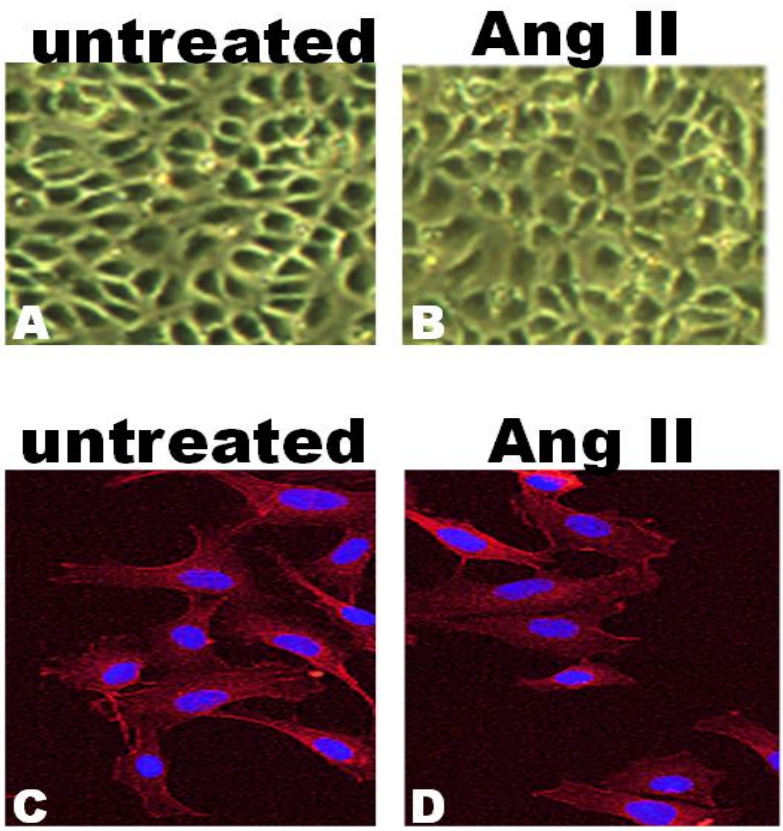

Figure 4. Effect of high Angiotensin II concentration on morphology of EA.hy926 cells. Top panel represents light photomicrograph of EA.hy926 cells (A) in the absence and (B) presence of Ang II (1 µM, 24 h). Ang II pre-treated cells remained adherent to their cell culture dishes and showed no morphological distortions relative to untreated cells. Bottom represents fluorescence photomicrograph of EA.hy926 cells (C) in the absence of Ang II and (D) presence of Ang II (1 µM, 24 h). There were no observable distortions in the architecture of the nuclei and actin cytoskeleton in Ang II pre-treated cells relative to untreated cells.

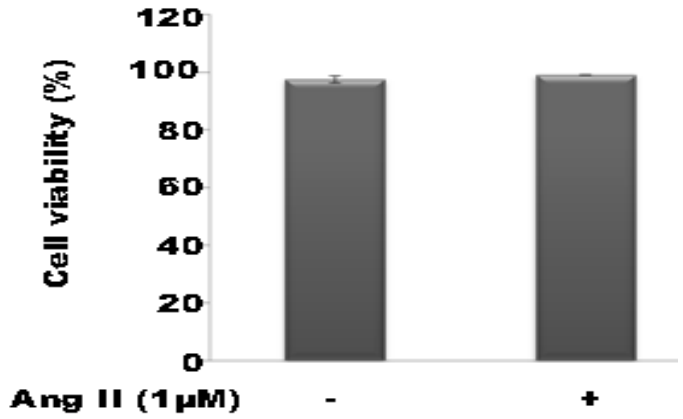

Figure 5. Effects of Ang II on the Viability of EA.hy926 cells. Trypan blue dye exclusion assay of EA.hy926 cells pre-treated with or without Ang II (1 µM, 24 h). Ang II pre-treatment did not affect the cell viability ($P>0.05$, Student's t test. The result represents the mean ± SEM of 3 separate experiments.

role in essential hypertension and insulin-resistant states such as obesity and type II diabetes mellitus (Schalkwijk and Stehouwer, 2005; Fontes-Guerra et al., 2015; Prieto et al., 2014; Tangvarasittichai, 2015). Hence, impaired NO-dependent vasodilation is a common feature in cardiovascular morbidities associated with these disease phenotypes (Giles et al., 2012; Mohan et al., 2010). Besides its role in regulating vascular tone, the anti-coagulating, anti-inflammatory and anti-apoptotic functions of NO produced by endothelial cells are important for maintenance of cardiovascular health (Ahiuwalia et al., 2004; Lamb and Goldstein, 2008; Randomski et al., 1990; Esmon and Esmon, 2011). The results from the Western blot experiments showing how 24 h Ang II (either 0.1 or 1 µM) pre-incubation of EA.hy926 differentially affected their response to insulin-treatment (100 nM, 30 min) were revealed because of the contrasting outcomes (Figure 2A and B). With the 0.1 µM Ang II pre-treatment prior to insulin stimulation, the eNOS Ser-1177 and AKT Ser-473 phosphorylation responses were not significantly altered relative to cells treated with only insulin. On the contrary, EA.hy926 cells pre-incubated with the 1 µM Ang II concentration for 24 h prior to stimulating with insulin, showed diminished insulin-mediated phosphorylation of AKT at Ser-473 and eNOS at Ser-1177 compared to none-Ang II treated cells. One previous study suggested that pre-incubation of primary HUVECs with 0.1 µM Ang II for a shorter time duration of 30 min prior to stimulation with insulin, impaired the insulin-PI3kinase signalling response via a different mechanism involving negative regulation of ins-PI3K-AKT signalling (Andreozzi et al., 2004), but no record can be found describing the effects of 24 h Ang II pre-incubation of EA.hy926 on insulin signalling via this pathway. Another study which reported the Ang II induced differential insulin action in rat skeletal muscles was conducted *in vivo* (Surapongchai et al., 2017). Although it suggested that a lower concentration of 100 ng/ml was more effective than 500 ng/ml at impairing insulin action, the findings does not invalidate the results of this *in vitro* study as the investigators did not determine the equilibrium Ang II concentration at the tissue level, prior to homogenization. The 1 µM concentration of Ang II had been previously reported to induce apoptosis in cultured HUVECs using detection by flow cytometry (Xiao et al., 2015) or the DNA fragmentation test (Dimmeler et al., 1997), an effect that was linked to attenuated endothelial NO production in both studies. Even though the former study also reported a differential proapototic effects following 24 h pre-incubation of HbmECs with 0.1 and 1 µM Ang II concentrations, it failed to extend the evaluation to any concentration-dependent effect in terms of changes in the levels of phosphorylated AKT Ser-473, phosphorylated eNOS Ser-1177 nor NO output of basal and agonist treated cells like in the present study, as the 0.1 µM concentration was adopted in the subsequent parts of the report. Contrary to the aforementioned proapoptotic studies, this study has not observed any significant alteration in the percentage cell viability of dish-adherent EA.hy926 post 24 h incubation with the 1 µM Ang II, using the Trypan blue viability assay technique (Figure 5). Although this dye exclusion viability assay is a less sensitive assay than ELISA and flow cytometry used in both previously reported studies. The Ang II pre-treated cells appeared normal under the light microscope; they neither showed significant distortion in their nuclei nor actin-cytoskeletal architecture compared to untreated cells (Figure 4C and D). Taken together, these data seem to suggest that EA.hy926 cells well-tolerated 24 h pre-incubation with the 1 µM Ang II concentration and this concentration seems to be more effective at modelling insulin-resistance in cultured ECs than the 0.1 µM concentration used in earlier *in vitro* acute drug exposure experiment (Andreozzi et al., 2004). Although it would seem to be in line with a recent study involving a different agonist from insulin (Xiao et al., 2015; Li et al., 2016), the observation in both studies differ in several aspects. While in the present study basal phosphorylated AKT Ser-473, phosphorylated eNOS Ser-1177 and NOS activity increased following 24 h pre-treatment with Ang II in a concentration dependent manner (Figure 3). Xiao et al. (2015) reported it diminished as the concentration of Ang II was increased. The observation in this study is novel as it highlights the significant contribution of accentuated basal NOS activation to the attenuated response to insulin by Ang II pre-treated EA.hy926 cells, when the data are normalized as ratios of treatment-matched controls.

The mechanisms underlying the contrasting outcomes of pre-treatment with the two different concentrations of Ang II on EA.hy926 response to insulin as reported in this study were not elucidated, but several possibilities could be inferred. Firstly, endothelial cells have been reported to produce angiotensin converting enzyme 2 (ACE 2), capable of degrading Ang II *in vitro* (Lovren et al., 2008). It is possible that with the smaller concentration of 0.1 µM, most of the added Ang II is degraded from the starvation medium, in the course of the 24 h pre-incubation of the cells. On the contrary, in cells treated with 1 µM Ang II, the effect was sustainable throughout the pre-incubation period due to the availability of some intact Ang II in the medium at the point of stimulation with insulin, despite the degradative action of the endothelial ACE 2. To exclude this as a possible underlying mechanism behind the Ang II-induced differential response of EA.hy926 cells to insulin, further work would have to be carried out to measure the residual Ang II concentration in the starvation medium at the end of the 24 h incubation period. ACE 2 degrades Ang II to yield Ang (1-7) which can activate the PI3K-AKT-eNOS signalling (Yang et al., 2012; Shi et al., 2015; Xiao et al., 2015). More Ang(1-7) could have been formed with the higher concentration of 1 µM Ang II, resulting in the relatively higher basal phosphorylated eNOS Ser-1177 levels and NOS activity in 1 µM Ang II pre-treated cells as compared to untreated and 0.1 µM Ang II pre-treated

cells (Figure 2A and B).

Another possible mechanism underlying the contrasting outcome seen here with the two different pre-treatment concentrations of Ang II could be differential expression of the agonist receptor subtypes. To exclude this, it would be necessary to experimentally compare AT1 and AT2 receptor proteins expression by EA.hy926 cells incubated under the two different Ang II concentrations. While relative AT1R predominance will favour an impaired insulin-response, a relatively predominant expression of AT2R could either enhance or impair insulin response depending on whether the data is normalized as a ratio of treatment-matched controls or not. This hypothesis could be further investigated by assessing changes in the receptor subtype expression under both treatment conditions at both protein and gene levels. Thirdly, it is also possible that differences in the extent of activation of NADPH oxidase system by Ang II in the two different conditioned starvation media, prior to stimulating with insulin, contributed to the different experimental outcomes. ROS are known to activate other serine/ threonine kinase pathways such as JUNK and MAPK that negatively regulate the insulin-PI3Kinase signalling (Andreozzi et al., 2004). Another interesting observation in this study was the fact that the basal eNOS Ser-1177 phosphorylation was higher in the Ang II induced insulin-resistant EA.hy926 versus none Ang II-treated controls (Figure 3A). This response could be reactive and could support the hypothesis of differential expression of the Ang II receptor subtypes, as Ang II acting via AT2R could induce eNOS Ser-1177 phosphorylation via the Bradykinin activation pathway (Yayama et al., 2006) or ACE 2 mediated production of Ang (1-7) from Ang II concentration dependently (Tassone et al., 2013; Li et al., 2016; Xiao et al., 2015). Incremental changes in basal phosphorylation of eNOS at Ser 1177 in the 1 µM Ang II compared to 0.1 µM pre-treated cells were observed. The incremental trend seen in the Ang II induced eNOS phosphorylation may be due to differences in either the residual Ang II or generated Ang (1-7) concentration following ACE 2 degradative action. Similar observation was made with the NOS activity of 1 µM Ang II pre-treated cells being significantly higher than basal activity (Figure 3B).

In conclusion, while 24 h pre-incubation of EA.hy926 with 1 µM concentration of Ang II significantly attenuated both the insulin-mediated phosphorylation of eNOS and NOS activity, the 0.1 µM concentration was not so effective in this study. The reactive enhancement of basal eNOS activation following 24 h pre-incubation with Ang II is thought to have made important contributions to the observed impaired response to insulin by EA.hy926 cells, when the data are expressed as a percentage of treatment-matched controls, as in the present study. The cells also tolerated the 1 µM Ang II concentration well enough, suggesting a suitable *in vitro* cell culture model of endothelial insulin-resistance.

Investigating the relative expression of the AT1 and AT2 receptor protein as well as the quantification of the residual Ang II and Ang (1-7) generated following the degradative actions of ACE 2, in the cell starvation media at the end of the 24 h incubation period, could further validate this limited study.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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REFERENCES

Abbasi F, Kohli P, Reaven GM, Knowles JW (2016). Hypertriglyceridemia: A simple approach to identify insulin resistance and enhanced cardio-metabolic risk in patients with prediabetes. Diabetes Research and Clinical Practice 120:156-161.

Ahluwalia A, Foster P, Scotland RS, McLean PG, Mathur A, Perretti M, Moncada S, Hobbs AJ (2004). Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. Proceedings of the National Academy of Sciences of the United States of America 101(5):1386-1391.

Altman SA, Randers L, Rao G (1993). Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. Biotechnology Progress 9(6):671-674.

Andreozzi F, Laratta E, Sciacqua A, Perticone F, Sesti G (2004). Angiotensin II impairs the insulin signaling pathway promoting production of nitric oxide by inducing phosphorylation of insulin receptor substrate-1 on Ser312 and Ser616 in human umbilical vein endothelial cells. Circulation Research 94(9):1211-1218.

Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B (2006). Recent advances in the relationship between obesity, inflammation, and insulin resistance. European Cytokine Network 17(1):4-12.

Brasier AR (2010). The nuclear factor-κB–interleukin-6 signalling pathway mediating vascular inflammation. Cardiovascular Research 86(2):211-218.

Calles-Escandon J, Cipolla M (2001). Diabetes and endothelial dysfunction: a clinical perspective. Endocrine Reviews 22(1):36-52.

Cefalu WT (2001). Insulin resistance: Cellular and clinical concepts. Experimental Biology and Medicine 226(1):3-26.

Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard- Herman M, Herrington D, Vallance P (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow- mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of the American College of Cardiology 39(2):257-265.

Dimmeler S, Rippmann V, Weiland U, Haendeler J, Zeiher A M (1997). Angiotensin II induces apoptosis of human endothelial cells. Circulation Research 81(6):970-976.

Esmon CT, Esmon NL (2011). The link between vascular features and thrombosis. Annu. Physiological Reviews 73:503-514.

Fontes-Guerra PC, Cardoso CR, Muxfeldt ES, Salles GF (2015). Nitroglycerin-mediated, but not flow-mediated vasodilation, is associated with blunted nocturnal blood pressure fall in patients with

resistant hypertension. Journal of Hypertension 33(8):1666-1675.
Giles TD, Sander GE, Nossaman BD, Kadowitz PJ (2012). Impaired vasodilation in the pathogenesis of hypertension: focus on nitric oxide, endothelial-derived hyperpolarizing factors, and prostaglandins. Journal of Clinical Hypertension 14(4):198-205.
Greenfield JR, Campbell LV (2004). Insulin resistance and obesity. Clinics in Dermatology 22(4):289-295.
Haffner SM, Mykkänen L, Festa A, Burke JP, Stern MP (2000). Insulin-resistant prediabetic subjects have more atherogenic risk factors than insulin-sensitive prediabetic subjects. Circulation 101(9): 975-980.
Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, (2011). Impaired insulin signaling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. Cell Metabolism 13(3):294-307.
Lamb RE, Goldstein BJ (2008). Modulating an oxidative-inflammatory cascade: potential new treatment strategy for improving glucose metabolism, insulin resistance, and vascular function. International Journal of Clinical Practice 62(7):1087-1095.
Li J, Geng XY, Cong XL (2016). PGC-1α ameliorates AngiotensinII-induced eNOS dysfunction in human aortic endothelial cells. Vascular Pharmacology 83:90-97.
Lincoln TM, Dey N, Sellak H (2001). Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. Journal of Applied Physiology 91(3):1421-1430.
Ling WC, Lau YS, Murugan DD, Vanhoutte PM, Mustafa MR (2015). Sodium nitrite causes relaxation of the isolated rat aorta: By stimulating both endothelial NO synthase and activating soluble guanylyl cyclase in vascular smooth muscle. Vascular Pharmacology 74:87-92.
Lizcano JM, Alessi DR (2002). The insulin signalling pathway. Current Biology 12(7):R236-R238.
Lovren F, Pan Y, Quan A, Teoh H, Wang G, Shukla PC, Levitt KS, Oudit GY, Al-Omran M, Stewart DJ, Slutsky AS (2008). Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. American Journal of Physiology-Heart and Circulatory Physiology 295(4):1377-1384.
Mehta PK, Griendling KK (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am. J. Physiol. Journal of Cellular Physiology 292(1)82-97.
Mohan V, Venkatraman JV, Pradeepa R (2010). Epidemiology of cardiovascular disease in type 2 diabetes: the Indian scenario. Journal of Diabetes Science and Technology 4(1):158-170.
Mombouli JV, Vanhoutte PM (1999). Endothelial dysfunction: from physiology to therapy. Journal of Molecular and Cellular Cardiology 31(1):61-74.
Montagnani M, Chen H, Barr VA, Quon MJ (2001). Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser1179. Journal of Biological Chemistry 276(32):30392-30398.
Muniyappa R, Montagnani M, Koh KK, Quon MJ (2007). Cardiovascular actions of insulin. Endocrine Reviews 28(5):463-491.
Nolan CJ, Damm P, Prentki M (2011). Type 2 diabetes across generations: from pathophysiology to prevention and management. The Lancet 378(9786):169-181.
Notas G, Nifli AP, Kampa M, Vercauteren J, Kouroumalis E, Castanas E (2006). Resveratrol exerts its antiproliferative effect on HepG2 hepatocellular carcinoma cells, by inducing cell cycle arrest, and NOS activation. Biochim. Biophys. Acta (BBA)-General Subjects 1760(11):1657-1666.
Pavo I, Jermendy G, Varkonyi TT, Kerenyi Z, Gyimesi A, Shoustov S, Shestakova M, Herz M, Johns D, Schluchter BJ, Festa A (2003). Effect of pioglitazone compared with metformin on glycemic control and indicators of insulin sensitivity in recently diagnosed patients with type 2 diabetes. Journal of Clinical Endocrinology and Metabolism 88(4):1637-1645.
Prieto D, Contreras C, Sánchez A (2014). Endothelial dysfunction, obesity and insulin resistance. Current Vascular Pharmacology 12(3):412-426.
Radomski MW, Palmer RM, Moncada S (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proceedings of the National Academy of Sciences 87(13):5193-5197.
Repetto S, Salani B, Maggi D, Cordera R (2005). Insulin and IGF-I phosphorylate eNOS in HUVECs by a caveolin-1 dependent mechanism. Biochemical and Biophysical Research Communications 337(3):849-852.
Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M, Schalkwijk CG, Stehouwer CD (2005). Vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clinical Science 109(2):143-159.
Shi Y, Lo CS, Padda R, Abdo S, Chenier I, Filep JG, Ingelfinger JR, Zhang SL, Chan JS (2015). Angiotensin-(1–7) prevents systemic hypertension, attenuates oxidative stress and tubulointerstitial fibrosis, and normalizes renal angiotensin- converting enzyme 2 and Mas receptor expression in diabetic mice. Clinical Science 128(10):649-663.
Shiuchi T, Iwai M, Li HS, Wu L, Min LJ, Li JM, Horiuchi M (2004). Angiotensin II type-1 receptor blocker valsartan enhances insulin sensitivity in skeletal muscles of diabetic mice. Hypertension 43(5):1003-1010.
Shoelson SE, Lee J, Goldfine AB (2006). Inflammation and insulin resistance. Journal of Clinical Investigation 116(7):1793-1801.
Somlyo AP, Somlyo AV (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiological Reviews 83(4):1325-1358.
Strober W (2001). Trypan blue exclusion test of cell viability. Current Protocols in Immunology, John Wiley and Sons, Appendix 3B.
Surapongchai J, Prasannarong M, Bupha-Intr T, Saengsirisuwan V (2017). Angiotensin II induces differential insulin action in rat skeletal muscle. Journal of Endocrinology 232(3):547-560.
Tangvarasittichai S (2015). Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World Journal of Diabetes 6(3):456-80.
Tassone EJ, Sciacqua A, Andreozzi F, Presta I, Perticone M, Carnevale D, Casaburo M, Hribal ML, Sesti G, Perticone F (2013). Angiotensin (1–7) counteracts the negative effect of angiotensin II on insulin signalling in HUVECs. Cardiovascular Research 99(1):129-136.
Wei Y, Sowers JR, Clark SE, Li W, Ferrario CM, Stump CS (2008). Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-κB activation via NADPH oxidase. American Journal of Physiology-Endocrinology and Metabolism 294(2):345-351.
Wheatcroft SB, Williams IL, Shah AM, Kearney MT (2003). Pathophysiological implications of insulin resistance on vascular endothelial function. Diabetes medications 20(4):255-268.
Xiao X, Zhang C, Ma X, Miao H, Wang J, Liu L, Chen S, Zeng R, Chen Y, Bihl JC (2015). Angiotensin-(1–7) counteracts angiotensin II-induced dysfunction in cerebral endothelial cells via modulating Nox2/ROS and PI3K/NO pathways. Experimental Cell Research 336(1):58-65.
Yang HY, Bian YF, Zhang HP, Gao F, Xiao CS, Liang B, Li J, Zhang NN, Yang ZM (2012). Angiotensin-(1–7) treatment ameliorates angiotensin II-induced apoptosis of human umbilical vein endothelial cells. Clinical and Experimental Pharmacology and Physiology 39(12):1004-1010.
Yayama K, Hiyoshi H, Imazu D, Okamoto H (2006). Angiotensin II stimulates endothelial NO synthase phosphorylation in thoracic aorta of mice with abdominal aortic banding via type 2 receptor. Hypertension 48(5):958-964.
Yuan Q, Yang J, Santulli G, Reiken SR, Wronska A, Kim MM, Osborne BW, Lacampagne A, Yin Y, Mark AR (2016). Maintenance of normal blood pressure is dependent on IP3R1-mediated regulation of eNOS. Proceedings of the National Academy of Sciences 113(30):8532-8537.
Zhao Y, Vanhoutte PM, Leung SW (2015). Vascular nitric oxide: Beyond eNOS. Journal of Pharmacological Sciences 129(2):83-94.