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Angiotensin-induced EGF receptor transactivation inhibits insulin signaling in C9 hepatic cells

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ABSTRACT

To investigate the potential interactions between the angiotensin II (Ang II) and insulin signaling systems, regulation of IRS-1 phosphorylation and insulin-induced Akt activation by Ang II were examined in clone 9 (C9) hepatocytes. In these cells, Ang II specifically inhibited activation of insulin-induced Akt Thr^{308} and its immediate downstream substrate GSK-3α/β in a time-dependent fashion, with ~70% reduction at 15 min. These inhibitory actions were associated with increased IRS-1 phosphorylation of Ser^{636}/Ser^{639} that was prevented by selective blockade of EGFR tyrosine kinase activity with AG1478. Previous studies have shown that insulin-induced phosphorylation of IRS-1 on Ser^{636}/Ser^{639} is mediated mainly by the PI3K/mTOR/S6K-1 sequence. Studies with specific inhibitors of PI3K (wortmannin) and mTOR (rapamycin) revealed that Ang II stimulates IRS-1 phosphorylation of Ser^{636}/Ser^{639} via the PI3K/mTOR/S6K-1 pathway. Both inhibitors blocked the effect of Ang II on insulin-induced activation of Akt. Studies using the specific MEK inhibitor, PD98059, revealed that ERK1/2 activation also mediates Ang II-induced S6K-1 and IRS-1 phosphorylation, and the impairment of Akt Thr^{308} and GSK-3α/β phosphorylation. Further studies with selective inhibitors showed that PI3K activation was upstream of ERK, suggesting a new mechanism for Ang II-induced impairment of insulin signaling. These findings indicate that Ang II has a significant role in the development of insulin resistance by a mechanism that involves EGFR transactivation and the PI3K/ERK1/2/mTOR-S6K-1 pathway.

1. Introduction

Insulin, the most potent anabolic hormone, is essential for optimal tissue development and growth, and the maintenance of glucose homeostasis. Insulin action is mediated through a complex network of signaling events initiated when the hormone binds to its cell-surface receptors. Activation of the insulin receptor (IR) triggers its intrinsic protein-tyrosine (Tyr) kinase activity, resulting in autophosphorylation of several IR Tyr residues and the recruitment and phosphorylation of IR substrate (IRS) proteins [1]. After Tyr phosphorylation, IRS acts as docking protein for several Src homology 2 (SH2) domain-containing molecules, including Grb2, the small adapter protein Nck, the tyrosine phosphatase Syp, phosphatidylinositol 3-kinase (PI3K), and others [1,2].

PI3K has a central role downstream of the IRS proteins in the activation and regulation of many insulin-induced metabolic processes. During its association with IRS, PI3K promotes the activation of Akt (PKB) that regulates multiple biological processes. These include stimulation of glucose transport, glycogen and protein synthesis, cellular proliferation and survival through regulation of additional kinases such as the glycogen synthase kinase-3α/β (GSK-3α/β), FOXO1, BAD, TSC2 and AS160 [1–5].

The duration and extent of signals induced by insulin are tightly regulated to promote optimal insulin actions in the body, and impaired generation of these signals causes a common pathological state termed insulin resistance. This occurs in a wide variety of pathological states, including obesity, hypertension, chronic infection and cardiovascular diseases, and is a central component of non-insulin dependent diabetes mellitus or Type 2 Diabetes Mellitus (DM2) [6–9].

It has been proposed that multiple phosphorylation of IRS proteins in serine/threonine (Ser/Thr) residues has a key role in the inhibition of insulin signaling by both physiological and pathophysiological activation of a negative feedback mechanism [10].

Abbreviations: Ang II, angiotensin II; AT_1R, angiotensin type 1 receptor; EGF, epidermal growth factor; GPCRs, G protein-coupled receptors; GSK-3, glycogen synthase kinase-3; IRS, insulin receptor substrate; IR, insulin receptor; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; RAS, renin–angiotensin system; S6K-1, ribosomal S6 kinase-1.

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Interestingly, many factors that enhance Ser/Thr phosphorylation of IRS proteins have been implicated in the development of insulin resistance. These include endothelin [11], TNFα [12], free fatty acids [13] and Ang II [14].

Ang II, the major effector hormone of the renin–angiotensin system (RAS), has an important role in the regulation of vascular and renal homeostasis. The actions of Ang II are initiated by its interaction with two GPCRs, the AT_1 and the AT_2 receptor subtypes (AT_1R and AT_2R) [15]. The majority of the biological and pathological actions of Ang II are mediated by the AT_1R, which signals via $G_{q/11}$ to activate phospholipase C-β. The subsequent generation of second messengers such as diacylglycerol (DAG) and inositol trisphosphate (IP3), in turn stimulate the activity of the protein PKC and mobilize Ca^{2+} from intracellular reservoirs [15]. This signaling pathway is the primary transduction mechanism initiated by the AT_1R in its major physiological tissues, including adrenal, neuronal, cardiac, renal, hepatic and smooth muscle cells [15,16]. In recent years, several reports have shown that AT_1Rs are also connected to signaling pathways usually associated with growth factor and cytokine receptors. This occurs mainly through AT_1R coupling to transactivation of epidermal growth factor receptors (EGFRs) to mediate critical cellular events such as growth, proliferative and antiproliferative effects, and migration [15,17–19].

Recent clinical and pharmacological studies have shown that Ang II induces insulin resistance, and that Ang II-converting enzyme inhibitors (ACEIs) and AT_1R blockers (ARBs) improve insulin sensitivity [5,20–22]. These findings suggest that overactivity of the RAS impairs insulin signaling and contributes to insulin resistance. Furthermore, Ang II also induces Ser phosphorylation of IRS-1 by ERK1/2 and PKC at Ser^{312} and Ser^{616}, leading to the impairment of PI3K and Akt activation [5,14,23–25]. Although phosphorylation of IRS-1 at Ser^{312} and Ser^{616} impairs insulin signaling, recent reports indicate that phosphorylation of IRS-1 on Ser^{636}/Ser^{639} has a major inhibitory action on the development of DM2 in humans [26]. In this context, Khamzina et al. [27] reported that insulin activation of the mammalian target of rapamycin (mTOR) and ribosomal S6 kinase-1 (S6K-1) inhibits insulin signaling to the PI3K/Akt pathway in hepatic cells, possibly by increased inhibitory phosphorylation of IRS-1 on Ser^{636}/Ser^{639} [27]. They also showed that mTOR and S6K-1 activation are increased in the liver and skeletal muscle of high fat-fed, obese rats, implicating these Ser/Thr kinases as potential mediators of insulin resistance [27].

The actions of Ang II on insulin sensitivity have been mainly described in cardiovascular cell models, since insulin resistance has been considered as an important risk factor in the development of cardiovascular diseases such as hypertension and atherosclerosis [14,24,25,28]. Although in insulin resistant states, insulin action is also impaired in the liver, skeletal muscle and adipose tissue, relatively few studies have explored the effect of Ang II on the development of insulin resistance in those tissues [3,29–31]. In this context, hepatic insulin resistance is an important underlying cause of the metabolic syndrome that manifests itself in diseases such as DM2, atherosclerosis and non-alcoholic fatty liver disease [32]. In the present study, an analysis of Ang II action in a hepatic cell line was performed to seek insights into the molecular mechanism by which Ang II interferes with insulin action leading to impairment of insulin.

2. Materials and methods

2.1. Reagents and antibodies

F-12K nutrient mixture (Kaighn's modification), fetal bovine serum (FBS), trypsin, and antibiotic/amphotericin B solutions and EGF were from Invitrogen-GIBCO (Carlsbad, CA, USA). Ang II was from BACHEM (Torrance, CA, USA); insulin, wortmannin, LY-294002 [2-(4-morpholinyl)-8-phenyl-(4H)-1-benzopyran-4-one], AG1478 (tyrphostin), GM6001 and rapamycin were from Sigma (St. Louis, MO, USA). Bisindolylmaleimide I, PD98059 and lactacystin were from Calbiochem (La Jolla, CA, USA). Losartan (DuP 753) was a generous gift from DuPont (Wilmington, DE, USA). ECL reagents were from GE Healthcare (Buckinghamshire, UK) or Immobilon™ Millipore Corporation (Billerica, MA, USA). Anti-phospho-ERK1/2 Thr^{202}/Tyr^{204} (anti-p-ERK1/2 Thr^{202}/Tyr^{204}), anti-phospho-IRS-1 Ser^{636}/Ser^{639} (anti-p-IRS-1 Ser^{636}/Ser^{639}), anti-IRS-1, anti-GSK-3β and anti-phospho-GSK-3α/β $Ser^{21/9}$ (anti-p-GSKα/β $Ser^{21/9}$) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-p70 S6K-1 Thr^{421}/Ser^{424} (anti-p-S6K-1 Thr^{421}/Ser^{424}), anti-phospho-Akt Thr^{308} (anti-p-Akt Thr^{308}), anti-IR (β-subunit), anti-p70 S6K-1 (anti-S6K-1) and anti-ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt Ser^{473} (anti-p-Akt Ser^{473}) was from UPSTATE (Lake Placid, NY, USA). Polyclonal and monoclonal antibodies were purchased from Invitrogen-Zymed (Carlsbad, CA, USA).

2.2. Cell culture

C9 rat liver epithelial cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were grown in Ham's F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (v/v) FBS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C in 95% air and 5% CO_2. For all studies, C9 cells between passages 3 and 12 were used because they exhibit maximum expression of their endogenous AT_1 receptors [17]. For experiments, cells were re-seeded on six- and twelve-well plates. Before experiments, C9 cells were maintained in low serum medium for 16 h, and then switched to serum-free medium for 3 h.

2.3. Immunoblot analysis

C9 cells were grown in six- or twelve-well plates in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% FBS. At 70–80% confluence, C9 cells were grown in 2% serum/F-12K nutrient mixture for 16 h, then media were changed to serum-free media for 3 h before treatment with the indicated ligands and inhibitors. After treatment, cells were placed on ice, the media were aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer 1×. The samples were briefly sonicated, heated at 99 °C, and centrifuged for 5 min at 12,000 rpm. The supernatant was electrophoresed on SDS-PAGE (8 or 10%) gels and transferred to PVDF nylon membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with ECL [enhanced chemiluminescence reagent]. Autoradiograms were scanned using the GS-800 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA, USA), and the labeled bands were quantified using the Quantity One 4.6.3 software program (Bio-Rad, Hercules, CA, USA).

2.4. Statistical analysis

Measurements of intensity from Western blots were analyzed using either one- or two-way ANOVA with Dunnet and Bonferroni's post-test using PRISM, version 4.0 (GraphPad Software, San Diego, CA, USA). In all cases, $p < 0.05$ was considered to be significant. Resultant data were plotted on bar graphs, with data expressed as mean ± S.E.M. percentage from at least three separate experiments, and representative blots are shown as needed.

3. Results

3.1. Insulin induces Akt phosphorylation at Thr^{308} and Ser^{473}

We first determined the effect of insulin on phosphorylation of Akt at Thr^{308} and Ser^{473} in hepatic C9 cells, where insulin caused rapid and marked phosphorylation of Akt at both sites (Fig. 1A). However, important differences in the maximum effect and temporality of phosphorylation were observed. Insulin-induced Akt Ser^{473} phosphorylation was relatively sustained, reaching a maximum (~200%) at 5 min, and persisted for up to 60 min or longer. In contrast, insulin-induced Akt Thr^{308} phosphorylation reached a maximum (about 350%) after 15 min and then declined over 30–60 min without reaching the basal phosphorylation level of Akt.

To determine the ability of Akt to regulate its immediate downstream substrate, glycogen synthase kinase-3α/β (GSK-3α/β), we evaluated phosphorylation and inactivation of GSK-3α/β in C9 cells. GSK-3α/β is a key regulatory enzyme of glycogen synthesis and represents one of the main insulin substrates in hepatic tissue. As expected, insulin caused rapid GSK-3α/β phosphorylation that reached a maximum (~200%) at 15 min and declined over 45–60 min without reaching the basal phosphorylation level (Fig. 1B).

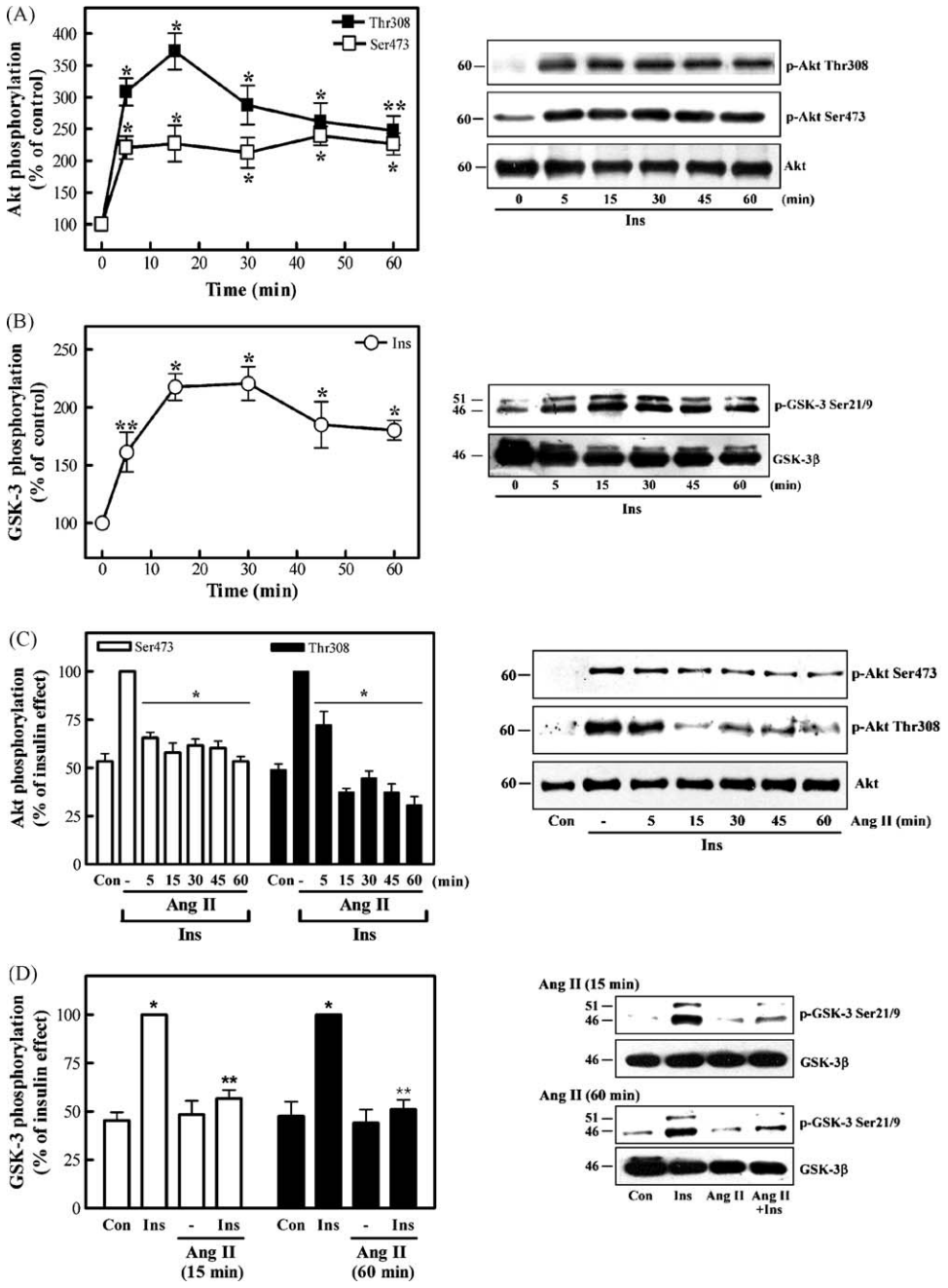

Fig. 1. Effect of Ang II on insulin-induced Akt and GSK-3 phosphorylation. C9 cells were treated with 100 nM insulin (A and B) for the indicated times, or pretreated with 100 nM Ang II from 5 to 60 min and then stimulated with 100 nM insulin for an additional 15 min (C and D). Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt Ser^{473}, anti-p-Akt Thr^{308} or anti-p-GSK-3α/β Ser$^{21/9}$ as described in Section 2. Vertical lines represent the S.E.M. The right panels show representative immunoblots. Western blots were also probed for total Akt and GSK-3β, showing equal loading. (A) $^{*}p < 0.001$ and $^{**}p < 0.01$ *vs* time 0 (control). (B) $^{*}p < 0.01$ and $^{**}p < 0.05$ *vs* time 0 (control). (C) $^{*}p < 0.001$ *vs* insulin (-). (D) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* insulin. Con, control; Ins, insulin.

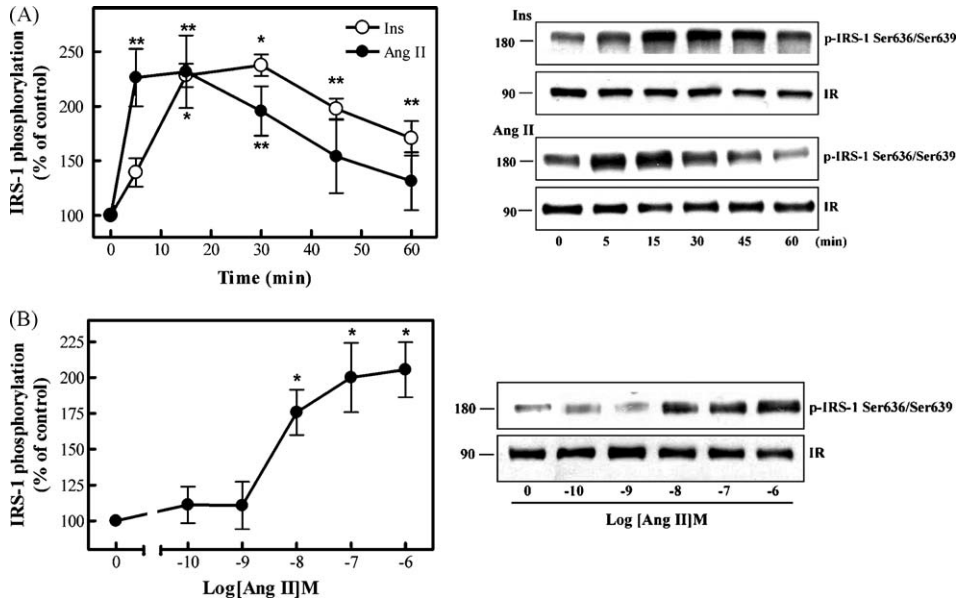

Fig. 2. Ang II induces IRS-1 Ser^{636}/Ser^{639} phosphorylation. (A) Cells were stimulated with 100 nM insulin or 100 nM Ang II for the indicated times. (B) Cells were exposed to the indicated concentration of Ang II for 15 min. Total cell lysates were separated by SDS-PAGE, and analyzed by immunoblotting with anti-p-IRS-1 Ser^{636}/Ser^{639} as described in Section 2. Vertical lines represent the S.E.M. The right panels show representative immunoblots. Western blots were also probed for total IR showing equal loading. (A) $^{*}p < 0.01$ and $^{**}p < 0.05$ *vs* time 0 (control). (B) $^{*}p < 0.05$ *vs* time 0 (control). Ins, insulin.

3.2. Ang II desensitizes insulin signaling

Since several reports have demonstrated that impairment of insulin-induced Akt phosphorylation is an important event in insulin resistance [30,32,34], we determined whether Ang II impairs insulin-induced Akt phosphorylation in C9 cells. As shown in Fig. 1C, incubation of cells with 100 nM Ang II from 5 to 60 min, followed by addition of 100 nM insulin for 15 min, reduced insulin-induced Akt phosphorylation on both Ser^{473} and Thr^{308}. Interestingly, the impairment of Akt phosphorylation by Ang II on these two sites was different: while Ang II inhibited Thr^{308} phosphorylation by about 70% ($p < 0.001$), Ser^{473} phosphorylation was inhibited by only 40% ($p < 0.001$). These data indicate for the first time that Ang II differentially affects the phosphorylation of Akt at Thr^{308}, a crucial residue involved in the Akt-induced metabolic actions of insulin [31,34].

To determine whether the effect of Ang II on insulin-induced Akt phosphorylation affects GSK-3α/β regulation, we examined the phosphorylation state of GSK-3α/β. As expected, phosphorylation of GSK-3α/β by insulin was diminished by pretreatment with Ang II for 15 or 60 min (Fig. 1D), indicating the ability of Ang II to desensitize insulin signaling and its downstream effectors.

3.3. Ang II induces phosphorylation of IRS-1 at Ser^{636}/Ser^{639}

Previous studies have shown the relevance of phosphorylation of IRS at Ser^{636}/Ser^{639} as a key mechanism in the impairment of insulin actions [35,36]. We therefore determined whether Ang II could induce IRS-1 phosphorylation in C9 cells. We first examined the effect of insulin on IRS-1 Ser^{636}/Ser^{639} phosphorylation in a control experiment, since this constitutes a mechanism of homologous desensitization in several cell types. As shown in Fig. 2A, IRS-1 phosphorylation at Ser^{636}/Ser^{639} was increased by insulin, reaching maximal activation (~230%) at 15 min and then declining over the next 45 min without reaching the basal level (~170% at 60 min). Interestingly, Ang II also increased IRS-1 Ser^{636}/Ser^{639} phosphorylation in a time- and concentration-dependent manner. Ang II promoted IRS-1 phosphorylation with a maximal increase of ~2-fold at 100 nM (Fig. 2B) that rapidly peaked at 5 min (~230%) and then declined after 15 min in a time-dependent manner (Fig. 2A).

3.4. Ang II causes phosphorylation of IRS-1 through the mTOR/S6K-1 pathway

Activation of mTOR/p70 S6 kinase-1 has emerged as the critical event in rendering IRS-1 unresponsive to insulin, mainly through phosphorylation of IRS-1 on Ser^{636}/Ser^{639} [27,37]. To determine whether p70 S6 kinase-1 (S6K-1) might be the kinase involved in insulin- and Ang II-induced IRS-1 Ser^{636}/Ser^{639}phosphorylation, we used a phospho-specific antibody that detects S6K-1 when it is phosphorylated at Thr^{421}/Ser^{424} in the auto-inhibitory domain [38]. C9 cells exposed to insulin showed a time- and dose-dependent S6K-1 phosphorylation that reached a maximum at 100 nM (~250%) (Fig. 3A) after 15 min stimulation that was maintained up to 60 min (Fig. 3B). In contrast, although Ang II increased phosphorylation of S6K-1, the maximum effect observed was lower than that caused by insulin, in a range between 1 and 1000 nM (~200%) (Fig. 3A). Interestingly, Ang II-induced S6K-1 phosphorylation peaked at 5 min and then decreased in a time-dependent manner (Fig. 3B).

S6K-1 is a downstream target of mTOR, a critical kinase involved in metabolic disorders and tumorigenesis [39]. To determine whether IRS-1 Ser^{636}/Ser^{639} phosphorylation is mediated by mTOR/S6K-1, C9 cells were preincubated with the specific mTOR inhibitor, rapamycin (100 nM) for 30 min, and then stimulated with insulin and Ang II. As expected, insulin- and Ang II-induced S6K-1 Thr^{421}/Ser^{424} phosphorylation was completely inhibited by rapamycin treatment. Furthermore, rapamycin also inhibited insulin- and Ang II-induced IRS-1 Ser^{636}/Ser^{639} phosphorylation (Fig. 3C). These data suggest that in C9 cells the mTOR/S6K-1 pathway is necessary for IRS-1 Ser^{636}/Ser^{639} phosphorylation caused by insulin and Ang II.

The classical pathway that has been described for mTOR/S6K-1 activation implies PI3K/Akt activation [40]. For this reason, we determined whether insulin- and Ang II-induced S6K-1 and IRS-1 phosphorylation were PI3K-dependent. As shown in Fig. 3D, when cells were exposed to 100 nM wortmannin (a selective PI3K

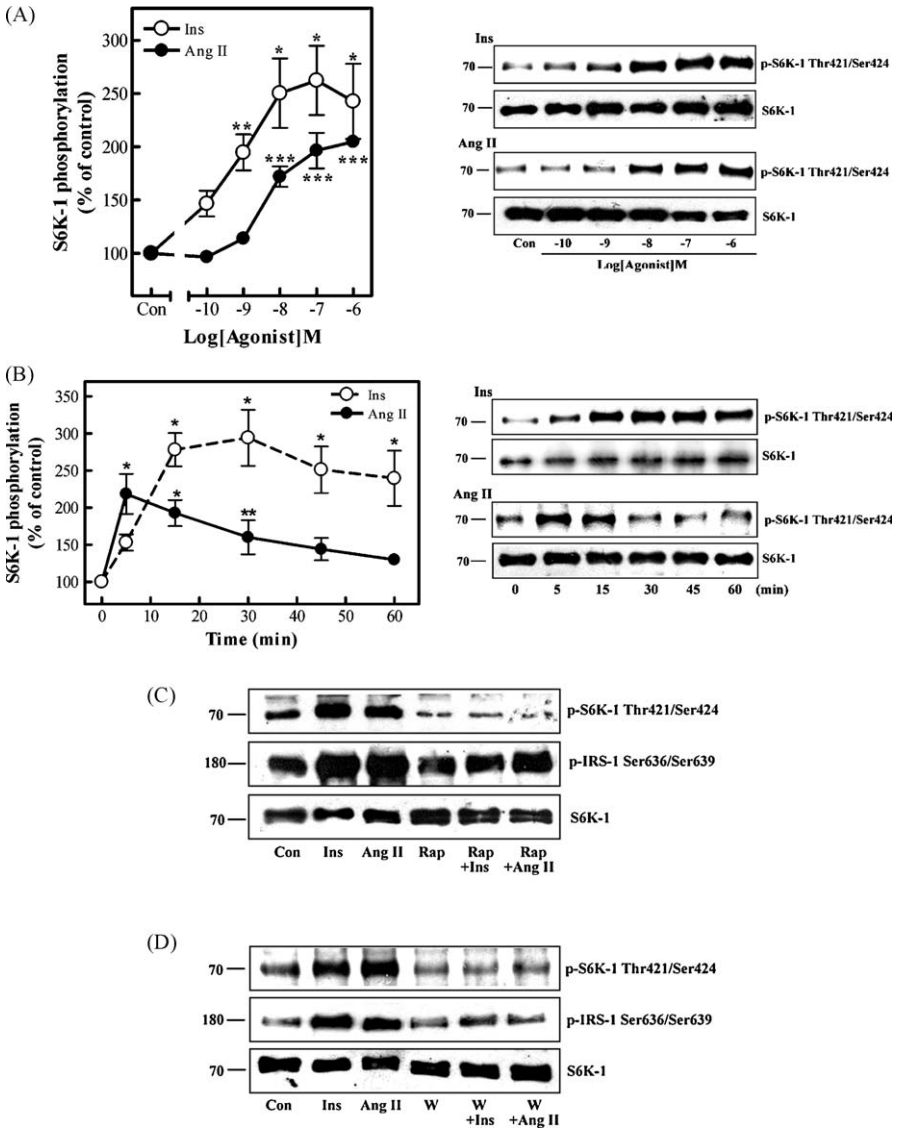

Fig. 3. PI3K/Akt/S6K-1 pathway mediates Ang II-induced IRS-1 Ser^{636}/Ser^{639} phosphorylation. (A) Cells were exposed to the indicated concentrations of Ang II or insulin for 15 min. (B) Cells were stimulated with 100 nM insulin or 100 nM Ang II for the indicated times. (C and D) Cells were pretreated with or without 100 nM rapamycin (C) or 100 nM wortmannin (D) for 30 min, before stimulation with 100 nM Ang II or 100 nM insulin for 15 min. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-S6K-1 Thr^{421}/Ser^{424} or anti-p-IRS-1 Ser^{636}/Ser^{639} as described under Section 2. Vertical lines represent the S.E.M. The panels show representative immunoblots. Western blots were also probed for total S6K-1 showing equal loading. (A) $^{*}p < 0.01$, $^{**}p < 0.05$, and $^{***}p < 0.001$ *vs* control. (B) $^{*}p < 0.01$ and $^{**}p < 0.05$ *vs* time 0 (control). Con, control; Ins, insulin; Rap, rapamycin; W, wortmannin.

inhibitor) for 30 min, the phosphorylation of S6K-1 Thr^{421}/Ser^{424} and IRS-1 Ser^{636}/Ser^{639} by both hormones was totally inhibited. Similar results were obtained when LY-294002, another selective PI3K inhibitor, was employed (data not shown). This indicated that insulin and Ang II exert their effects on IRS-1 phosphorylation through the PI3K/mTOR/S6K-1 signaling pathway.

3.5. Ang II promotes S6K-1 and IRS-1 phosphorylation by EGF receptor transactivation

The above results have shown that Ang II induces IRS-1 Ser^{636}/Ser^{639} phosphorylation through the PI3K/mTOR/S6K-1 pathway, similar to the manner in which insulin does so. However, there remains the question of how Ang II activates the PI3K/mTOR/S6K-1 pathway and induces IRS-1 Ser^{636}/Ser^{639} phosphorylation and Akt inactivation in C9 cells.

In this context, previous studies in C9 cells have shown that Ang II-induced activation of the AT_1R causes PI3K/Akt activation by transactivating the EGFR [41,42]. This transactivation pathway is mediated by PKC/Src-dependent activation of transmembrane matrix metalloproteases (MMPs), which release heparin-binding EGF (HB-EGF) that causes activation of the EGFR [19,43]. To determine whether Ang II-induced IRS-1 and S6K-1 phosphorylation are due to AT_1R-mediated EGFR transactivation, cells were exposed to the AT_1 receptor antagonist DuP 753, and specific inhibitors of EGFR tyrosine kinase activity (AG1478), MMPs (Galardin, GM6001) and PKC (Bisindolylmaleimide I, BIM) for 30 min, in the presence or absence of 100 nM Ang II or 100 nM insulin. Consistent with our previous reports showing that Ang II exerts its effects through activation of AT_1Rs in C9 cells [16,42], Ang II-induced S6K-1 and -IRS-1 phosphorylation was completely inhibited with 10 μM DuP 753 (Fig. 4A and B, respectively). However, phosphorylation of both proteins was not altered when cells were stimulated with insulin in the presence of an AT_1R antagonist (Fig. 4A and B). The use of specific inhibitors to block AT_1R-induced EGFR transactivation confirmed that Ang II/AT_1R-

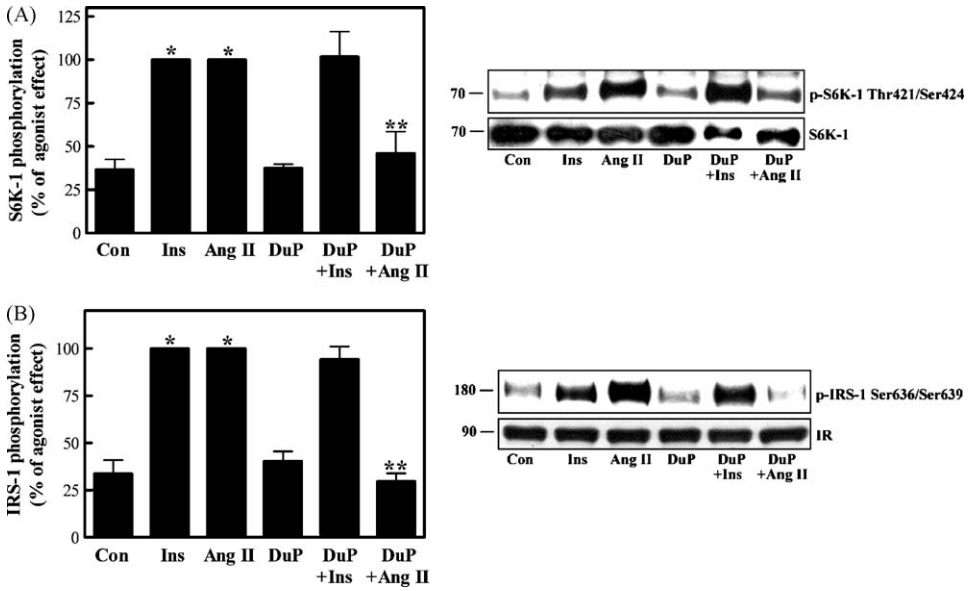

Fig. 4. Ang II acts through the AT_1 receptor. Cells were pretreated with or without 10 μM DuP 753 for 30 min, before stimulation with 100 nM Ang II or 100 nM insulin for 15 min. Total cell lysates were separated by SDS-PAGE and analyzed with anti-p-S6K-1 Thr^{421}/Ser^{424} (A) or anti-p-IRS-1 Ser^{636}/Ser^{639} (B) as described in Section 2. Vertical lines represent the S.E.M. The right panels show representative immunoblots for A and B. Western blots were also probed for total S6K-1 and IR showing equal loading. (A) $^{*}p < 0.01$ *vs* control; $^{**}p < 0.01$ *vs* Ang II. (B) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* Ang II. Con, control; Ins, insulin; DuP, DuP 753.

induced S6K-1 Thr^{421}/Ser^{424} and IRS-1 Ser^{636}/Ser^{639} phosphorylation are dependent on activation of PKC, MMPs and EGFR transactivation (Fig. 5A–D).

3.6. Activation of ERK1/2 by Ang II promotes S6K-1 and IRS-1 phosphorylation

We next evaluated the role of ERK1/2 activation in Ang II-induced S6K-1 and IRS-1 phosphorylation, since recent evidence suggests that ERK1/2 represents a direct upstream S6K-1 kinase involved in its phosphorylation and activation [44,45]. For this purpose, C9 cells were pretreated with the selective MEK1/2 inhibitor PD98059 (1 μM), and stimulated with 100 nM Ang II or 100 nM insulin for 15 min. As expected, both insulin- and Ang II-induced ERK1/2 phosphorylation were completely inhibited when cells were exposed to PD98059 (Fig. 6A). Interestingly, the MEK1/2 inhibitor also completely inhibited only the Ang II-induced IRS-1 and S6K-1 phosphorylation and not those induced by insulin (Fig. 6B and C, respectively). These data strongly suggest a critical role of ERK1/2 in Ang II-induced S6K-1 and IRS-1 phosphorylation. Since our data did not rule out the involvement of PI3K in the activation of S6K-1, we next determined if ERK1/2 is a downstream effector of the PI3K/Akt pathway. For this we examined the effect of wortmannin on Ang II-induced ERK1/2 phosphorylation. As shown in Fig. 7, wortmannin blocked Ang II-induced ERK1/2 phosphorylation, but had no effect on insulin-induced ERK1/2 phosphorylation. Taken together, these data suggest that Ang II-induced ERK1/2 phosphorylation is downstream of PI3K and its activation is necessary for Ang II-induced S6K-1 Thr^{421}/Ser^{424} and IRS-1 Ser^{636}/Ser^{639} phosphorylation.

3.7. The inhibitory effect of Ang II on Akt and GSK-3 is mediated by IRS-1 Ser^{636}/Ser^{639} phosphorylation

Our results demonstrate that Ang II-induced S6K-1 activation and the subsequent phosphorylation of IRS-1 Ser^{636}/Ser^{639} are mediated by EGFR transactivation through PI3K/ERK/mTOR. To determine whether this signaling mechanism is responsible for inhibition of Akt and GSK-3α/β, C9 cells were exposed to specific kinase inhibitors (1 μM AG1478, 1 μM GM6001, 1 μM BIM), then treated with 100 nM Ang II for 15 or 60 min and stimulated with 100 nM insulin for an additional 15 min. Insulin-stimulated Akt Thr^{308} (Fig. 8) and GSK-3α/β (data not shown) phosphorylation was not affected, indicating that Ang II/AT_1R-mediated EGF receptor transactivation is necessary for the inhibitory effect of Ang II on insulin-induced Akt Thr^{308} and GSK-3α/β phosphorylation.

Since wortmannin and LY-294002 inhibit at the level of PI3K to modulate Akt activity, we could not directly evaluate the involvement of PI3K in Ang II-induced Akt Thr^{308} inhibition because both compounds also inhibit insulin-induced Akt Thr^{308} (Fig. 8) and GSK-3α/β (data not shown) phosphorylation [31]. However, rapamycin, which inhibits the downstream effector of PI3K, mTOR/S6K-1, blocked the inhibitory effect of Ang II (Figs. 8 and 9), suggesting the involvement of the PI3K/mTOR/S6K-1 pathway in the inhibitory effect on Akt Thr^{308} and GSK-3α/β phosphorylation.

The role of MEK/ERK in the Ang II-induced activation of S6K-1 and IRS-1 phosphorylation observed above suggested its participation in the development of insulin resistance. To investigate the role of MEK/ERK in the Ang II-induced inhibition of Akt activity and GSK-3α/β, C9 cells were exposed to PD98059 for 30 min, treated with Ang II (100 nM for 15 or 60 min) and stimulated with insulin for 15 min. The MEK inhibitor was found to inhibit the effect of Ang II (Figs. 8 and 9), indicating that its inhibitory effect on Akt Thr^{308} and GSK-3α/β phosphorylation involves the MEK/ERK1/2 pathway.

It has been recently shown that Ser^{636}/Ser^{639} phosphorylation of IRS-1 triggers its degradation in response to insulin, as a homologous desensitization mechanism [35,36]. To determine if this mechanism is also involved in Ang II-induced Akt Thr^{308} inhibition, we evaluated the effect of insulin or Ang II treatment on IRS-1 protein integrity. When C9 cells were exposed to 100 nM insulin or 100 nM Ang II, and IRS-1 protein integrity was evaluated with an anti-IRS-1 antibody, insulin treatment reduced IRS-1 protein by about 44% as detected by Western blot analysis. Interestingly, Ang II treatment from 60 to 180 min also diminished IRS-1 protein by about 40% (Fig. 10A). When cells were pretreated with specific inhibitors of Ang II signaling, such as DuP 753,

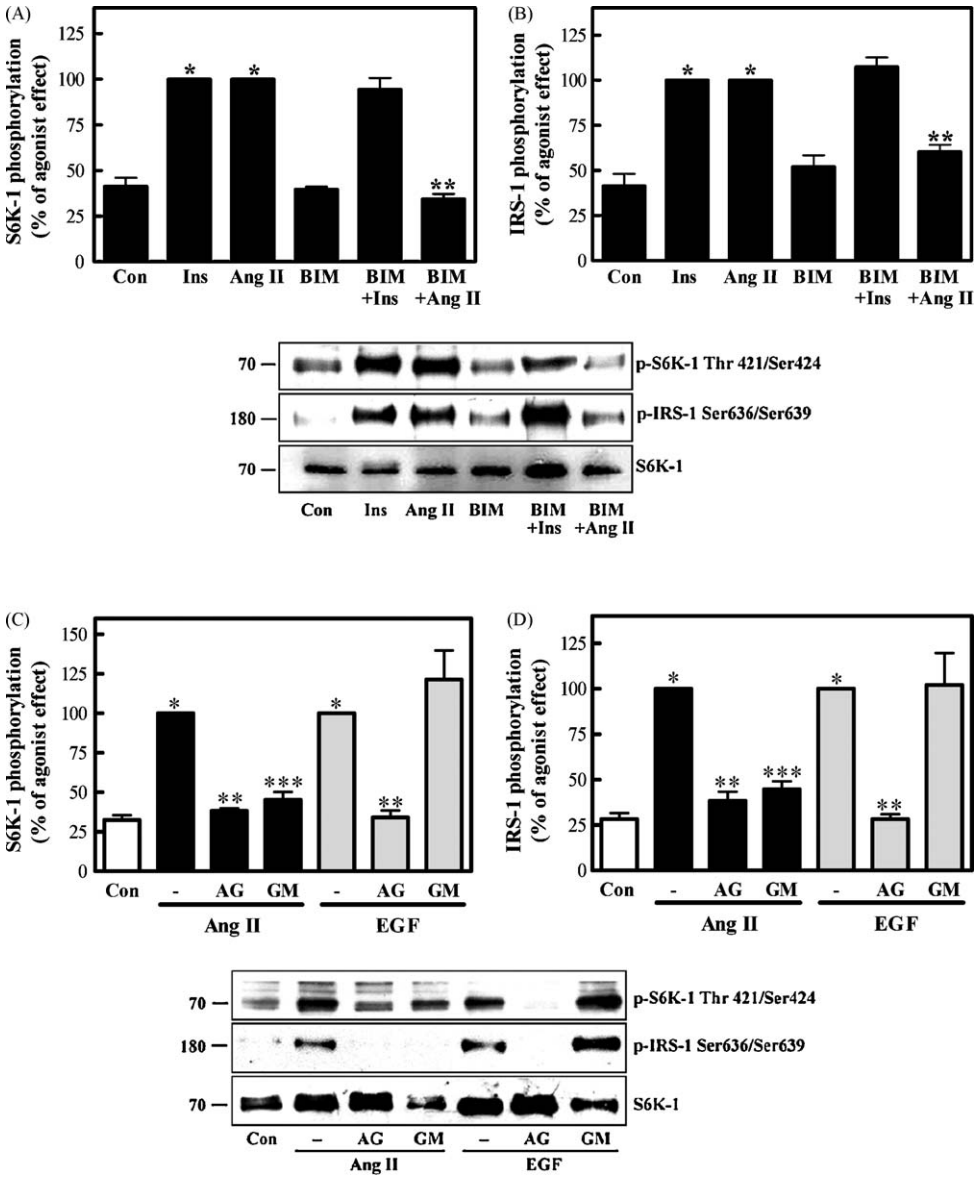

Fig. 5. Transactivation of EGFRs by Ang II mediates IRS-1 and S6K-1 phosphorylation. C9 cells were pretreated with 1 μM BIM (A and B), 1 μM AG1478 or 1 μM GM6001 (C and D) for 30 min, before stimulation with 100 nM Ang II (A–D) or 100 nM insulin (A and B) for 15 min, or 10 ng/ml EGF for 5 min (C and D). Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-S6K-1 Thr^{421}/Ser^{424} (A and C) or anti-p-IRS-1 Ser^{636}/Ser^{639} (B and D) as described in Section 2. Vertical lines represent the S.E.M. The lower panels show representative immunoblots for A and B and C and D. Western blots were also probed for total S6K-1 showing equal loading. (A) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* Ang II. (B) $^{*}p < 0.01$ *vs* control; $^{**}p < 0.05$ *vs* Ang II. (C and D) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* Ang II or EGF (-); $^{***}p < 0.01$ *vs* Ang II (-). Con, control; Ins, Insulin; AG, AG1478; GM, GM6001.

PD98059 or rapamycin, the degradative effect of Ang II on IRS-1 protein was inhibited (Fig. 10C).

It is known that various cellular proteins of the insulin signaling process, including IRS-1, are tightly regulated by proteolysis through proteasome in response to external signaling molecules [35,36]. For this reason, we sought to determine whether proteasome-mediated degradation is involved in the insulin- and Ang II-dependent decline of the IRS-1 level. To address this question, serum-starved C9 cells were cultured with Ang II in the presence or absence of the proteasome inhibitor, lactacystin. As shown in Fig. 10B and C, such proteasome inhibition caused marked stabilization of IRS-1 despite insulin and Ang II stimulation. This finding indicated that IRS-1 is regulated through proteasome-mediated degradation, which is triggered by Ang II stimulation. This decrease in IRS-1 protein may explain the inhibitory effect of Ang II treatment for 60 min on Akt Thr^{308} phosphorylation, although IRS-1 $Ser^{636/639}$ phosphorylation is absent.

4. Discussion

In the present study, we employed the rat hepatic C9 cell model, to investigate the effect of Ang II on insulin signaling, focusing on the insulin-induced regulation of Akt and GSK-3α/β, key proteins involved in insulin signaling. The C9 cells employed in our study are derived from the normal rat liver and retain an epithelial phenotype. They also express endogenous AT_1Rs and IRs and provide a useful model for studies on the individual and interacting signaling mechanisms of GPCRs and RTKs [16–19,43,46–49]. Our results show that Ang II, upon transactivation of the EGFR, inhibits insulin-induced Akt activation through phosphorylation of IRS-1 at Ser^{636}/Ser^{639} and its subsequent proteasomal degradation by a mechanism that depends on the PI3K/ERK1/2/mTOR/S6K-1 signaling pathway.

Activation of Akt by insulin is a multistep process that for full activation requires hierarchical phosphorylation of two residues,

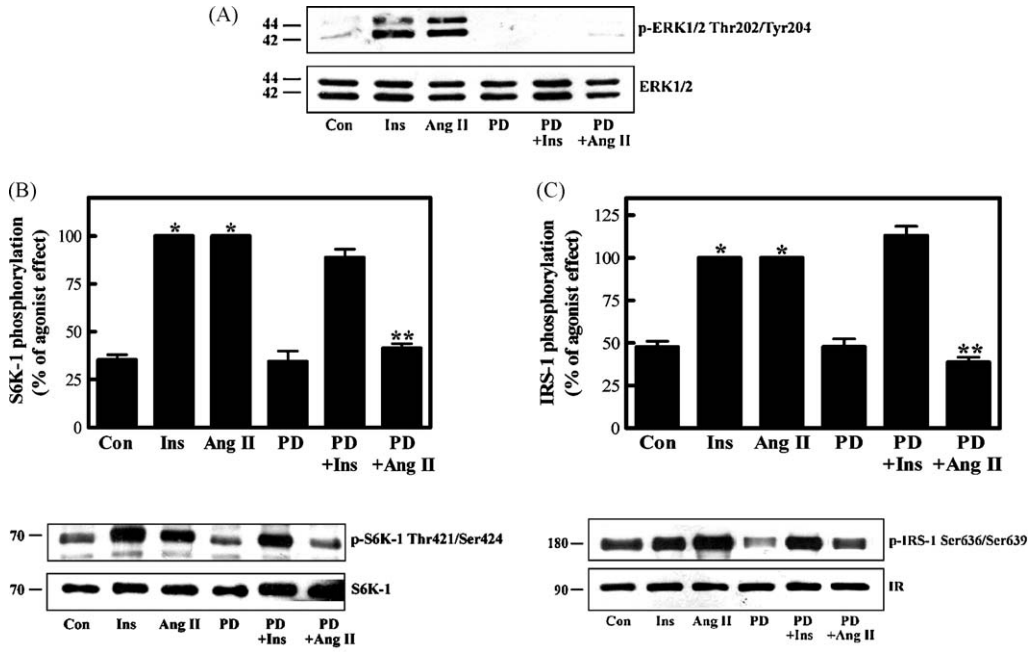

Fig. 6. ERK1/2 activation is involved in Ang II-induced IRS-1 Ser^{636}/Ser^{639} and S6K-1 Thr^{421}/Ser^{424} phosphorylation. C9 cells were pretreated with or without 1 μM PD98059 for 30 min, before stimulation with 100 nM Ang II or 100 nM insulin for 15 min. Total cell lysates were separated by SDS-PAGE and analyzed with anti-p-ERK1/2 Thr^{202}/Tyr^{204} (A), anti-p-S6K-1 Thr^{421}/Ser^{424} (B) or anti-p-IRS-1 Ser^{636}/Ser^{639} (C), as described in Section 2. Vertical lines represent the S.E.M. The lower panels show representative immunoblots for B and C. Western blots were also probed for total ERK1/2, S6K-1 and IR showing equal loading. (B and C) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* Ang II. Con, control; Ins, insulin; PD, PD98059.

the Thr^{308} in the activation loop within the kinase domain, and the Ser^{473} in the C-terminal of the kinase domain [4]. The phosphorylation of both sites is mediated by the actions of PDK1 and PDK2 (mTORC2), respectively [31,50–52]. Interestingly, several reports have suggested that each phosphorylation site performs different tasks in insulin action. Mutation of Thr^{308} to Ala blocks its activation, indicating that phosphorylation of this residue is required for Akt activation. On the other hand, mutation of Ser^{473} into Ala only partially inhibits Akt activation, suggesting that Ser^{473} phosphorylation is required for maximal kinase activation [50,53,54]. Moreover, several studies have demonstrated that phosphorylation of Thr^{308} is necessary for insulin-induced glucose uptake, whereas phosphorylation of Ser^{473} is not required [31,34,55].

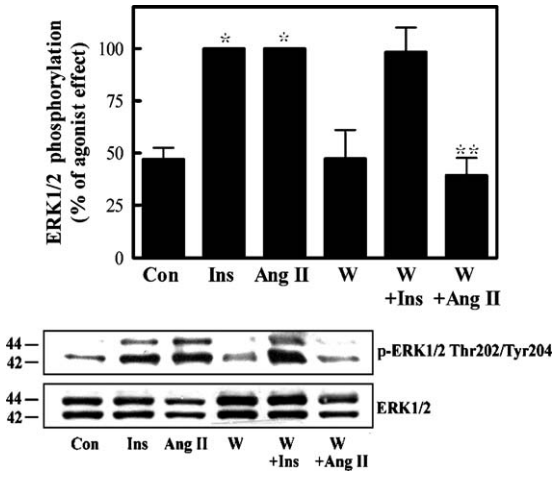

Fig. 7. Ang II-induced ERK1/2 activation is downstream of PI3K. C9 cells were pretreated with or without 100 nM wortmannin for 30 min before stimulation with 100 nM Ang II or 100 nM insulin for 15 min. Total cell lysates were separated by SDS-PAGE, and analyzed with anti-p-ERK1/2 Thr^{202}/Tyr^{204} as described in Section 2. Vertical lines represent the S.E.M. The lower panel shows a representative immunoblot. Western blots were also probed for total IR showing equal loading. $^{*}p < 0.01$ *vs* control; $^{**}p < 0.001$ *vs* Ang II. Con, control; Ins, insulin; W, wortmannin.

We observed in C9 cells that insulin induced, as expected, phosphorylation of Akt at Thr^{308} and Ser^{473}. When cells were exposed to Ang II, phosphorylation at both sites was impaired. Interestingly, the inhibitory effect of Ang II was higher in insulin-induced Thr^{308} phosphorylation than in Ser^{473}. In this context, Akt phosphorylation at Thr^{308} has been implicated in the metabolic actions of insulin. Bayascas et al. [31] reported that knock-in mice expressing a mutant of PDK1 incapable of binding phosphoinositides were unable to activate Akt. Inhibition of Akt action resulted from reduced phosphorylation of Thr^{308}, without affecting Ser^{473} phosphorylation. Interestingly, the knock-in mice are significantly small, insulin resistant, and hyperinsulinemic, correlating these abnormalities with defective PDK1 and the impairment of Akt phosphorylation at Thr^{308} [31]. Similarly, Kondapaka et al. [34] reported that 7-hydroxystaurosporine induces clinical insulin resistance by blocking Akt activation and subsequent GLUT4 translocation in response to insulin, and this effect appears to occur by inhibiting Thr^{308} phosphorylation without affecting Ser^{473} phosphorylation. Considering that phosphorylation of Akt at Thr^{308} appears to have important roles in insulin action, the ability of Ang II to impair insulin-induced phosphorylation at Thr^{308} suggests that it could be important in impairment of the metabolic actions of insulin.

One of the major targets of activated Akt is GSK-3 [56,57], which has an important role in the regulation of glycogen synthesis via inhibitory phosphorylation of glycogen synthase (GS). Upon insulin-mediated phosphorylation on $Ser^{21/9}$ of the two isoforms of GSK-3, GSK-3α and GSK-3β, respectively, GSK-3 is inactivated [56,57]. This inactivation, in parallel to protein phosphatase-1 activation, relieves the inhibitory phosphorylation of GS, which becomes activated and promotes glycogen synthesis [57,58]. To evaluate the metabolic impairment caused by Ang II as a consequence of its inhibitory effect on Akt, we determined its effect on insulin-induced GSK-3α/β $Ser^{21/9}$ phosphorylation. Our results showed that AT_1R activation in C9 cells causes a reduction

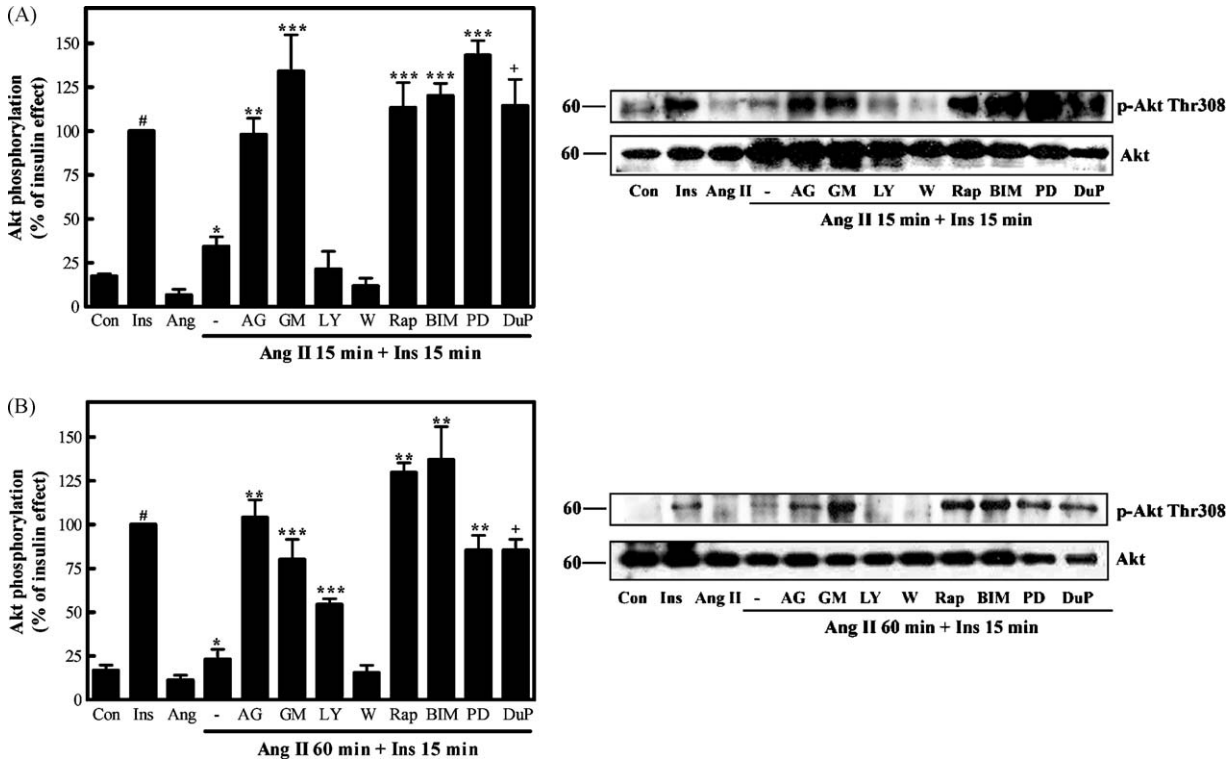

Fig. 8. Inhibition of IRS-1 Ser^{636}/Ser^{639} reverses inhibition of insulin-induced Akt Thr^{308} phosphorylation elicited by Ang II treatment for 15 min. C9 cells were pretreated with or without: 1 μM AG1478, 1 μM GM6001, 10 μM LY-294002, 100 nM wortmannin, 100 nM rapamycin, 1 μM BIM, 1 μM PD98059, or 10 μM DuP 753, for 30 min before treatment with 100 nM Ang II for 15 min (A) or 60 min (B) and then stimulated with 100 nM insulin for an additional 15 min. Total cell lysates were separated by SDS-PAGE, and analyzed with anti-p-Akt^{308} as described in Section 2. Vertical lines represent the S.E.M. The right panels show a representative immunoblot for A and B. Western blots were also probed for total Akt showing equal loading. (A) $^{\#}p < 0.001$ *vs* control; $^{*}p < 0.001$ *vs* insulin; $^{**}p < 0.05$, $^{***}p < 0.001$, and $^{+}p < 0.01$ *vs* Ang II + Ins (-). (B) $^{\#}p < 0.001$ *vs* control; $^{*}p < 0.001$ *vs* insulin; $^{**}p < 0.001$, $^{***}p < 0.05$, and $^{+}p < 0.01$ *vs* Ang II + Ins (-). Con, control; Ins, insulin; AG, AG1478; GM, GM6001; LY, LY-294002; W, wortmannin; Rap, rapamycin; PD, PD98059; DuP, DuP 753.

in insulin-induced GSK-3α/β $Ser^{21/9}$ phosphorylation, affecting hepatic glycogen synthesis, and are consistent with the results observed in the transgenic REN2 rat, an experimental model of excessive tissue local RAS activity with severe cardiovascular and metabolic defects, where insulin-stimulated glucose-transport activity, GSK-3 phosphorylation and glycogen synthesis are substantially reduced [59].

Initial attempts to unravel the molecular mechanism of insulin resistance strongly suggested that a defect responsible for this is located at the post-receptor level of insulin signaling [29].

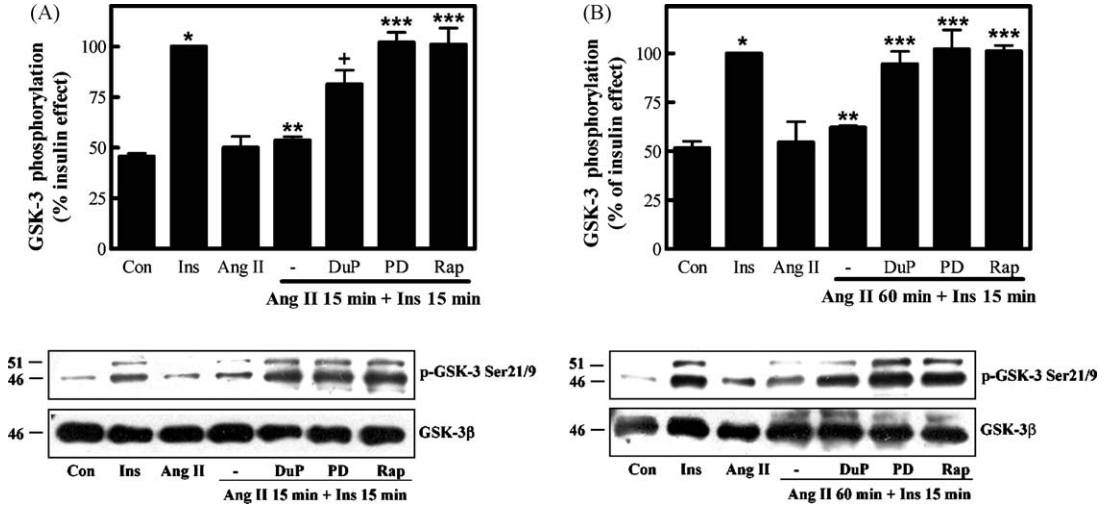

Fig. 9. Inhibition of IRS-1 Ser^{636}/Ser^{639} reverses inhibition of insulin-induced GSK-3α/β $Ser^{21/9}$ phosphorylation elicited by Ang II treatment. C9 cells were pretreated with or without: 10 μM DuP 753, 100 nM rapamycin or 1 μM PD98059, for 30 min before treatment with 100 nM Ang II for 15 min (A) or 100 nM Ang II for 60 min (B) and then stimulated with 100 nM insulin for an additional 15 min. Total cell lysates were separated by SDS-PAGE, and analyzed with anti-pGSK-3α/β $Ser^{21/9}$ as described in Section 2. Vertical lines represent the S.E.M. The lower panels show representative immunoblots for A and B. Western blots were also probed for total GSK-3β showing equal loading. (A) $^{*}p < 0.001$ *vs* control; $^{**}p < 0.001$ *vs* Ins; $^{+}p < 0.05$ and $^{***}p < 0.001$ *vs* Ang II + Ins (-). (B) $^{*}p < 0.01$ *vs* control; $^{**}p < 0.01$ *vs* Ins; $^{***}p < 0.05$ *vs* Ang II + Ins (-). Con, control; Ins, insulin; DuP, DuP 753; PD, PD98059; Rap, rapamycin.

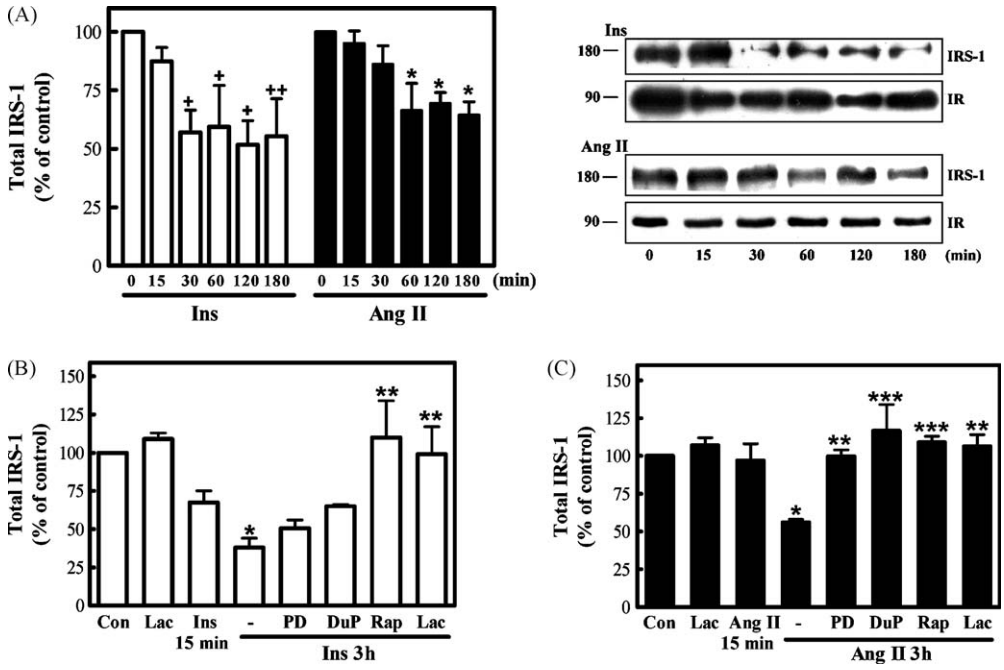

Fig. 10. Ang II triggers degradation of IRS. (A) Cells were stimulated with 100 nM insulin or 100 nM Ang II from 15 to 180 min. Cells were pretreated with or without: 40 μM lactacystin, 10 μM DuP 753, 100 nM rapamycin or 1 μM PD98059, for 30 min before treatment with 100 nM insulin (B) or 100 nM Ang II (C) for 15 or 180 min. Total cell lysates were separated by SDS-PAGE, and analyzed with anti-IRS-1 as described under Section 2. Vertical lines represent the S.E.M. The right panel shows a representative blot for A. Western blots were also probed for total IR or total RAF-1 showing equal loading. (A) $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{*}p < 0.05$ *vs* time 0 (control). (B) $^{*}p < 0.01$ *vs* control; $^{**}p < 0.001$ *vs* insulin 3 h (-). (C) $^{*}p < 0.05$ *vs* control; $^{**}p < 0.05$ *vs* Ang II 3 h (-); $^{***}p < 0.01$ *vs* Ang II 3 h (-). Con, control; Lac, lactacystin; Ins, insulin; PD, PD98059; DuP, DuP 753; Rap, rapamycin.

A mechanism that recently emerged as a potential explanation proposed that serine phosphorylation of IRS-1 proteins can reduce their ability to attract PI3K, minimizing its activation [29,60]. A number of serine kinases that phosphorylate serine residues of IRS-1 and weaken insulin signal transduction have been identified, such as the insulin-induced activation of mTOR and S6K-1 [12,33,61].

The mTOR pathway and its downstream target S6K-1 integrate insulin and nutrient signaling in numerous cell types. Recent studies suggest that this pathway also acts as a homologous desensitization mechanism (negative feedback loop) that negatively regulates insulin signaling to PI3K/Akt in insulin target tissues [27,33,62]. In this context, Um et al. [33] recently demonstrated that S6K-1-deficient mice are hypersensitive to insulin due to loss of the negative feedback loop from S6K-1 to IRS-1, and are protected from age- and diet-induced obesity [33]. In genetic models of obesity, such as K/KAy and ob/ob mice, insulin signaling is suppressed with increased phosphorylation of Ser^{307} and Ser^{636}/Ser^{639} in IRS-1 [33]. In such mice, the activities of JNK and mTOR/S6K-1, which can phosphorylate serine residue(s) of IRS-1, were reported to be elevated [33,63]. Khamzina et al. [27] also showed that the mTOR/S6K-1 cascade was over-stimulated and insulin-induced Akt activation was inhibited in the livers of rats fed with a high fat diet.

Because of the relevance of the PI3K/S6K-1 pathway in the regulation of insulin signaling, we determined whether Ang II could induce S6K-1 activation in C9 cells and if this may lead to IRS-1 Ser^{636}/Ser^{639} phosphorylation and thus explain the inhibitory effect on Akt and its downstream substrate GSK-3. We observed that Ang II induced a transitory increase in IRS-1 Ser^{636}/Ser^{639} and S6K-1 Thr^{421}/Ser^{424} phosphorylation. Ang II-induced phosphorylation of both proteins was rapamycin- and wortmannin-dependent, suggesting the involvement of the PI3K/mTOR/S6K-1 pathway.

Our results are in agreement with previous findings that have shown that Ang II induces IRS-1 serine phosphorylation as a key mechanism to desensitize insulin signaling. For example, Izawa et al. [24] found that Ang II-induced JNK and ERK1/2 activation causes serine phosphorylation of IRS-1 at both 307 and 616, and inhibits insulin-induced IRS-1 tyrosine phosphorylation in RASMC. In HUVEC cells, Andreozzi et al. [23] reported that Ang II activates JNK and MAP-kinase pathways, leading to increased serine phosphorylation of IRS (Ser^{312} and Ser^{616}, respectively), impaired insulin-induced PI3K/Akt/eNOS signaling pathway, and endothelial dysfunction. In rat aortic smooth muscle cells (RASMCs), it has been shown that Ang II impairs insulin-mediated IRS-1 tyrosine phosphorylation by a mechanism that involves an increased phosphorylation of the IR, IRS and the p85 subunit of PI3K on Ser residues [64]. In addition, Motley et al. showed that Ang II inhibits insulin-induced activation of Akt by blocking IRS-1 function through PKCα activation [25]. However, none of these studies has pointed out the role of Ang II-induced S6K-1 activation in the regulation of insulin signaling through phosphorylation of IRS-1 at Ser^{636}/Ser^{639}.

We have previously described that Ang II induces PI3K and ERK1/2 activation in C9 cells by a mechanism that depends exclusively on EGFR transactivation [16,18,19,65]. Thus, we also determined whether EGFR transactivation could unleash the Ang II-induced activation of PI3K/mTOR/S6K-1 pathway to desensitize insulin signaling. Our results showed that Ang II induces EGF receptor transactivation that leads to the phosphorylation of S6K-1 and IRS-1 by the mechanism previously described that requires PKC/Src/MMP activation and release of the HB-EGF endogenous ligand of EGFR [19]. Although the ability of Ang II to activate S6K-1 through EGFR transactivation was previously described [66,67], our results demonstrate for the first time that Ang II-induced S6K-1 activation is able to impair insulin signaling by increasing serine phosphorylation of IRS-1, specifically at Ser^{636}/Ser^{639}.

Many recent studies have been focused on the mechanisms involved in the regulation of S6K-1. New insights into this research suggest the involvement of different pathways, including the classical PI3K/mTOR and the PKC/cRAF/MEK1/2/ERK1/2 sequence.

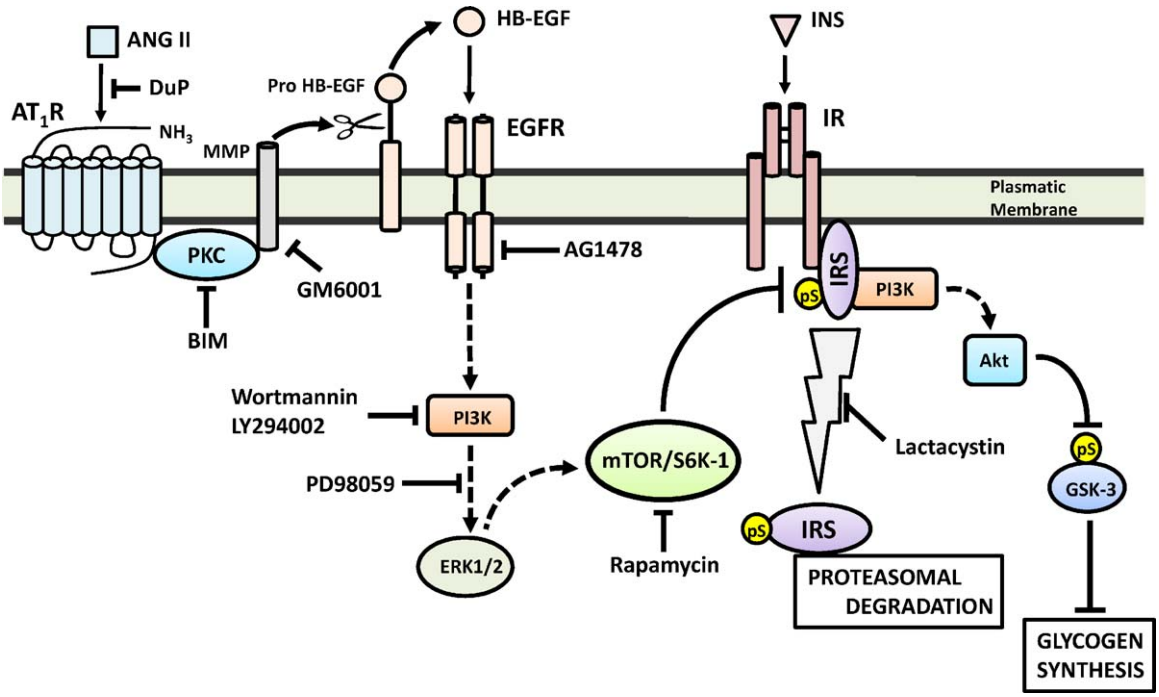

Fig. 11. A model is presented whereby serine phosphorylation of IRS-1 serves as a mediator of Ang II to inhibit insulin-induced Akt Thr^{308} phosphorylation. When C9 cells are stimulated with Ang II, the endogenous AT_1R is activated leading to PKC/MMP-dependent release of the HB-EGF ligand, which in turn activates the EGFR. Such EGFR transactivation leads to activation of the PI3K/ERK1/2/mTOR/S6K-1 pathway, where S6K-1 has an important role in the phosphorylation and degradation of IRS-1, which impairs insulin-induced PI3K and Akt activation.

Since AT_1Rs expressed in the C9 cell model are coupled to both signaling pathways, we tested for cross-talk between the ERK and the S6K-1 pathways and found that the specific PI3K inhibitors, wortmannin and LY-294002, inhibited Ang II-induced ERK1/2 activation. Interestingly, we found that the MEK1/2 inhibitor PD98059 could inhibit not only Ang II-induced ERK1/2 phosphorylation but also the S6K-1 and IRS-1 phosphorylation. These data revealed that ERK1/2 could have a role in Ang II-induced S6K-1 and IRS-1 phosphorylation. Although we demonstrate here that Ang II-induced ERK1/2 can be a downstream target of PI3K, previous reports have shown PI3K dependence for ERK1/2 activation. In this context, Tanski et al. [68] showed that smooth muscle cell migration induced by sphingosine-1-phosphate is dependent on ERK1/2 activation that is mediated by PI3K. Similarly, Punn et al. [69] showed in human embryonic kidney 293 cells that UCN-1, acting through its CRH-R_1-α receptor, induces ERK1/2 activation that is dependent on both PI3K/Akt and EGF receptor activation. These data provide evidence for a new mechanism for Ang II-induced S6K-1 activation that leads to IRS-1 Ser^{636}/Ser^{639} phosphorylation, involving AT_1 receptor mediated-EGFR transactivation and subsequent activation of the PI3K/ERK1/2/mTOR/S6K-1 pathway.

We report here that Ang II-induced IRS-1 Ser^{636}/Ser^{639} phosphorylation by S6K-1 is involved in the inhibition of Akt. The importance of the mTOR/S6K-1 pathway in the development of insulin resistance has been previously described. Ueno et al. [70] showed that chronic hyperinsulinemia itself decreases insulin signaling in the liver and muscle of rats, and increases mTOR/S6K-1 activity and Ser phosphorylation of IRS-1/2. In addition, rapamycin-induced inhibition of the mTOR signaling pathway prevents the insulin resistance caused by chronic hyperinsulinemia in liver and muscle [70].

We observed that at 60 min Ang II was unable to induce IRS-1 phosphorylation, while retaining the inhibitory effect on Akt activation. This can be explained because Ang II can induce IRS-1 protein degradation as observed in Fig. 10. Previous reports have shown that Ser/Thr phosphorylation of IRS-1 protein can induce its degradation, specifically by a mechanism that involves IRS-1 phosphorylation at Ser^{636}/Ser^{639} [36]. In addition, it has been suggested that insulin-induced IRS-1 degradation is mediated by a rapamycin-sensitive pathway [35].

In summary, our findings indicate that Ang II impairs insulin-induced Akt Thr^{308} phosphorylation by increasing IRS-1 Ser^{636}/Ser^{639} phosphorylation and IRS-1 protein degradation, by a mechanism dependent on EGF transactivation that leads to PI3K/ERK1/2/mTOR/S6K-1 activation (Fig. 11). These findings provide evidence that defines a role of Ang II in the development of insulin resistance.

Conflict of interest

None.

Acknowledgements

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