Angiotensin II inhibits insulin-stimulated phosphorylation of eukaryotic initiation factor 4E-binding protein-1 in proximal tubular epithelial cells

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Interaction between angiotensin II, which binds a G-protein-coupled receptor, and insulin, a ligand for receptor tyrosine kinase, was examined in renal proximal tubular epithelial cells. Augmented protein translation by insulin involves activation of eukaryotic initiation factor 4E (eIF4E) which follows the release of the factor from a heterodimeric complex by phosphorylation of its binding protein, 4E-BP1. Angiotensin II (1 nM) or insulin (1 nM) individually stimulated 4E-BP1 phosphorylation. However, pre-incubation with angiotensin II abrogated insulin-induced phosphorylation of 4E-BP1, resulting in persistent binding to eIF4E. Although angiotensin II and insulin individually activated phosphoinositide 3-kinase and extracellular signal-regulated kinase (ERK)-1/-2-type mitogen-activated protein (MAP) kinase, pre-incubation with angiotensin II abolished insulin-induced stimulation of these kinases, suggesting more

proximal events in insulin signalling may be intercepted. Pre-treatment with angiotensin II markedly inhibited insulin-stimulated tyrosine phosphorylation of insulin-receptor β -chain and insulin-receptor substrate 1. Losartan prevented angiotensin II inhibition of insulin-induced ERK-1/-2-type MAP kinase activation and 4E-BP1 phosphorylation, suggesting mediation of the effect of angiotensin II by its type 1 receptor. Insulin-stimulated *de novo* protein synthesis was also abolished by pre-incubation with angiotensin II. These data show that angiotensin II inhibits 4E-BP1 phosphorylation and stimulation of protein synthesis induced by insulin by interfering with proximal events in insulin signalling. Our data provide a mechanistic basis for insulin insensitivity induced by angiotensin II.

Key words: 4E-BP1 phosphorylation, receptor cross-talk.

INTRODUCTION

Recent investigations have revealed important interactions between signalling pathways of G-protein-coupled receptors and receptor tyrosine kinases [1]. In vascular smooth-muscle cells, angiotensin II, which acts via a G-protein-coupled receptor, transactivates platelet-derived-growth-factor (PDGF) receptor, a receptor tyrosine kinase, in the absence of PDGF, via activation of Shc-Grb2 complex [2]. In cardiac fibroblasts, angiotensin II stimulation of extracellular signal-regulated kinase (ERK)-1/-2-type mitogen-activated protein (MAP) kinase is dependent on transactivation of epidermal-growth-factor receptor, another receptor tyrosine kinase [3]. The proximal tubular epithelial (PTE) cells are known to express receptors for both angiotensin II and insulin. However, the potential interaction between the two agonists has not been studied, although they individually regulate several common tubular functions such as sodium reabsorption [4,5]. It is likely that interaction between angiotensin II and insulin involves signalling pathways recruited by these agonists. Angiotensin II binds to two types of G-protein-coupled receptor. Angiotensin II type 1 receptor (AT₁ receptor) is known to activate tyrosine kinases such as Src and Janus kinases and receptor tyrosine kinases such as the epidermal-growth-factor receptor [6]. Insulin receptor is a tyrosine kinase and most of the downstream events in insulin signalling are mediated by docking proteins such as insulin-receptor substrate (IRS)-1 or -2 that bind to the receptor [7]. Tyrosine phosphorylation of IRS proteins by

insulin receptor is followed by activation of a variety of signalling pathways including the phosphoinositide 3-kinase (PI 3-kinase) pathway and the Raf/MEK/ERK-1/-2-type MAP kinase pathway (where MEK is MAP kinase/ERK kinase). Interestingly, phosphorylation of IRS-1 and activation of PI 3-kinase and ERK-type MAP kinases also occur following binding of angiotensin II to its AT₁ receptor [8]. The similarities in pathways recruited by the two agonists suggest that potential interaction between insulin and angiotensin II may involve signalling events.

Angiotensin II and insulin play major roles in the pathogenesis and management of diabetic kidney disease, respectively. Angiotensin II participates in the haemodynamic and cellular processes that lead to matrix expansion, reduction in filtration function and proteinuria in diabetic kidney disease [9]. Strict control of plasma glucose concentration by insulin administration has been shown to significantly reduce microvascular complications of diabetes, including kidney disease [10]. However, the potential interaction between angiotensin II and insulin has not been well studied in the renal tissue.

In the present study, our aim was to investigate angiotensin II and insulin interaction in PTE cells by targeting an important biological effect of insulin, i.e. protein synthesis. In this regard, in addition to *de novo* protein synthesis, we examined a crucial step in protein translation regulated by insulin. Insulin is a potent stimulus for both the initiation and the elongation phases of translation [11]. The initiation phase is critically controlled by the mRNA cap-binding protein, eukaryotic initiation factor 4E

Abbreviations used: AT₁ receptor, angiotensin II type 1 receptor; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E-binding protein 1; ERK, extracellular signal-regulated kinase; IRS, insulin-receptor substrate; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; MBP, myelin basic protein; PI 3-kinase, phosphoinositide 3-kinase; TBST, Tris-buffered saline with 0.1% Tween 20; ACE, angiotensin-converting enzyme; PTE, proximal tubular epithelial; Dulbecco's MEM, Dulbecco's modified Eagle's medium; m⁷ GTP, 7-methyl GTP.

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(eIF4E). In the resting state of cells, eIF4E is held in an inactive complex by its binding protein, 4E-BP1, and is released upon phosphorylation of the latter [12]. Phosphorylation of eIF4E has been shown to augment its stimulatory effects on the initiation phase of protein translation [13]. Insulin strongly induces phosphorylation of both eIF4E and 4E-BP1 [14–16]. We employed insulin regulation of 4E-BP1 phosphorylation and *de novo* protein synthesis as tools to study angiotensin II modulation of insulin responsiveness in PTE cells.

EXPERIMENTAL

Cell culture

Simian virus 40-transformed murine PTE cells (kindly provided by Dr Eric Neilson, Vanderbilt University, Nashville, TN, U.S.A.) were grown in Dulbecco's modified Eagle's medium (Dulbecco's MEM) containing 7% fetal bovine serum, 5 mM glucose and no insulin [17]. Confluent monolayers of cells were serum-deprived in Dulbecco's MEM for 18 h before the experiment.

In vivo ^{32}P labelling

PTE cells were labelled with [^{32}P]P_i as described recently [16]. Briefly, PTE cells were grown in Dulbecco's MEM containing 7% fetal bovine serum. At 70–80% confluence, cells were serum-deprived for 18 h. The serum-starved cells were labelled with 200 $\mu\text{Ci}/\text{ml}$ [^{32}P]P_i (NEN Life Science Products, Boston, MA, U.S.A.) for 6 h at 37 °C with or without 1 nM insulin and 1 nM angiotensin II. After treatment, medium was removed, cells were rinsed with cold PBS and lysed in 500 μl of RIPA buffer (50 mM Tris/HCl, pH 7.4, 140 mM NaCl, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1% Nonidet P-40, 1 mM EGTA, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ leupeptin) for 30 min on ice. Cell lysates were cleared by centrifugation at 15 700 *g* for 20 min at 4 °C. Cell lysates containing equal amounts of protein from controls and each treatment were incubated with 5 μl of specific antibodies against 4E-BP1 or IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 3 h at 4 °C with gentle rotation. Protein A-Sepharose beads (20 μl) were added and incubation was continued for 1 h. Beads were collected by centrifugation and washed four times with cold lysis buffer and once with cold PBS. The beads were resuspended in Laemmli buffer at 95 °C for 5 min. Proteins were resolved by SDS/PAGE, using a 15% gel for 4E-BP1 analysis and a 7.5% gel for IRS-1 analysis. Gels were dried and exposed to Kodak Biomax MR X-ray film with an image-intensifying screen.

Analysis of 4E-BP1 phosphorylation [16]

Quiescent serum-deprived PTE cells were incubated with or without 1 nM insulin and 1 nM angiotensin II for the indicated times at 37 °C. Cells were washed twice with PBS, collected in 500 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 mM β -glycerophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 1 mM EGTA, 2 mM benzamidine, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin and 1 $\mu\text{g}/\text{ml}$ leupeptin), and lysed by three freeze–thaw cycles. Cell debris was removed by centrifugation at 15 700 *g* for 5 min and protein concentration was measured using Bio-Rad protein assay reagent. Equal amounts of lysate proteins (300 μg) were boiled for 7 min and then cooled on ice prior to centrifugation at 15 700 *g* for 5 min at 4 °C. Heat-soluble proteins were precipitated by addition of trichloroacetic acid to a final concentration of 15%. After 3 h of incubation at 4 °C, protein precipitates were

centrifuged for 10 min, the supernatants removed and the remaining trichloroacetic acid was extracted with diethylether. The final pellets were resuspended in Laemmli sample buffer. Samples were boiled for 5 min and proteins were separated on a 15% acrylamide gel followed by electrotransfer to a nitrocellulose membrane. The membrane was rinsed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and blocked with 2% non-fat milk powder in TBST. After rinsing with TBST buffer, membranes were incubated with anti-4E-BP1 antibody (40 ng/ml; Santa Cruz Biotechnology) or anti-phospho-4E-BP1 antibody (1:2000 dilution; New England Biolabs, Beverly, MA, U.S.A.). The antibody against phospho-4E-BP1 reacts only with 4E-BP1 that is phosphorylated at Thr-70. The membranes were washed twice with TBST buffer followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The antigen–antibody complexes were detected using the chemiluminescence reagent kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

Interaction between eIF4E and 4E-BP1

7-Methyl GTP (m^7 GTP)–Sepharose affinity chromatography was performed as described previously [18]. A slurry of m^7 GTP–Sepharose CL-6B (25 μl ; Amersham Pharmacia Biotech) was added to 300 μg of protein. The lysates were then rotated for 1 h at 4 °C. The m^7 GTP–Sepharose was pelleted by centrifugation at 1000 *g* for 2 min. The beads were then washed three times in RIPA buffer. For SDS/PAGE, proteins were removed from the m^7 GTP matrix by boiling it in SDS loading buffer. Proteins were separated on a 15% acrylamide gel followed by electrotransfer to a nitrocellulose membrane. The membrane was rinsed with TBST and blocked with 2% non-fat milk powder in TBST. Membranes were incubated with anti-4E-BP1 antibody (40 ng/ml). Membranes were washed twice with TBST buffer followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The antigen–antibody complexes were detected using chemiluminescence reagent kit. Membranes were stripped and reprobed with anti-eIF4E antibody (Santa Cruz Biotechnology).

PI 3-kinase assay

Control and treated PTE cells were homogenized in RIPA buffer. Equal amounts of homogenates (500 μg) were immunoprecipitated using anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.), as described previously [16,19]. Immunobeads were incubated with 10 μg of PtdIns for 10 min at 25 °C in the PI 3-kinase assay buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid. Following addition of 20 mM MgCl_2 and 5 μCi of [γ - ^{32}P]ATP to the assay mixture, the reactants were further incubated for 10 min at 25 °C. The reaction was stopped by the addition of a mixture of chloroform/methanol/11.6 M HCl in the proportions 50:100:1. The reactants were extracted with 100 μl of chloroform and the organic layer was washed with methanol/1 M HCl in a 1:1 ratio. The reaction products were dried, resuspended in chloroform and separated by TLC using the solvent chloroform/methanol/28% ammonium hydroxide/water in the ratio 129:114:15:21. The spots were visualized by autoradiography.

ERK-1/-2-type MAP kinase assay

Equal amounts (100 μg) of PTE cell lysates from control and treated cells were immunoprecipitated by incubating with anti-ERK-1 antibody (Santa Cruz Biotechnology) at 4 °C for 3 h, as

described previously [16]. Protein A–Sepharose beads were added and incubated at 4 °C for 1 h. The immunobeads were washed three times in RIPA buffer and twice in PBS. Beads were incubated with 2.5 mg/ml of the substrate, myelin basic protein (MBP), 25 μ M unlabelled ATP and 1 μ Ci of [γ - 32 P]ATP in MAP kinase buffer (10 mM Hepes, pH 7.4, 10 mM MgCl₂, 0.5 mM dithiothreitol and 0.5 mM sodium orthovanadate) for 30 min at 30 °C. Reaction was stopped by addition of sample buffer and the reaction mixture was boiled for 5 min and subjected to SDS/PAGE (15 % gel). The gel was dried and exposed to Kodak Biomax MR X-ray film with an image-intensifying screen. Western blotting with the anti-ERK-1 antibody was performed on an aliquot of assay lysates to assess loading.

Immunoblotting with anti-phosphotyrosine antibody

Quiescent serum-deprived PTE cells were incubated with or without 1 nM insulin and 1 nM angiotensin II at 37 °C. Equal amounts of protein from the cell lysates were immunoprecipitated with specific antibodies against insulin-receptor β -chain (Santa Cruz Biotechnology) or IRS-1 (Upstate Biotechnology), and the immune complexes were separated by SDS/PAGE (7.5 % gel). Proteins were transferred to nitrocellulose membrane and the membrane was incubated with a monoclonal anti-phosphotyrosine antibody (1:2000 dilution; Upstate Biotechnology). Reactive bands were detected by chemiluminescence followed by autoradiography.

Protein-synthesis assay

Serum-starved cells were incubated with angiotensin II and/or insulin, as described above, followed by incubation with [35 S]-methionine at 10 μ Ci/ml for 60 min. The cells were then washed in PBS and lysed in RIPA buffer. Protein (20 μ g) was spotted on to 3 MM paper (Whatman International, Maidstone, Kent, U.K.). The filters were then washed by boiling for 1 min in 10 % trichloroacetic acid containing 0.1 g/l methionine. This was repeated three times. Filters were then dried and immersed in liquid scintillation medium before determining radioactivity by counting [20].

Statistics

Data were expressed as means $\pm$ S.E.M. and analysed by ANOVA for comparison between multiple groups; $P < 0.05$ was considered significant.

RESULTS

Angiotensin II inhibits insulin-induced 4E-BP1 phosphorylation

Angiotensin II and insulin are known to individually augment phosphorylation of 4E-BP1 [15,21]. We examined whether pre-treatment of PTE cells with angiotensin II would modify insulin-induced 4E-BP1 phosphorylation. Quiescent PTE cells were stimulated with 1 nM insulin with or without pre-incubation with 1 nM angiotensin II, and cell lysates were subjected to immunoblot analysis with phospho-4E-BP1 antibody, which reacts selectively with phosphorylated species of 4E-BP1. Preliminary experiments were performed to determine the optimal concentration and duration of pre-incubation with angiotensin II that inhibited the insulin effect on 4E-BP1 phosphorylation. As shown in Figure 1(a), addition of angiotensin II or insulin individually augmented phosphorylation of 4E-BP1 as assessed by immunoblotting with phospho-4E-BP1 antibody. Pre-treatment with angiotensin II completely inhibited the insulin-

Figure 1 Angiotensin II inhibits insulin-induced 4E-BP1 phosphorylation

PTE cells were serum-starved for 18 h and stimulated with 1 nM insulin with or without a 5 min pre-incubation with 1 nM angiotensin II (Ang II). (a) Cell lysates were run on a SDS-polyacrylamide gel (15%) followed by Western blotting using anti-phospho-4E-BP1 antibody. (b) Growth-arrested PTE cells were labelled with 200 μ Ci/ml [32 P]P_i for 6 h and treated as above. Equal amounts of protein from control and each treatment were immunoprecipitated with anti-4E-BP1 antibody, and the immune complexes were separated by SDS/PAGE (15% gel). Phosphorylated 4E-BP1 was visualized by autoradiography. (c) Cell lysates (300 μ g) were mixed with 25 μ l of a slurry of m⁷ GTP–Sepharose CL-6B for 1 h at 4 °C. For SDS/PAGE, proteins were removed from the m⁷ GTP matrix by boiling it in SDS loading buffer. Proteins were separated by SDS/PAGE (15% gel) and immunoblotted with antibody against eIF4E or 4E-BP1.

induced 4E-BP1 phosphorylation. [32 P]P_i labelling further confirmed these data. There was an increase in incorporation of 32 P label into phosphorylated 4E-BP1 when cells were treated with angiotensin II or insulin alone, whereas pre-incubation with angiotensin II abolished the insulin-induced increase in label incorporation (Figure 1b).

Phosphorylation of 4E-BP1 leads to reduction in its affinity for eIF4E, resulting in dissociation of the heterodimeric complex and release of eIF4E. We examined whether angiotensin II pre-incubation could affect insulin regulation of eIF4E–4E-BP1 interaction by employing m⁷ GTP–Sepharose, which selectively binds to eIF4E. Stimulation of 4E-BP1 phosphorylation by either angiotensin II or insulin resulted in reduction in the amount of 4E-BP1 co-purifying with eIF4E (Figure 1c). Pre-incubation with angiotensin II prevented insulin-induced dissociation of the eIF4E–4E-BP1 complex and 4E-BP1 remained associated with eIF4E, the amount being comparable with untreated control.

These findings demonstrate that prior exposure to angiotensin II inhibits insulin-induced 4E-BP1 phosphorylation and dissociation of the eIF4E–4E-BP1 dimeric complex, and suggest that cross-talk exists between angiotensin II- and insulin-signalling pathways in PTE cells.

Angiotensin II pre-treatment inhibits insulin-induced PI 3-kinase activity

Next, we examined events in insulin-signalling pathways as potential sites of interference by angiotensin II. Insulin-induced

Figure 2 Angiotensin II pretreatment inhibits insulin-induced PI 3-kinase activity

(a) Serum-starved PTE cells were stimulated with 1 nM insulin with or without a 5 min pre-incubation with 1 nM angiotensin II (Ang II). Equal amounts of protein from the control and each treatment were immunoprecipitated with anti-phosphotyrosine antibodies, and kinase activity in immune complexes was measured by an assay using PtdIns as a substrate. Phospholipids were separated by TLC and visualized by autoradiography. PIP indicates the migration of PtdIns3P, an end product of PI 3-kinase activity. (b) Histogram of densitometric analysis of phosphorylated PtdIns3P from three independent experiments is shown. * $P < 0.001$ and ** $P < 0.01$ compared with the control; \$ $P < 0.001$ compared with insulin alone.

PI 3-kinase activity is required for mediating insulin-induced 4E-BP1 phosphorylation [15,16]. Accordingly, we investigated regulation of insulin-induced PI 3-kinase activity by angiotensin II. Anti-phosphotyrosine immunoprecipitates from cells incubated with insulin or angiotensin II were employed in an *in vitro* kinase assay using PtdIns as a substrate. Insulin augmented the activity of PI 3-kinase by approx. 2.3 ± 0.07 -fold when compared with control ($P < 0.001$; Figure 2). Angiotensin II alone increased the kinase activity by nearly 2 ± 0.1 -fold ($P < 0.01$), consistent with previous reports [22]. However, pretreatment with angiotensin II completely abolished the insulin-induced PI 3-kinase activity.

Figure 3 Angiotensin II inhibits insulin-induced ERK-1/-2-type MAP kinase activity

(a) Equal amounts (100 μ g) of cell lysates from control and treated cells were immunoprecipitated with anti-ERK-1 antibodies and immune complexes were used in an *in vitro* kinase assay using MBP as a substrate. Phosphorylated MBP was separated by SDS/PAGE (15% gel) followed by autoradiography. The lower panel shows a Western blot with anti-ERK-1 antibody that was done to assess loading. Ang II, angiotensin II. (b) The histogram represents the densitometric analysis of MBP phosphorylation from three individual experiments; * $P < 0.001$ and ** $P < 0.05$ compared with the control; \$ $P < 0.001$ compared with insulin alone. (c) PTE cells were incubated with 1 nM angiotensin II for 5 min with or without 5 min pre-incubation with 1 nM insulin. Equal amounts (100 μ g) of cell lysates from control and treated cells were processed for ERK activity as in (a).

Angiotensin II pre-incubation inhibits insulin-induced ERK-1/-2-type MAP kinase activity

ERK activation may occur via the Ras/Raf/MEK pathway, independent of PI 3-kinase activation. We have recently shown that phosphorylation of 4E-BP1 induced by insulin requires activation of ERK-1/-2-type MAP kinase [16]. Therefore, we explored the role of ERK-1/-2-type MAP kinase in the angiotensin II regulation of insulin-induced 4E-BP1 phosphorylation. ERK-1/-2-type MAP kinase activity was examined in an *in vitro* assay using MBP as a substrate. Insulin significantly stimulated

Figure 4 Angiotensin II inhibits insulin-induced tyrosine phosphorylation of IRS-1 and insulin-receptor β -chain

(a) Serum-starved PTE cells were labelled with 200 μ Ci/ml [32 P] P_i for 6 h and stimulated with 1 nM insulin with or without a 5 min pre-incubation with 1 nM angiotensin II (Ang II). Equal amounts of protein from control and each treatment were immunoprecipitated with anti-IRS-1 antibody, and the immune complexes were separated by SDS/PAGE. Phosphorylated IRS-1 was visualized by autoradiography. (b) Serum-starved PTE cells were incubated with 1 nM insulin with or without a 5 min pre-incubation with 1 nM angiotensin II. Equal amounts of protein (500 μ g) from control and each treatment were immunoprecipitated (IP) with anti-IRS-1 antibody, and the immune complexes were separated by SDS/PAGE (7.5% gel). The proteins were transferred to a nitrocellulose membrane. The membrane was incubated with a 1:2000 dilution of a monoclonal anti-phosphotyrosine antibody and reactive bands were detected by chemiluminescence followed by autoradiography. A representative blot from three experiments is shown. Lower panel: the membrane was stripped and reprobed with antibodies to assess loading. (c) Serum-starved PTE cells were incubated with 1 nM insulin with or without a 5 min pre-incubation with 1 nM angiotensin II. Equal amounts of protein (400 μ g) from control and each treatment were immunoprecipitated with anti-insulin-receptor β -chain antibody, and the immune complexes were separated by SDS/PAGE (7.5% gel). The proteins were transferred to a nitrocellulose membrane. The membrane was incubated and reactive bands were detected as for (b). A representative blot from two experiments is shown. (b, c) Lower panels: the membranes were stripped and reprobed with antibodies to (b) IRS-1 and (c) insulin-receptor β -chain to assess loading.

the ERK-1/-2-type MAP kinase activity by 2.2 ± 0.2 -fold, compared with the control ($P < 0.001$; Figure 3). Angiotensin II alone increased ERK-1/-2-type MAP kinase activity by 1.7 ± 0.1 -fold ($P < 0.05$). However, pretreatment with angiotensin II completely inhibited the insulin-induced ERK-1/-2-type MAP kinase activity. Additionally, we examined the effect of pre-incubation with insulin on signalling events stimulated by angiotensin II. We examined ERK activity as an index of this inter-

action. Insulin appeared to weakly potentiate angiotensin II stimulation of ERK activity (Figure 3c).

Angiotensin II inhibits insulin-induced tyrosine phosphorylation of IRS-1 and insulin receptor

Angiotensin II inhibition of insulin-induced activation of PI 3-kinase and ERK-1/-2-type MAP kinase suggested that it might be interfering with more proximal events in insulin signalling. Accordingly, we investigated whether angiotensin II regulated IRS-1 activation by insulin. IRS-1, the principal substrate for insulin receptor, is phosphorylated on multiple tyrosine residues by the activated insulin receptor [7]. IRS-1 is also phosphorylated by angiotensin II [8]. To investigate potential regulation of insulin-induced IRS-1 phosphorylation by angiotensin II in PTE cells, serum-starved cells were labelled with [32 P] P_i and stimulated with insulin with or without pre-incubation with angiotensin II. Treatment with insulin resulted in an approx. 3-fold increase in IRS-1 phosphorylation relative to the control (Figure 4a). Angiotensin II also stimulated IRS-1 phosphorylation by itself (Figure 4a). Pre-incubation of PTE cells with angiotensin II followed by addition of insulin, however, resulted in a 70–80% decrease in insulin-induced IRS-1 phosphorylation. The increase in 32 P-label incorporation into IRS-1 could be due to either an increase in serine/threonine phosphorylation or augmented tyrosine phosphorylation or both. Activation of insulin receptor and subsequent phenotypic effects of insulin such as protein synthesis depend on tyrosine phosphorylation of IRS-1. Accordingly, we studied the effect of angiotensin II on tyrosine phosphorylation of IRS-1 induced by insulin. Following incubation of PTE cells with or without insulin and/or angiotensin II, IRS-1 was immunoprecipitated and immunoblotted with anti-phosphotyrosine antibody. Insulin alone and angiotensin II alone augmented IRS-1 tyrosine phosphorylation (Figure 4b). However, pretreatment with angiotensin II abolished insulin-induced IRS-1 tyrosine phosphorylation, confirming data obtained by 32 P labelling.

We next examined a more proximal event in insulin signalling and tested whether angiotensin II regulated insulin-receptor activation following binding with insulin. Cell homogenates were immunoprecipitated with an antibody against insulin-receptor β -chain and immunoblotted with an anti-phosphotyrosine antibody. As shown in Figure 4(c), insulin alone increased tyrosine phosphorylation of the insulin-receptor β -chain, which was inhibited by pre-incubation with angiotensin II. Angiotensin II by itself did not have any effect on insulin-receptor phosphorylation. These data show that angiotensin II affects very early events in the insulin-signalling pathway.

Role of AT₁ receptor in angiotensin II regulation of insulin-stimulated ERK-1/-2-type MAP kinase activity

Angiotensin II activation of PI 3-kinase [22], phospholipase C [23] and MAP kinase [24] has been linked to the activation of AT₁ receptor, a prototypical G-protein-coupled receptor. We examined whether AT₁ receptor was involved in angiotensin II regulation of insulin-induced 4E-BP1 phosphorylation. First, we tested the role of AT₁ receptor in ERK-1/-2-type MAP kinase activation. Serum-starved cells were incubated with or without losartan, a blocker of AT₁ receptor, and the cells were treated with 1 nM angiotensin II for 5 min. Anti-ERK-1 antibody immunoprecipitates were used in an *in vitro* kinase assay with MBP as a substrate. Losartan markedly reduced angiotensin II-induced ERK-1/-2-type MAP kinase activation (Figure 5). Although there was a trend towards an increase in ERK activity

Figure 5 Angiotensin II-induced ERK-1/-2-type MAP kinase activity is AT₁ receptor-dependent

(a) Serum-starved PTE cells were treated with 1 nM angiotensin II (Ang II) for 5 min with or without pre-incubation for 30 min with 10 μ M losartan, an AT₁ receptor antagonist. Cell homogenates were processed for ERK-1/-2-type MAP kinase activity, as described in Figure 3. The lower panel shows a Western blot with anti-ERK-1 antibody that was done to assess loading. A representative blot of three individual experiments is shown. (b) The histogram represents composite results from three individual experiments. ERK-1 activity is expressed as a percentage relative to the control (means $\pm$ S.E.M.); * P < 0.001 compared with the control; \$ P < 0.001 compared with angiotensin II alone.

in cells incubated with losartan alone, composite data from three experiments did not reach statistical significance. Next, we examined the effect of losartan on angiotensin II regulation of insulin-induced ERK-1/-2-type MAP kinase activation. Insulin alone significantly stimulated ERK-1/-2-type MAP kinase activity, which was markedly reduced by pretreatment with angiotensin II (Figure 6). However, pre-incubation with losartan significantly reversed angiotensin II inhibition of insulin-stimulated ERK-1/-2-type MAP kinase activation (Figure 6). These data demonstrate that AT₁ receptor is involved in angiotensin II inhibition of insulin-induced ERK-1/-2-type MAP kinase activation.

AT₁ receptor is involved in angiotensin II inhibition of insulin-induced 4E-BP1 phosphorylation

Next we studied the role of AT₁ receptor in angiotensin II regulation of insulin-induced 4E-BP1 phosphorylation. Quiescent PTE cells were incubated with or without losartan and stimulated with 1 nM insulin with or without pre-incubation with 1 nM angiotensin II. Cell lysates were immunoblotted with phospho-specific 4E-BP1 antibody. Individual treatment

Figure 6 AT₁ receptor is involved in angiotensin II regulation of insulin-stimulated ERK-1/-2-type MAP kinase activity

(a) Serum-starved PTE cells were incubated for 30 min with or without 10 μ M losartan and treated with 1 nM insulin for 10 min with or without pre-incubation with 1 nM angiotensin II (Ang II) for 5 min. Cell homogenates were processed for ERK-1/-2-type MAP kinase activity, as described in Figure 3. The lower panel shows a Western blot with anti-ERK-1 antibody that was done to assess loading. A representative blot from three experiments is shown. (b) The histogram represents the composite results from three individual experiments. ERK-1/-2-type MAP kinase activity is expressed as a percentage relative to the control (means $\pm$ S.E.M.); * P < 0.001 compared with the control; \$ P < 0.001 compared with insulin alone; @ P < 0.001 compared with angiotensin II + insulin.

with insulin or angiotensin II resulted in phosphorylation of 4E-BP1 (Figure 7, compare lanes 2 and 3 with lane 1). Pre-incubation of angiotensin II completely inhibited insulin-induced 4E-BP1 phosphorylation (Figure 7, compare lanes 4 and 2). In the presence of losartan, angiotensin II failed to phosphorylate 4E-BP1 (results not shown). Inhibitory activity of angiotensin II on insulin-stimulated 4E-BP1 phosphorylation was blocked by the AT₁ receptor antagonist, losartan (Figure 7, compare lanes 6 and 4), restoring the insulin effect. These results demonstrate that AT₁ receptor is involved in angiotensin II inhibition of insulin-induced 4E-BP1 phosphorylation.

Angiotensin II abolishes insulin-stimulated protein synthesis

The metabolic end point of 4E-BP1 phosphorylation is the stimulation of protein synthesis. We evaluated whether interference of insulin-induced 4E-BP1 phosphorylation has impli-

Figure 7 AT_1 receptor is involved in angiotensin II inhibition of insulin-induced 4E-BP1 phosphorylation

Serum-starved PTE cells were incubated for 30 min with or without 10 μ M losartan and treated with 1 nM insulin for 10 min with or without pre-incubation with 1 nM angiotensin II (Ang II) for 5 min. Cell lysates were separated by SDS/PAGE (15% gel) followed by Western blotting using anti-phospho-4E-BP1 antibodies.

Figure 8 Angiotensin II inhibits insulin-stimulated protein synthesis

Serum-starved PTE cells were incubated with 1 nM insulin for 10 min with or without pre-incubation with 1 nM angiotensin II (Ang II) for 5 min. Cells were then labelled with 10 μ Ci/ml [35 S]methionine for 60 min. Trichloroacetic acid-precipitable radioactivity was estimated as described in the Experimental section. Data are normalized to the control at 100%. Combined data from two experiments with triplicates at each point in each experiment are shown; * $P < 0.001$ compared with the control.

cations for *de novo* protein synthesis. Studies on incorporation of [35 S]methionine into proteins showed that angiotensin II or insulin individually stimulated protein synthesis significantly (Figure 8). However, insulin-induced protein synthesis was completely blocked by pre-incubation with angiotensin II. These data suggest that angiotensin II inhibition of insulin-stimulated 4E-BP1 phosphorylation has physiological implications for *de novo* protein synthesis in PTE cells.

DISCUSSION

We have demonstrated that treatment of PTE cells with insulin or angiotensin II leads to increased phosphorylation of 4E-BP1, dissociation of 4E-BP1 from eIF4E–4E-BP1 heterodimeric complex and stimulation of *de novo* protein synthesis. Insulin or angiotensin II treatment activated both PI 3-kinase and ERK-1/-2-type MAP kinase pathways. However, pretreatment with

angiotensin II abolished the following insulin-induced events: tyrosine phosphorylation of insulin-receptor β -chain and IRS-1, activation of PI 3-kinase and ERK kinase, 4E-BP1 phosphorylation, its dissociation from eIF4E, and an increase in protein synthesis. PI 3-kinase and ERK represent two main pathways involved in 4E-BP1 phosphorylation [16,25,26]. Inhibitory activity of angiotensin II on insulin-stimulated 4E-BP1 phosphorylation was blocked by losartan, demonstrating involvement of the AT_1 receptor subtype in cross-talk between angiotensin II- and insulin-signalling systems.

PI 3-kinase activation is required to mediate insulin-receptor signalling events leading to 4E-BP1 phosphorylation [15]. As angiotensin II inhibited insulin-induced 4E-BP1 phosphorylation, we examined whether it affected PI 3-kinase activation by insulin. Consistent with this hypothesis, we found that pre-incubation of angiotensin II completely blocked the insulin-induced PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Analogous to smooth-muscle cells [26], we have recently observed that, in PTE cells, insulin-induced 4E-BP1 phosphorylation is dependent on ERK-1/-2-type MAP kinase, and that activation of the latter by insulin requires prior PI 3-kinase activation [16]. Accordingly, we examined the role of ERK-1/-2-type MAP kinase in angiotensin II regulation of insulin-stimulated ERK-1/-2-type MAP kinase activation. Although insulin and angiotensin II independently augmented ERK-1/-2-type MAP kinase activity, pre-incubation with angiotensin II abolished insulin-stimulated ERK-1/-2-type MAP kinase activation. Thus angiotensin II inhibited the two main pathways involved in 4E-BP1 phosphorylation promoted by insulin.

Inhibition of insulin-stimulated PI 3-kinase and ERK-1/-2-type MAP kinase activation suggested that angiotensin II might interrupt more proximal events in insulin signalling. Therefore, we examined the status of IRS-1 and insulin-receptor β -chain tyrosine phosphorylation. Pre-incubation of cells with angiotensin II markedly inhibited insulin-induced IRS-1 tyrosine phosphorylation when compared with cells incubated with insulin alone. Furthermore, insulin-induced tyrosine phosphorylation of the insulin-receptor β -chain was also inhibited by prior exposure of cells to angiotensin II. Mechanisms underlying angiotensin II interference with insulin activation of IRS-1 could involve angiotensin II induction of serine phosphorylation of the insulin receptor or IRS-1, as shown previously in vascular smooth-muscle cells [27]. Angiotensin II activates protein kinase C [28], which in turn may promote serine phosphorylation of the insulin-signalling proteins. Additionally, reduction in tyrosine phosphorylation of IRS-1 and insulin-receptor β -chain promoted by insulin could also be due to activation of tyrosine phosphatases such as SHP (Src homology 2 domain-containing phosphatase)-1 or SHP-2 by angiotensin II [29]. Our observations also suggest that angiotensin II and insulin may share common signalling

elements leading to 4E-BP1 phosphorylation. Recruitment of IRS-1 and kinases activated downstream of IRS-1, i.e. PI 3-kinase and, perhaps, ERK-1/2 MAP kinase, by angiotensin II may limit access of these pathways to insulin added after angiotensin II. This may also explain the absence of any additive effects when the two agonists are added together, although each individually augments 4E-BP1 phosphorylation. Next, we examined the angiotensin II receptor subtype involved in cross-talk with insulin receptor. Angiotensin II activates a G-protein-coupled AT₁ receptor that is necessary for Janus kinase (JAK) 2-, IRS-1- and PI 3-kinase-mediated signalling [8]. We found that losartan significantly counteracted angiotensin II inhibition of insulin-stimulated 4E-BP1 phosphorylation, indicating the involvement of the AT₁ receptor.

The consequences of cross-talk between G-protein-coupled receptors and receptor tyrosine kinases appear to be cell-specific. In rat mesangial cells, angiotensin II significantly enhanced insulin induction of collagen I and IV mRNA [30]. Additive effects on protein and DNA synthesis have also been reported with angiotensin II and insulin-like growth factor 1 [31]. In contrast to the enhancing effect of angiotensin II on biological effects of receptor tyrosine kinases, Folli et al. have reported inhibition of insulin-signalling events following pre-incubation of smooth-muscle cells with angiotensin II that was unaffected by AT₁ receptor blocker, losartan [27]. However, in this study, the effect of angiotensin II on a specific biological event regulated by insulin was not studied [27].

In our studies, we could relate the regulatory effects of angiotensin II on insulin signalling to insulin-stimulated phosphorylation of 4E-BP1, an important regulatory step in protein translation, and to *de novo* protein synthesis. Phosphorylation of 4E-BP1 occurs on a minimum of six serine/threonine residues [32], leading to its dissociation from eIF4E. Angiotensin II or insulin promoted the dissociation of the eIF4E–4E-BP1 heterodimeric complex, releasing eIF4E to augment translation of capped mRNAs. Pre-incubation with angiotensin II inhibited insulin-induced eIF4E–4E-BP1 dissociation. This could occur by abolition of activation of insulin-receptor signalling events leading to 4E-BP1 phosphorylation, as demonstrated by neutralization of insulin stimulation of PI 3-kinase and ERK pathways. An alternative mechanism could involve induction of phosphorylation of an amino acid residue by angiotensin II, leading to conformational changes in 4E-BP1 and interference with phosphorylation of aforementioned serine/threonine residues targeted by insulin. Inhibition of insulin-stimulated protein synthesis by angiotensin II has implications for cell function. Potentially, angiotensin II could inhibit insulin regulation of cellular processes that require protein synthesis, e.g. GLUT4 synthesis [33]. Although insulin is acknowledged as a potent stimulus for protein translation, the eIF4E pathway has not been well studied in the context of regulation of glucose metabolism by insulin. The eIF4E system is particularly involved in the control of translation of mRNAs that have complex 5' untranslated regions [12]. It is unclear whether mRNAs of factors important in glucose metabolism that are regulated by insulin possess complex 5' untranslated regions and whether their synthesis is under control of the eIF4E system.

The angiotensin II–insulin interaction reported here has potential biological significance. Angiotensin II participates in the haemodynamic and cellular processes that lead to matrix expansion, reduction in filtration function and proteinuria in diabetic kidney disease [9]. Furthermore, angiotensin-converting enzyme (ACE) inhibitors and AT₁ receptor blockers have been shown to have beneficial effects on the course of diabetic kidney disease [34,35]. ACE inhibitors have also been reported to

improve insulin sensitivity in states of insulin resistance such as type 2 diabetes and essential hypertension [36], implying that angiotensin II may oppose actions of insulin. In recent clinical studies, appearance of type 2 diabetes was reduced in high-risk patients receiving ACE inhibitors, suggesting that these agents may improve insulin sensitivity [37,38]. Achievement of normal plasma-glucose concentrations by rigorous administration of insulin has been shown to significantly reduce microvascular complications of diabetes, including renal disease [10]. Our observations provide a potential mechanistic basis for the decrease in insulin sensitivity caused by angiotensin II. Additionally, they suggest that suppression of angiotensin II may be important to facilitate actions of insulin in diabetes to achieve optimal control of plasma-glucose levels and thereby ameliorate diabetic complications, including diabetic kidney disease.

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REFERENCES

- Luttrell, L. M., Daaka, Y. and Lefkowitz, R. J. (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Opin. Cell. Biol.* **11**, 177–183
- Linseman, D. A., Benjamin, C. W. and Jones, D. A. (1995) Convergence of angiotensin II and platelet-derived growth factor receptor signalling cascades in vascular smooth muscle cells. *J. Biol. Chem.* **270**, 12563–12568
- Moriguchi, Y., Matsubara, H., Mori, Y., Murasawa, S., Masaki, H., Maruyama, K., Tsutsumi, Y., Shibasaki, Y., Tanaka, Y., Nakajima, T. et al. (1999) Angiotensin II-induced transactivation of epidermal growth factor receptor regulates fibronectin and transforming growth factor-beta synthesis via transcriptional and posttranscriptional mechanisms. *Circ. Res.* **84**, 1073–1084
- Schuster, V. L., Kokko, J. P. and Jacobson, H. R. (1984) Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules. *J. Clin. Invest.* **73**, 507–515
- Quinones-Galvan, A. and Ferrannini, E. (1997) Renal effects of insulin in man. *J. Nephrol.* **10**, 188–191
- Haendeler, J. and Berk, B. C. (2000) Angiotensin II mediated signal transduction. Important role for tyrosine kinases. *Regul. Peptides* **95**, 1–7
- White, M. F. and Yenush, L. (1998) The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. *Curr. Top. Microbiol. Immunol.* **228**, 179–208
- Saad, M. J., Velloso, L. A. and Carvalho, C. R. (1995) Angiotensin II induces tyrosine phosphorylation of insulin receptor substrate 1 and its association with phosphatidylinositol 3-kinase in rat heart. *Biochem. J.* **310**, 741–744
- Leehey, D. J., Singh, A. K., Alavi, N. and Singh, R. (2000) Role of angiotensin II in diabetic nephropathy. *Kidney Int.* **58**, 93–98
- DCCT Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications of insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **329**, 977–986
- Proud, C. G. and Denton, R. M. (1997) Molecular mechanisms for the control of translation by initiation. *Biochem. J.* **328**, 329–341
- Gingras, A. C., Raught, B. and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68**, 913–963
- Minich, W. B., Balasta, M. L., Goss, D. J. and Rhoads, R. E. (1994) Chromatographic resolution of *in vivo* phosphorylated eukaryotic translation initiation factor eIF4E; increased cap affinity of the phosphorylated form. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7668–7672
- Morley, S. J. and Traugh, J. A. (1990) Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters. *J. Biol. Chem.* **265**, 10611–10616
- Mendez, R., Myers, Jr, M. G., White, M. F. and Rhoads, R. E. (1996) Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **16**, 2857–2864
- Bhandari, B., Fellers, D., Senthil, D., Faulkner, J. L., Gingras, A.-C., Abboud, H. E., Ghosh-Choudhury, G., Sonenberg, N. and Kasinath, B. S. (2001) Insulin induced phosphorylation of a protein translation repressor, 4E-BP1, an eIF4E binding protein, in renal epithelial cells involves diverse signaling pathways. *Kidney Int.* **59**, 866–875

- 17 Yee, J., Kuncio, G. S., Bhandari, B., Shihab, F. S. and Neilson, E. G. (1997) Identification of promoter activity and differential expression of transcripts encoding the murine stromelysin-1 gene in renal cells. *Kidney Int.* **52**, 120–129
- 18 Flynn, A. and Proud, C. G. (1996) Insulin and phorbol ester stimulate initiation factor eIF-4E phosphorylation by distinct pathways in Chinese hamster ovary cells overexpressing the insulin receptor. *Eur. J. Biochem.* **236**, 40–47
- 19 Ghosh-Choudhury, G., Biswas, P., Grandaliano, G., Fouqueray, B., Harvey, S. A. and Abboud, H. E. (1994) PDGF-mediated activation of phosphatidylinositol 3 kinase in human mesangial cells. *Kidney Int.* **46**, 37–47
- 20 Herbert, T. P., Kilhams, G. R., Batty, I. H. and Proud, C. G. (2000) Distinct signalling pathways mediate insulin and phorbol ester-stimulated eukaryotic initiation factor 4F assembly and protein synthesis in HEK 293 Cells. *J. Biol. Chem.* **275**, 11249–11256
- 21 Fleurent, M., Gingras, A. C., Sonenberg, N. and Meloche, S. (1997) Angiotensin II stimulates phosphorylation of the translational repressor 4E-binding protein 1 by a mitogen-activated protein kinase-independent mechanism. *J. Biol. Chem.* **272**, 4006–4012
- 22 Seward, L. and Zahradka, P. (1997) Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle cells. *Circ. Res.* **81**, 249–257
- 23 Velloso, L. A., Folli, F., Sun, X. J., White, M. F., Saad, M. J. and Kahn, C. R. (1996) Cross-talk between the insulin and angiotensin signaling systems. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12490–12495
- 24 Ishida, Y., Kawahara, Y., Tsuda, T., Koide, M. and Yokoyama, M. (1992) Involvement of MAP kinase activators in angiotensin II-induced activation of MAP kinases in cultured vascular smooth muscle cells. *FEBS Lett.* **310**, 41–45
- 25 Von Manteuffel, S., Dennis, D. B., Pullen, N., Gingras, A. C., Sonenberg, N. and Thomas, G. (1997) The insulin induced signaling pathways leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70^{S6K}. *Mol. Cell. Biol.* **17**, 3426–3436
- 26 Rao, G. N., Madamanchi, N. R., Lele, M., Gadiparthi, L., Gingras, A. C., Eling, T. E. and Sonenberg, N. A. (1999) Potential role for extracellular signal related kinases in prostaglandin F_{2α}-induced protein synthesis in smooth muscle cells. *J. Biol. Chem.* **274**, 12925–12932
- 27 Folli, F., Kahn, C. R., Hansen, H., Bouchie, J. L. and Feener, E. P. (1997) Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *J. Clin. Invest.* **100**, 2158–2169
- 28 Dixon, B. S., Sharma, R. V., Dickerson, T. and Fortune, J. (1994) Bradykinin and angiotensin II: activation of protein kinase C in arterial smooth muscle. *Am. J. Physiol.* **266**, C1406–C1420
- 29 Marrero, M. B., Venema, V. J., Ju, H., Eaton, D. C. and Venema, R. C. (1998) Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. *Am. J. Physiol.* **275**, C1216–C1223
- 30 Anderson, P. W., Zhang, X. Y., Tian, J., Correale, J. D., Xi, X. P., Yang, D., Graf, K., Law, R. E. and Hsueh, W. A. (1996) Insulin and angiotensin II are additive in stimulating TGF-β1 and matrix mRNAs in mesangial cells. *Kidney Int.* **50**, 745–753
- 31 Gustafsson, T., Andersson, P., Chen, Y., Magnusson, J. O. and Arqvist, H. J. (1999) Interaction of angiotensin II and the insulin-like growth factor system in vascular smooth muscle cells. *Am. J. Physiol.* **277**, H499–H507
- 32 Yang, D. Q. and Kastan, M. B. (2000) Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. *Nat. Cell. Biol.* **2**, 893–898
- 33 Yu, Z. W., Buren, J., Enerback, S., Nilsson, E., Samuelsson, L. and Eriksson, J. W. (2001) Insulin can enhance GLUT4 gene expression in 3T3-F442A cells and this effect is mimicked by vanadate but counteracted by cAMP and high glucose – potential implications for insulin resistance. *Biochim. Biophys. Acta* **1535**, 174–185
- 34 Lewis, E. J., Hunsicker, L. G., Bain, R. P. and Rohde, R. D. (1993) The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N. Engl. J. Med.* **329**, 1456–1462
- 35 Kohzuki, M., Yasujima, M., Kanazawa, M., Yoshida, K., Fu, L. P., Obara, K., Saito, T. and Abe, K. (1995) Antihypertensive and renal protective effects of losartan in streptozotocin diabetic rats. *J. Hypertens.* **13**, 97–103
- 36 Torlone, E., Britta, M., Rambotti, A. M., Perriello, G., Santeusano, F., Brunetti, P. and Bolli, G. B. (1993) Improved insulin action and glycemic control after long-term angiotensin-converting enzyme inhibition in subjects with arterial hypertension and type II diabetes. *Diabetes Care* **16**, 1347–1355
- 37 Hansson, L., Lindholm, L. H., Niskanen, L., Lanke, J., Hedner, T., Niklason, A., Luomanmaki, K., Dahlöf, B., de Faire, U., Morlin, C. et al. (1999) Effect of angiotensin converting enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril Prevention Project (CAPP) randomized trial. *Lancet* **353**, 611–616
- 38 HOPE Study Investigators (2000) Effects of angiotensin converting enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *N. Engl. J. Med.* **342**, 145–153

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