ORIGINAL

Angiotensin II inhibits insulin-induced actin stress fiber formation and glucose uptake via ERK1/2

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Abstract : There is crosstalk in intracellular signaling between Angiotensin II (Ang II) and insulin. We hypothesized that the underlying mechanism might be related to changes in cytoskeleton. In the presence of 100 nM of Ang II, insulin-induced glucose uptake was decreased and insulin-induced actin filament organization was inhibited. PKC inhibitors, including GF 109203x and p38 MAPK inhibitor (SB 203580) neither improved insulin-induced actin reorganization nor glucose uptake. In contrast, the Ang II-induced inhibition of glucose uptake and actin filament disorganization was reversed by 10 μmol ERK 1/2 MAPK inhibitor (PD 98059). Pretreatment of Ang II increased ERK1/2 phosphorylation and inhibited insulin -induced Akt phosphorylation. The effect of Ang II on ERK1/2 phosphorylation was blocked by Ang II type 1 receptor antagonists, RNH 6270 and PD 98059 but not by SB 203580 or Guanosine-5'-O-(2-ThioDiphosphate), a G-protein inhibitor. We next tested the effect of broad-spectrum matrix metalloproteinase (MMP) inhibitor (GM 6001) on Ang II-inhibition of insulin signaling pathway. GM 6001 did not improve Ang II-induced actin filament disorganization and did not inhibit ERK1/2 phosphorylation. From these data in L6 myotube, we conclude that Ang II negatively regulates the insulin signal not through MMP signaling pathway but specifically through MMP-independent ERK 1/2 activation pathway, providing an alternative molecular mechanism for angiotensin-induced insulin resistance. J. Med. Invest. 54 : 19-27, February, 2007

Keywords **:** *insulin, angiotensin II, cytoskeleton, angiotensin receptor blockers, matrix metalloproteinase*

INTRODUCTION

Angiotensin II (Ang II) is a multifunctional peptide hormone that not only controls cardiovascular homeostasis but also promotes cell growth, migration and inflammation. Ang II is also known to inhibit insulin action on glucose uptake. Actin is a cellular protein essential for the motility and cell signaling of both non-muscle and muscle cells. In the muscle cell dynamic actin polymerization and de-polymerization is the basic mechanism of the cell signaling. The cytoskeleton is a filamentous network consisting largely of filamentous actin (F-actin), which provides a scaffold on which signaling proteins, such as

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GLUT4 translocation to generate internal stress and alter the amount of the glucose uptake of the cells(1-5). However, the intracellular signaling mechanisms governing on reorganization actin filaments and effect on insulin signaling pathway remains essentially undefined.

Many signal transduction pathways mediated by Ang II actions, including the activation of janus kinase, which phosphorylates and activates signal transducers, activators of transcription factors, and the stimulation of mitogen activated protein (MAP) kinase pathway(6-8). Ang II receptor are also coupled to a wide variety of signal transduction elements, including protein kinase C (PKC), c-Src tyrosine kinase, protein tyrosine phosphorylation, and extracellular signal-regulated kinase (ERK) 1/2(9).

In the present study, we investigate mechanism by which Ang II inhibits insulin-induced actin arrangement and glucose uptake in L6 myotube cells. The aims of this investigation were (a) to clarify mechanism of inhibition of glucose uptake by Ang II and its relation to actin stress fiber formation, (b) to determine the role of ERKs in insulin-induced actin organization and glucose uptake in skeletal muscle cells. The results indicate that insulin play an important role in insulin-induced actin stress fiber formation and organization as well as glucose uptake and that Ang II inhibit both actions, at least in part, through ERK 1/2 phosphorylation.

MATERIALS AND METHODS

Cells

In this study we used L6 myoblast (L6). In these cells, we studied the effect of pretreatment of Ang II on insulin signaling pathway using fluorescence microscopy and glucose uptake. The L6 cells were maintained in α-minimal essential medium (α-MEM, Sigma, Saint Louis, Missouri) supplemented with 10% fetal bovine serum (FBS, ICN Biomedical Inc., Aurora, Ohio) (vol/vol) and gentamycin 100U/ml in an atmosphere of 5% CO_2 at 37℃. For actin filament staining, the cells were treated with trypsin and transferred into indicated dish and incubated at 37℃ with 5% CO_2. After 5-7 days, the cells before reaching confluent were used for experiments.

Colorimetric assay of actin filament staining

Cells in 3cm dish with cover slip were incubated in 2ml Krebs Ringer Hepes (KRH) buffer for 30min at 37℃ and then incubated in 2ml 300nM insulin. After fixation with 2% paraformaldehyde (10min, room temperature), cells were washed with Phosphate Buffered Saline (PBS), and then incubated in 0.1% Triton X-100 in PBS for 10min at room temperature. Cells were washed with PBS, and then incubated in 1% BSA in PBS for 20min at room temperature. The cells were extensively washed with PBS buffer before and after introducing rhodamine-phalloidin (Molecular Probes Inc., Leiden, The Netherlands) for 20min at room temperature. Then, fluorescence microscopy was used for detection of actin filaments. To determine the actin filament status in cytoplasm, Adobe Photo Shop was used to select for stained cells. Selected regions were copied and pasted into a new RGB channel.

Determination of 2-deoxy-[^{3}H] D-glucose uptake in cells in culture

Glucose uptake was measured on differentiated cells grown in 24-well plates using 2-deoxy-[^{3}H] D-glucose (Muromachi, Japan). Briefly, the cells were pre-incubated at 37℃ for 4h in serum-free medium containing 10mM cold glucose for starvation after washing with serum-free medium. To ensure that the cells were in the basal state at the beginning of experiment, the cells were washed 3 times in KRH buffer (KRH/0.1% BSA) and pre-incubation cells with KRH/0.1% BSA at 37℃ for 20 min. Cells were treated with indicated concentrations of ligands for indicated periods at 37℃. Hexose uptake was measured at room temperature for 10 min in transport buffer containing : 0.5 M HEPES, 1 M $MgSO_4$, 1M $CaCl_2$, 2M KCl, 5M NaCl and 50μM 2-deoxy-[^{3}H] D-glucose (1mCi/ml). After cells were exposure to 2-deoxy-[^{3}H] D-glucose for 10min, the cells were washed 4 times in KRH/ 0.1% BSA/phloretin and solublized in 0.1% sodium dodecyl sulfate (SDS) ; the cell-associated radioactivity was measured. Cellular protein content was measured by the bicinchoninic acid (BCA) method.

Western blot analysis

Growth-arrested L6 cells were either left untreated or stimulated by Ang II, insulin, PD 98059, RNH 6270 and others. Cells were lysed with lysis buffer containing 20mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1% Sodium deoxycholate, 0.1% SDS, 1% Nonodet-P40, 2mM EDTA (2Na) and 1mM phenylmethylsulfonyl fluoride. Equal amounts of cellular protein were analyzed by SDS-polyacrylamide gel

electrophoresis (SDS-PAGE) and electro transferred to PVDF membranes, and probed with the indicated antibodies. The membranes were than subjected to immunoblotting with a rabbit polyclonal Akt antibody, an ERK 1/2 antibody, a phospho-Akt (Ser-473) antibody and a phospho-ERK 1/2 (phospho 44/42 MAP kinase, Thr 202 and Tyr 204 respectively) antibody. Secondary antibodies were coupled to horseradish peroxidase, HRP-conjugated goat anti-rabbit IgG, and Western blot detection was done by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). All of the antibodies were purchase from Santa Cruz Biotechnology Inc. (Santa Cruz, California).

Statistical analysis

Data are presented as the mean ±standard deviation and were performed using ANOVA plus Bonferroni multiple comparison tests. Values of $p< 0.05$ were considered significantly different.

RESULTS

Insulin stimulates actin stress fiber formation

Before insulin stimulation, the fluorescent intensity of actin fibers in the cytoplasm of L6 myotube cells was minimal. There was an increase in actin fiber polymerization after 10min incubation with 300 nM insulin (Fig. 1A upper panel), resulting in the reorientation of fibers with the long axis of the cells (stress fiber formation). Insulin also significantly increased glucose uptake (Fig. 1C).

Ang II inhibits actin stress fiber formation and glucose uptake through AT 1 receptor

Ang II is known to inhibit insulin signaling and glucose uptake. We examined the effect of pretreatment of Ang II on insulin-induced actin organization. The lower panel of Fig. 1A shows, when the cell was exposed to 100nM Ang II for 60 minutes, insulin-induced stress fiber formation was abolished. In

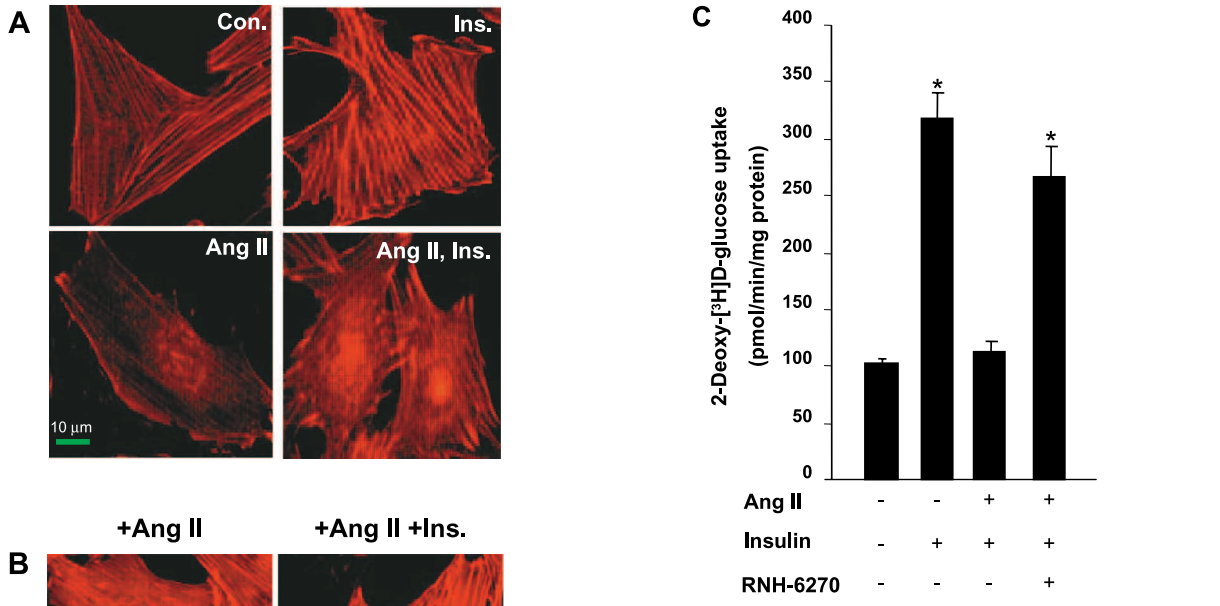

Fig. 1. Effect of Ang II on insulin-induced actin stress fiber formation and glucose uptake through AT 1 receptor.
Actin stress fibers were observed under a fluorescence microscope. A : Actin staining shows that insulin stimulates the formation of actin stress fibers (Ins., straight red fiber-like strands along the long axis) comparison with control (Con.) in L6 cells. Cells were preincubated with 100 nM Ang II for 60 min and stimulated by 300 nM insulin for 10 min. Insulin-induced formation of actin stress fibers in cells was completely blocked by pretreatment of Ang II (Ang II, Ins.) as well as Ang II (Ang II). B:The insulin-induced formation of actin stress fibers was not improved by treatment with 10 μM PD 123319 (an AT2 receptor blocker, 1B lower panel) for 30 min but completely reversed by treatment with 100 nM RNH-6270 (an AT 1 receptor blocker, 1 B upper panel). C : Cells were pretreated with RNH-6270 before treatment with Ang II or pretreatment of Ang II. The total amount of 2-deoxy-[^{3}H] D-glucose uptake into the cells during last 10 min incubation was measured as described in materials and method. Bar graph quantization of five experiments is shown ; significant differences (* $P< 0.05$) are indicated.

order to check whether this blocking effect of Ang II is mediated by AT1 receptor, we examined the effect of RNH-6270, an AT1 receptor blocker, an active form of olmesartan, on pretreatment of Ang II. When the cells were exposed to 100nM RNH-6270, blocking effect of Ang II on insulin-induced stress fiber formation was completely reversed (Fig. 1B upper panel). To verify whether altered Ang II signaling is due to changes in Ang II receptors status, we examined AT1/AT2 receptor antagonist, saralasin, and AT2 receptor antagonist, PD 123319, on pretreatment and treatment of Ang II. Ang II-induced effects were blocked by salarasin (data not down) but not significantly by PD 123319 (Fig. 1B lower panel). Figure 1C shows that glucose uptake was significantly ($p<0.001$) increased in the presence of 300nM insulin for 10 minutes. Pretreatment of Ang II significantly decreased insulin-induced glucose uptake. The inhibition of glucose uptake with Ang II was reversed by AT1 blocker, RNH 6270, suggesting that Ang II inhibits actin reorganization and glucose uptake through AT 1 receptor.

Ang II inhibited insulin-stimulated glucose uptake is not through GTP[S] pathway

We also investigated GTP[S] pathways, an important pathway of Ang II. Pretreatment of 100μM GDP-β-S, a G protein inhibitor, for 10 minutes did not reverse the inhibitory effect of Ang II on insulin-stimulated glucose uptake and actin stress fiber formation (Fig. 2A and 2B). However, in the presence of G protein inhibitor, actin intensities in the peripheral area of the cells (cortical actin filament)

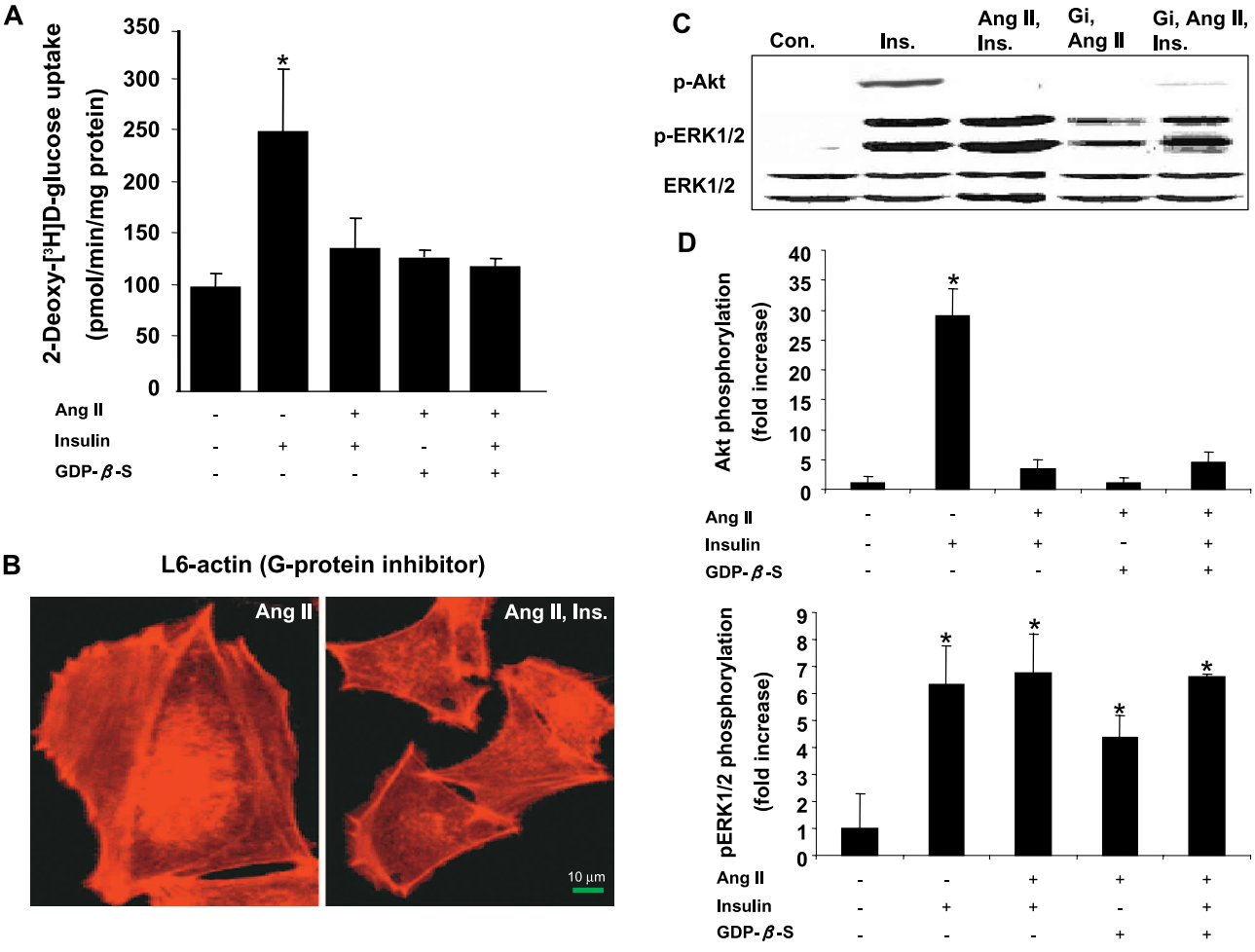

Fig. 2. Ang II inhibited insulin-stimulated glucose uptake is not through GTP[S] pathway.
A : In the presence of 100 μM [GDP-β-S], a G protein inhibitor, for 10 minutes, the inhibitory effect of pretreatment of Ang II on insulin-stimulated glucose uptake did not improve. B : G protein inhibitor did not return actin filament disarrangement by Ang II or pretreatment of Ang II (Ang II, Ins.). C and D : The effect of Ang II on insulin-induced Akt and ERK 1/2 phosphorylation and also influences of G-protein inhibitor (Gi) on Ang II impairment of Akt phosphorylation by insulin (Ins.). Cell lyses were immunoblotted by phospho-Akt (pAkt) and ERK 1/2 (pERK 1/2). Figure C shows representative blots and figure D shows the densitometry analyses. Values are normalized by arbitrarily setting the densitometry of control cells (without any stimulation) to 1(values are the mean ±SD, n=3). * $P<0.05$ vs. control cells.

become strong. Akt phosphorylation is a major pathway of insulin signaling on glucose uptake. Figures 2C and D (upper panels) show that Akt phosphorylation was blocked by Ang II. G protein inhibitor did not improve Ang II-induced inhibition of Akt phosphorylation. G protein inhibitor also did not inhibit effect of Ang II or insulin on ERK 1/2 phosphorylation in L6 cells (Fig. 2C and D lower panel) suggesting that Ang II inhibited insulin-induced Akt phosphorylation is independent on GTP[S] pathway.

Ang II inhibits insulin-induced actin stress fiber formation and glucose uptake through ERK 1/2 signaling pathway

Ang II is also coupled to PKC and MAP kinase, we studied the effect of various intracellular signaling using specific inhibitors. By the treatment of L6 cells with GF 109203X, a selective PKC inhibitor, and SB 203580, a p38 MAP kinase inhibitor, Ang II-induced disarrangement of actin stress fiber was not altered. PD 98059, a selective ERK 1/2 inhibitor, completely reversed Ang II-induced actin disarrangement (Fig. 3 A), suggesting that this inhibitory effect of Ang II was mediated by ERK 1/2 signaling pathway but not by PKC or p38 MAP kinase signaling pathways. Figure 3B shows the effect of inhibitors of Ang II signaling pathways on glucose uptake in the presence of insulin. Pretreatment of 10μM PD 98059 for 30 minutes, but not those of 10μM GF 109203X or 10μM SB 203580 improved glucose uptake. These results suggest that Ang II-induced suppression of insulin-stimulated glucose uptake is also mediated by ERK 1/2 signaling pathway.

ERK 1/2 phosphorylation by Ang II has negative effect on insulin-induced Akt phosphorylation

Ang II has been reported to inhibit insulin signaling at multiple levels in cells. Figures 4A and B show the effect of pretreatment of Ang II on insulin-induced Akt phosphorylation. Pretreatment of Ang II significantly inhibited insulin-induced Akt phosphorylation. Treatment of PD 98059, an ERK 1/2 inhibitor, improved inhibitory effect of Ang II on insulin-induced Akt phosphorylation.

We further studied relation between ERK 1/2 and

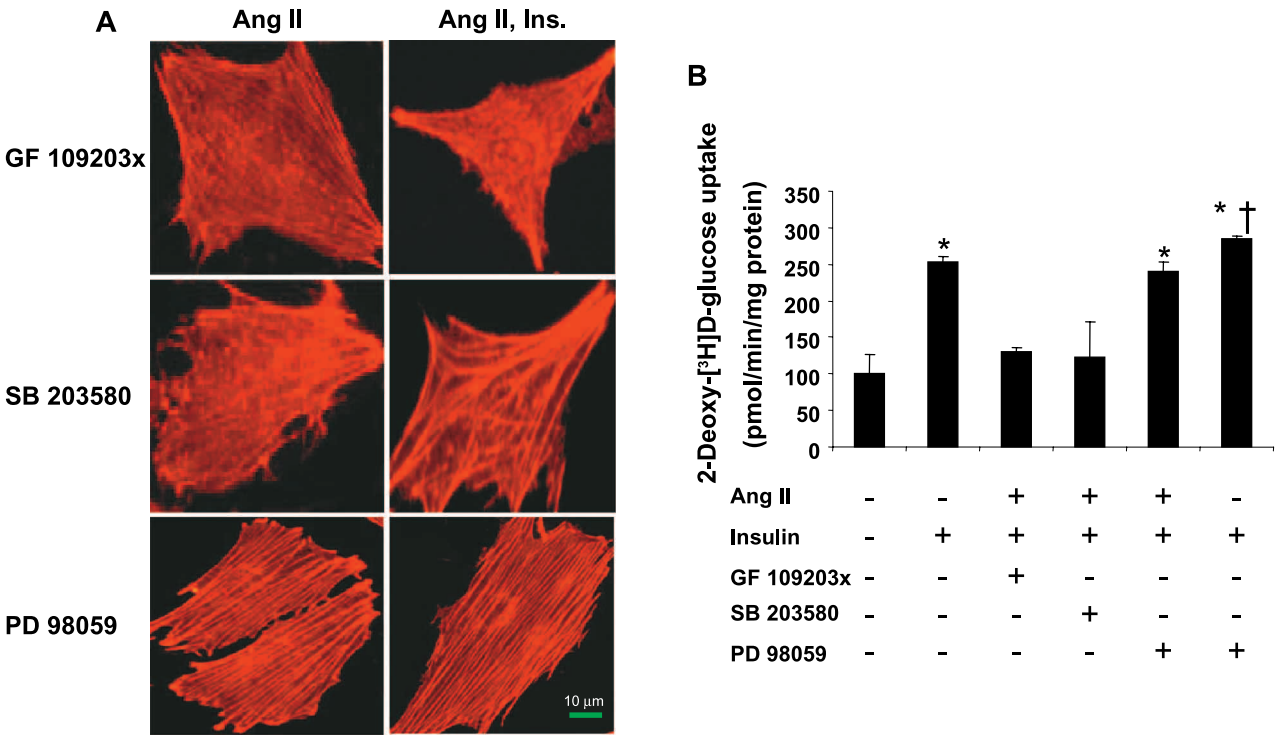

Fig. 3. Ang II inhibits insulin-induced actin stress fiber formation and glucose uptake through ERK 1/2 signaling pathway.
A : Actin staining by rhodamine-phalloidin shows the effects some of the elements on treatment of Ang II or pretreatment of Ang II (Ang II, Ins.) on insulin-induced actin stress fiber formation. Treatment of L 6 cells with 10 μM PD 98059, completely improved Ang II-induced disarrangement of actin stress fiber but not improved by 10 μM GF 109203 X or 10 μM SB 203580. B : The effect of Ang II signaling pathways blockers on glucose uptake in the presence of 300 nM insulin. Bar graph quantization of five experiments is shown ; significant differences (*P<0.05 vs. control cells, † P<0.05 vs. insulin) are indicated.

Akt phosphorylations. Expression of ERK 1/2 protein was detected as p42 and p44 ERKs. In figure 4 C, Ang II alone as well as insulin alone increased ERK 1/2 phosphorylation, and greater phosphorylation occurred by combination of Ang II and insulin. PD 98059 but not SB 203580, completely blocked ERK 1/2 phosphorylation by Ang II in L6 cells (Fig. 4D). In addition PD 98059 also increased phosphrylation of Akt in the presence of Ang II and insulin suggesting that suppression of ERK 1/2 phosphorylation might increase Akt phosphorylation, a major pathway for insulin-stimulated glucose uptake signaling. In the presence of insulin alone, PD 98059 slightly but significantly improved glucose uptake (Fig. 3B), suggesting that insulin enhances phosphorylation of Akt and increase glucose uptake through phosphatidylinositol 3-kinase (PI3-K) but on the other hand insulin might inhibit glucose uptake by ERK 1/2 phosphorylation.

Matrix Metalloproteinases (MMPs) are not involved in Ang II-induced inhibition of actin stress fiber formation

Several studies have shown that various MMPs play a critical role in the activation of the EGFR and ERK 1/2 in response to diverse environmental stimuli, including cigarette smoking and Ang II. We therefore hypothesized that EGFR activation may essential for inhibition by Ang II on actin stress fiber formation and glucose uptake in L6 cells. To examine this possibility, cells were treated with GM 6001, a compound that broadly inhibits MMP activation and specifically inhibits EGFR tyrosine kinase activity, for 10min before Ang II exposure and actin filaments were stained. We found that

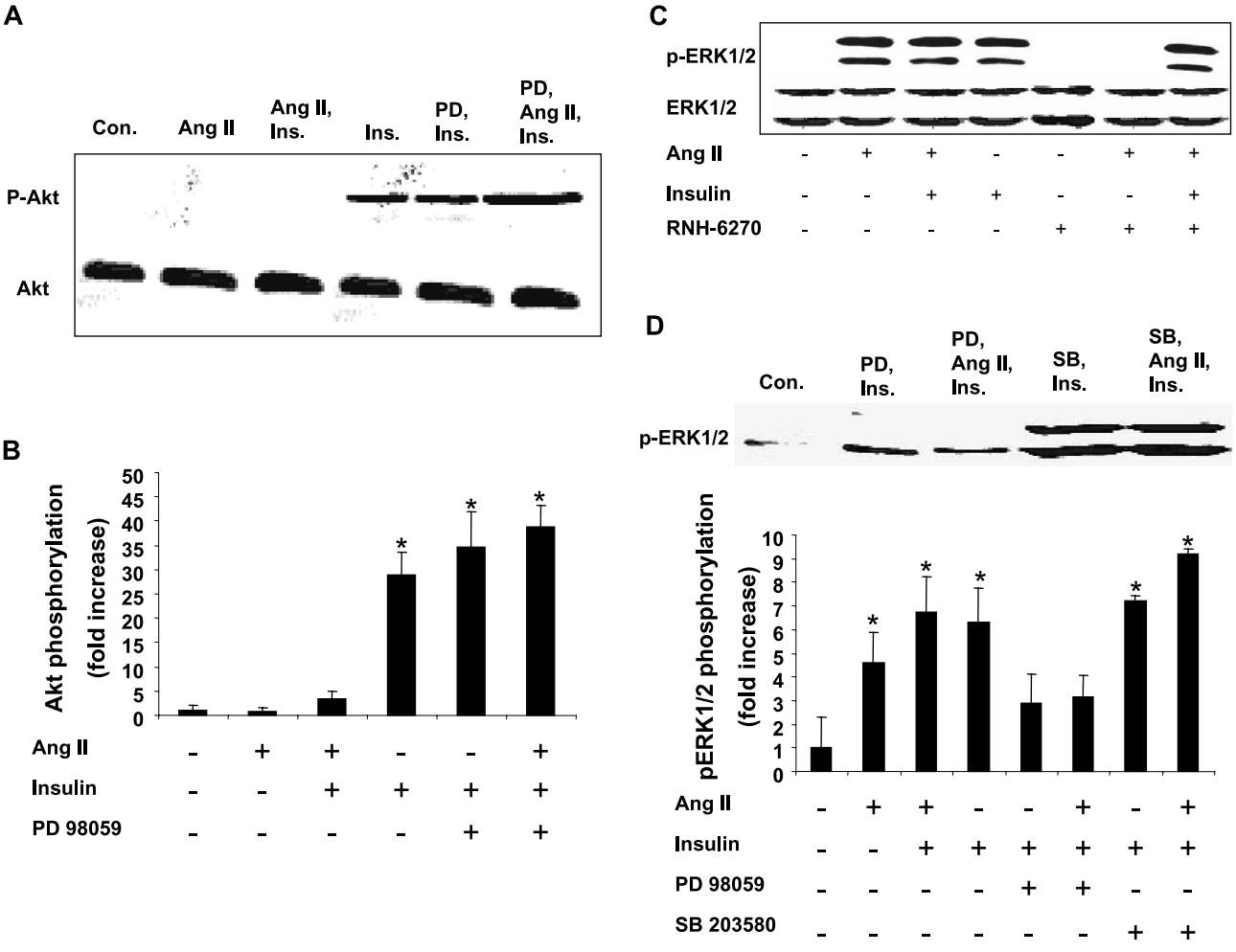

Fig. 4. Effect of RNH-6270 on phosphorylation of ERK 1/2.
L6 cells were preincubated with PD 98059 (PD) and SB 203580 (SB) for 30 min or 100 nM RNH-6270,followed with treatment of Ang II or pretreatment of Ang II (Ang II, Ins.) or treatment with insulin (Ins.). Cells were harvested, lysed and used for subsequent measurement of Akt phosphorylation (pAkt), total Akt (Akt), ERK 1/2 phosphorylation (pERK 1/2)and total ERK 1/2(ERK 1/2)as described in material and methods. A : Representative blots of pAkt and Akt, B : densitometry analyses of pAkt. C : Representative blots of pERK 1/2 and ERK 1/2. D : PD 98059 significantly inhibited ERK 1/2 phosphorylation but SB 203580, a p38 MAP kinase inhibitor, is no any effect on it. Figure D upper panel show representative blots and lower panel show the densitometry analyses. Values are normalized by arbitrarily setting the densitometry of control cells (without any stimulation) to 1(values are the mean± SD, n=3). *P<0.05 vs. control cells.

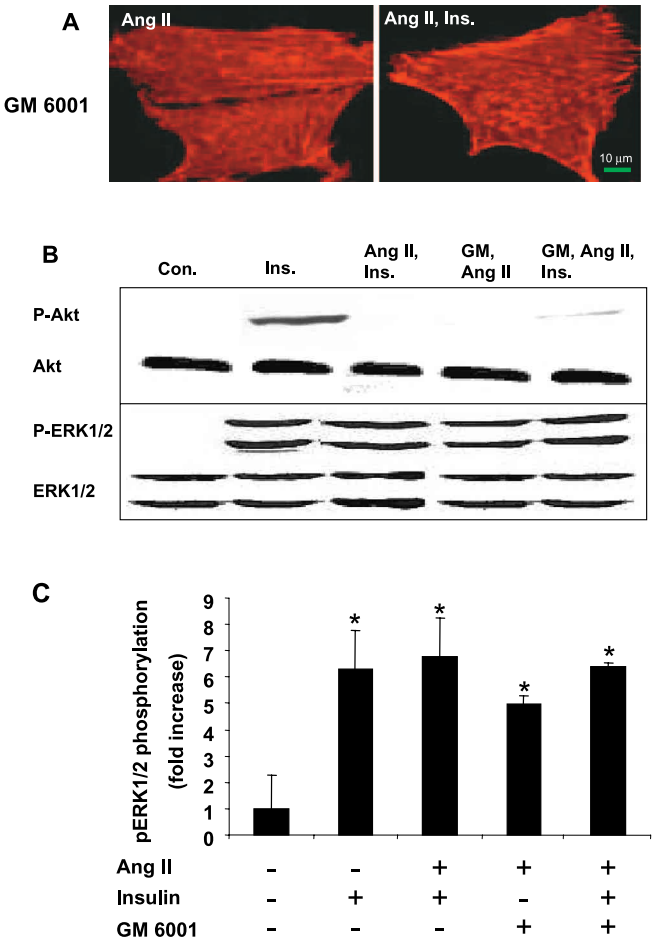

Fig. 5. MMPs are not involved in Ang II-induced inhibition of actin stress fiber formation.
L6 cells were treated with GM 6001(GM), a compound that broadly inhibits MMP activation, for 10 min before Ang II or pretreatment of Ang II (Ang II, Ins.). A : Actin filaments were observed under fluorescence microscope. B : Representative blots of Akt phosphorylation (pAkt), total Akt (Akt), ERK 1/2 phosphorylation (pERK 1/2) and total ERK 1/2(ERK 1/2). C : Densitometry analyses of pERK 1/2. Values are normalized by arbitrarily setting the densitometry of control cells (without any stimulation) to 1(values are the mean±SD, n=3). *P<0.05 vs. control cells.

GM 6001 did not affect on Ang II-induced inhibition of actin stress fiber formation (Fig. 5A). Western blotting data shows, MMP inhibitor did not reverse inhibitory effect of Ang II (Fig. 5B upper panel). Figures 5B lower panel and 5C show that GM 6001 did not inhibit effect of Ang II or insulin on ERK 1/2 phosphorylation in L6 cells. The results suggest that Ang II-inhibited insulin-induced actin organization and glucose uptake is independent on MMPs activity as well as EGFR transactivation pathway in L6 cells.

DISSCUSSION

In this study, we addressed the question of whether Ang II-induced alterations in insulin signaling contribute to impair glucose uptake and actin organization in skeletal muscle cells. Ang II inhibited insulin-induced actin reorganization and glucose uptake. Ang II also inhibited Akt phosphorylation, a key pathway of insulin-induced glucose uptake. Most notably is the observation that when ERK 1/2 activity is blocked by PD 98059, a selective inhibitor of the ERK 1/2, actin rearrangement and glucose uptake were improved in addition to Akt phosphorylation. Thus, the data suggest that ERK 1/2 signaling pathway is key mediators of Ang II - mediated actin filament disorganization and glucose uptake.

Molecular mechanisms whereby Ang II causes actin disarrangement have not been fully elucidated especially in skeletal muscle cells. The present study demonstrates that disarrangement of actin filament by Ang II results in impairing glucose uptake in L6 myotube cells. This occurred via AT1 receptor, as deduced by the ability of the AT1 antagonist, RNH-6270, to reverse the inhibitory effect of Ang II. This event was associated with impaired actin filament disarrangement through activating of ERK 1/2 phosphorylation. The results indicate that inhibition of ERK 1/2 signal transduction contributes to improve actin stress fiber formation and glucose uptake. To our knowledge, the present study demonstrates for the first time that Ang II modulates of ERK 1/2 and actin disarrangement, consequently decrease in glucose uptake.

Ang II has been shown to activate Akt via AT1 receptor in various cell types, including vascular smooth muscle cells (10, 11-13). However, we found opposite phenomenon and did not observe Ang II-induced Akt activation in our study. This discrepancy probably results from differences in cell types.

Ang II stimulated many intracellular signaling pathway ; G protein and MAP kinase activities in L6 myotube cells (14, 15). The present study showed that an Ang II effects on insulin-induced actin disorganization is not mediated by these signaling pathways. In addition, Ang II activates ERK 1/2 via independent signaling pathways involving PKC, guanosine 5'-O-(2-thiodiphosphate)-insensitive G protein and p38 MAP kinase.

Activation of angiotensin receptor with Ang II leads to reorganization of the actin cytoskeleton in L6 myotube cells. However, scare information is available on the cellular signaling pathways by Ang II (7, 9, 11-13, 16). The present studies suggest that pretreatment of Ang II on insulin disrupted stress fiber formation.

It is thought that AT1 receptor ligand binding causes activation of ERKs through three distinct signaling pathways. One pathway is via the Gq-PKC-dependent mechanism, another is via the Src-Ras-dependent mechanism, and the third pathway is by trans-activation of the epidermal growth factor receptor (6, 15-24).

MMPs are zinc-dependent endopeptidases comprising mainly the collagenases, gelatinases, stromelysins, and membrane-type MMP. EGFR phosphorylation and downstream signaling have been shown to be dependent on MMP activity in many systems. Ang II can transactivate receptor tyrosine kinases (RTK), even though Ang II does not bind directly to RTK. In the present study we provided evidences that Ang II induces ERK 1/2 activation not through Gq-dependent mechanisms or transactivation of the epidermal growth factor receptor in L6 cells. This phenomenon occurs in cross talk to Akt activation and consequently actin organization and glucose uptake from Src-dependent-ERK 1/2 signaling pathway. Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter regulating hormone action. In the case of Ang II, the crosstalk with insulin-mediated pathways resulted in negative interactions between Ang II and insulin signaling systems at the ERKs.

We also found that insulin causes ERK 1/2 phosphorylation as well as Ang II. Simultaneous stimulation with both hormones led to increased dually phosphorylated ERKs. In contrast, reduction of PDK-1 phosphorylation (data not shown) and Akt phosphorylation was observed compared with that after Ang II administration. The precise mechanism for this difference is not known ; however, possibilities include differential sites of serine and theronine phosphorylation on ERK 1/2 proteins after Ang II plus insulin treatment leading to an impairment of the PDK-1/Akt pathway. Interestingly, insulin action on glucose uptake was slightly but significantly enhanced by an ERK 1/2 inhibitor. Thus, phosphorylation of ERK 1/2 by insulin might have an inhibitory effect on glucose uptake or actin reorganization, although main pathway of insulin via PI3-K/Akt enhances glucose uptake and actin reorganization.

Finally, these data provide a novel insight into the Ang II signaling mechanism leading to inhibited insulin-induced actin fiber organization consequently glucose uptake. We establish a correlative relationship between a proximal signaling event (the activation of ERKs) and a distal cellular response (cytoskeleton organization). Furthermore, detecting a putative signaling pathway by which ERK 1/2 exerts this anti-diabetic effect in the clinically important skeletal muscle cell.

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