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, trans-activates 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 32P labelling**

PTE cells were labelled with [32P]PPi, 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 μCi/ml [32P]PPi (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 EDTA, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/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 EDTA, 2 mM benzamidine, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/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. 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 (m7 GTP)–Sepharose affinity chromatography was performed as described previously [18]. A slurry of m7 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 m7 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 m7 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 glycolbis(β-aminoethyl ether)-N,N,N’,N’-tetra-acetic acid. Following addition of 20 mM MgCl2, and 5 μCi of [γ-32P]ATP to the assay mixture, the reactions 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 reactions were extracted with 100 μl of chloroform and the organic layer was washed with methanol/1 M HCl in a 1:1 ratio. The organic layers were dried, redissolved 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
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followed by autoradiography. Reactive bands were detected by chemiluminescence phosphotyrosine antibody (1:2000 dilution; Upstate Biotechnology). The membrane was incubated with a monoclonal anti-immune complexes were separated by SDS/PAGE (7.5% gel). Proteins were transferred to nitrocellulose membrane and incubated with anti-phosphotyrosine antibody (1:2000 dilution; Santa Cruz Biotechnology) or IRS-1 (Upstate Biotechnology), and the membrane was incubated with a monoclonal anti-phosphotyrosine antibody (1:2000 dilution; Upstate Biotechnology). Reactive bands were detected by chemiluminescence following 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 incubated with a monoclonal anti-phosphotyrosine antibody (1:2000 dilution; Upstate Biotechnology). Western blotting with the anti-ERK-1 antibody was performed on an aliquot of assay lysates to assess loading.

**Protein-synthesis assay**

Serum-starved cells were incubated with angiotensin II and/or insulin, as described above, followed by incubation with [35S]-methionine at 10 μCi/ml for 60 min. The cells were then washed in PBS and lysed in RIPA buffer. Protein (20 μg) was spotted on to 3 MM paper (Whatman Intenational, 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 ± 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 and 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-induced 4E-BP1 phosphorylation. [32P]P labelling further confirmed these data. There was an increase in incorporation of 32P 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 P1 3-kinase activity**

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

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
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-

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 $[^32P]$P, 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 $[^32P]$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 $[^32P]$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 AT$_1$ 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 AT$_1$ receptor, a prototypical G-protein-coupled receptor. We examined whether AT$_1$ receptor was involved in angiotensin II regulation of insulin-induced 4E-BP1 phosphorylation. First, we tested the role of AT$_1$ receptor in ERK-1/-2-type MAP kinase activation. Serum-starved cells were incubated with or without losartan, a blocker of AT$_1$ 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...
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 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-
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 AT1 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 AT$_1$ 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 AT$_1$ 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 I [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 AT$_1$ 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 eIF-4E. 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 AT$_1$ 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

Identification of promoter activity and differential expression of transcripts encoding the murine stromelysin-1 gene in renal cells. Kidney Int. 52, 120–129


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