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Short communication

Epidermal growth factor receptor is indispensable for c-Fos expression and protein synthesis by angiotensin II

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Abstract

We have reported that angiotensin II induces the epidermal growth factor (EGF) receptor transactivation leading to extracellular signal-regulated kinase (ERK) activation in rat vascular smooth muscle cells. Here, we report that the EGF receptor kinase inhibitor AG1478 and the ERK kinase inhibitor PD98059 markedly inhibited angiotensin II-induced c-Fos expression and protein synthesis but not c-Jun expression in these cells. These data suggest that the EGF receptor transactivation and subsequent ERK activation are indispensable for angiotensin II-mediated growth promotion of vascular smooth muscle cells providing a new mechanistic insight whereby angiotensin II contributes abnormal vascular remodeling.

Keywords: Angiotensin II; Smooth muscle cell, vascular; Receptor, epidermal growth factor; Extracellular signal-regulated kinase; c-Fos; Protein synthesis

1. Introduction

Angiotensin II, a major effector peptide of the renin–angiotensin system, is now believed to play a critical role in the pathogenesis of cardiovascular remodeling. We and others have previously cloned the angiotensin AT_1 receptor (Inagami, 1995) which not only mediates diverse hemodynamic effects of angiotensin II but also promotes hypertrophy and/or hyperplasia of vascular smooth muscle cells (Berk and Corson, 1997; Griendling et al., 1997). In vascular smooth muscle cells, the AT_1 receptor activates phospholipase C, which initiates the generation of inositol trisphosphate and diacylglycerol, causing intracellular Ca^{2+} mobilization and protein kinase C activation, respectively. It also shares typical signaling events commonly evoked by growth factor receptors. Angiotensin II stimulates protein tyrosine kinases and extracellular signal-regulated kinases (ERK), and induces nuclear protooncogenes, c-*fos* and c-*jun*, in these cells (Berk and Corson, 1997; Griendling et al., 1997).

We have recently reported that angiotensin II transactivates the epidermal growth factor (EGF) receptor which serves as a scaffold for pre-activated tyrosine kinases and downstream adaptor proteins, leading to Ras/ERK activation in cultured rat vascular smooth muscle (Eguchi et al., 1996, 1998). To further gain insight into the molecular mechanism by which angiotensin II initiates vascular remodeling, we have investigated the roles of EGF receptor transactivation in angiotensin II-induced nuclear protooncogene expression and protein synthesis in these cells.

2. Materials and methods

2.1. Materials

Angiotensin II was purchased from Peninsula Laboratories. EGF and a homodimer of platelet-derived growth factor-B (PDGF-BB) were from Upstate Biotechnology. The EGF receptor tyrosine kinase inhibitor AG1478 and polyclonal anti-c-Jun antibodies were obtained from Cal-

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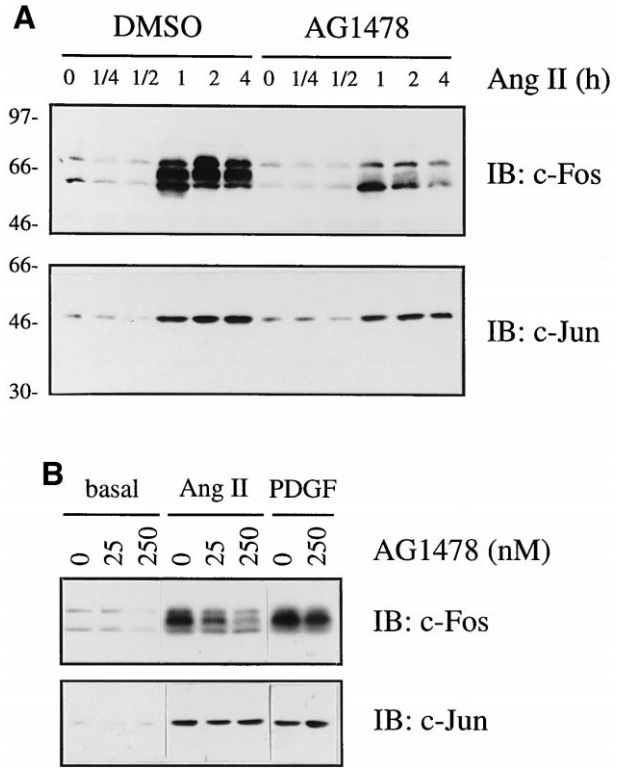

Fig. 1. Effect of AG1478 on angiotensin II-induced c-Fos and c-Jun expression in rat vascular smooth muscle cells. (A) Cells were pretreated with AG1478 (250 nM) or the vehicle dimethyl sulfoxide (DMSO: 0.1%) for 30 min and stimulated with angiotensin II (Ang II: 100 nM) for indicated durations. (B) Cells were pretreated with AG1478 at indicated concentrations for 30 min and stimulated with angiotensin II (Ang II: 100 nM) or PDGF-BB (PDGF: 100 ng/ml) for 1 h. Immunoblotting with antibodies for c-Fos and c-Jun was performed by repeated reprobing.

biochem. Polyclonal anti-c-Fos antibodies were purchased from Santa Cruz Biotechnology. The ERK kinase (MEK) inhibitor PD98059 was from New England Biolabs.

2.2. Cell culture

Vascular smooth muscle cells were prepared from the thoracic aorta of Sprague–Dawley rats by the explant method and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as previously described (Eguchi et al., 1996). The expression of angiotensin AT_1 but not AT_2 receptors was confirmed (Eguchi et al., 1996). Cells at ~80% confluence were made quiescent by incubation in serum-free medium for 3 days.

2.3. Immunoblotting

For immunoblot analysis, cells were directly lysed by Laemmli sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis, and immunoblotted as described previously (Eguchi et al., 1996).

2.4. Protein synthesis

Protein synthesis was assessed by incorporation of [^{3}H]leucine into the cells as previously described (Hirata et al., 1992). In brief, quiescent cells were incubated with angiotensin II and 1 μCi [^{3}H]leucine for 24 h. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter.

3. Results

To identify the role of EGF receptor transactivation by angiotensin II in its protooncogene expression, we examined the effects of the specific EGF receptor kinase inhibitor AG1478 (Levitzki and Gazit, 1995) on angiotensin II-induced c-Fos and c-Jun expression in vascular smooth muscle cells. Angiotensin II (100 nM) rapidly (within 1 h) induced c-Fos protein expression which was markedly inhibited by 250 nM AG1478 (Fig. 1A and B, upper

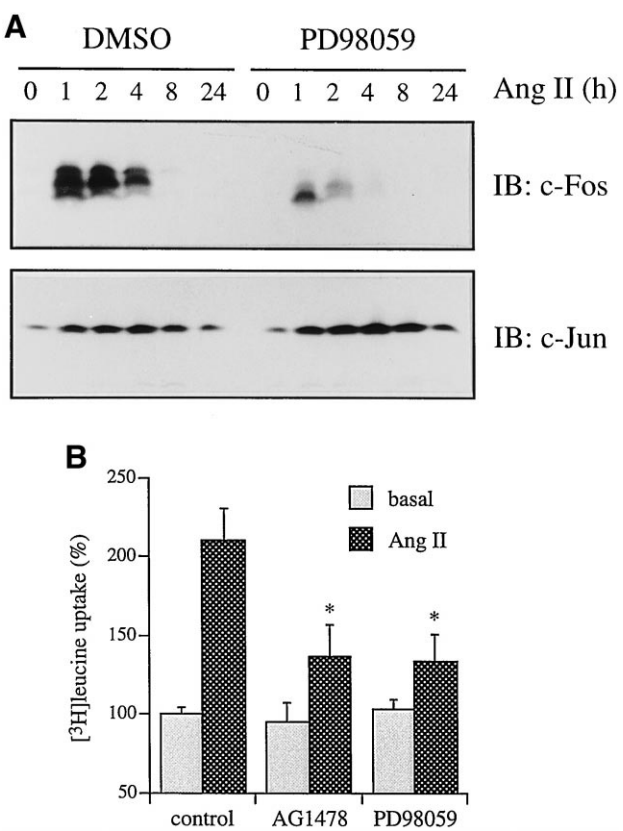

Fig. 2. Inhibitory effect of PD98059 on angiotensin II-induced protooncogene expression and that of AG1478 and PD98059 on angiotensin II-induced protein synthesis in rat vascular smooth muscle cells. (A) Cells were pretreated with PD98059 (25 μM) or the vehicle dimethyl sulfoxide (DMSO: 0.1%) for 30 min and stimulated with angiotensin II (Ang II: 100 nM) for indicated durations. (B) Cells were pretreated with AG1478 (250 nM) or PD98059 (25 μM) for 30 min and stimulated with angiotensin II (Ang II: 100 nM) in the presence of [^{3}H]leucine for 24 h. Data are presented as percent of the basal incorporation ($n = 4$, mean ± S.D.). Statistically different from controls: $^*P < 0.05$.

panel). By contrast, Ag1478 (250 nM) minimally influenced angiotensin II-induced c-Jun expression (Fig. 1A and B, lower panels). Marked inhibition of angiotensin II-induced c-Fos expression was observed at as low as 25 μM of AG1478. Both c-Fos and c-Jun induction by EGF was completely abolished by AG1478 (data not shown), whereas neither c-Fos nor c-Jun expression by PDGF was affected by AG1478 (Fig. 1B).

To determine whether angiotensin II-induced c-Fos expression is mediated by ERK presumably through the transactivation of EGF receptor, effect of the selective MEK inhibitor PD98059 (Alessi et al., 1995) on the protooncogenes expression was tested. Consonant with the effect of AG1478, PD98059 (25 μM) markedly inhibited angiotensin II-induced c-Fos expression, whereas it had no effect on the c-Jun expression (Fig. 2A).

To study whether EGF receptor and ERK are involved in angiotensin II-induced vascular hypertrophy, effects of AG1478 and PD98059 on angiotensin II-induced protein synthesis was examined. Pretreatment with these inhibitors partially but markedly inhibited angiotensin II-induced [^{3}H]leucine incorporation (Fig. 2B). AG1478 had no effect on the PDGF-induced incorporation (data not shown). These data indicate that angiotensin II-induced growth promoting effect of vascular smooth muscle cells is at least in part mediated through EGF receptor and its downstream effector, ERK.

4. Discussion

ERK phosphorylates and activates TCF/Elk1 thereby inducing c-Fos expression (Hill and Treisman, 1995). Dominant-negative mutants of either Ras or ERK were shown to inhibit angiotensin II-induced c-*fos* promoter transcriptional activity in H295R cells (Watanabe et al., 1996) and angiotensin II stimulates ERK mainly through EGF-independent EGF receptor transactivation in vascular smooth muscle cells (Eguchi et al., 1998). In the present study, angiotensin II-induced c-Fos expression was inhibited not only by the MEK inhibitor PD98059 but also by the EGF receptor kinase inhibitor AG1478. Previously, AG1478 was shown to inhibit the c-*fos* mRNA expression and DNA synthesis by G protein-coupled receptor agonists such as endothelin-1 and thrombin in Rat-1 fibroblasts (Daub et al., 1996). Recently, it was shown that when EGF receptor was expressed by transfection, lysophosphatidic acid was able to stimulate c-*fos* promoter transcriptional activity in B82L fibroblasts which lacks endogenous EGF receptor (Cunnick et al., 1998). Taken together, EGF receptor transactivation and subsequent ERK activation may be a common signaling mechanism of c-Fos induction shared by several G protein-coupled receptors in cells expressing EGF receptor.

The present study showed that neither AG1478 nor PD98059 affected angiotensin II-induced c-Jun expression in vascular smooth muscle cells. In liver epithelial cells, a non-receptor tyrosine kinase, PYK2, and c-Jun N-terminal kinase (JNK) are coordinately activated by angiotensin II (Li et al., 1997). In vascular smooth muscle cells, angiotensin II was showed to induce tyrosine kinase-dependent JNK activation (Schmitz et al., 1998) and we have recently shown that angiotensin II activates PYK2 (Eguchi et al., 1999). Therefore, whether PYK2 and JNK are involved in c-Jun induction by angiotensin II remains to be determined. Since it is well known that c-Fos and c-Jun need to dimerize with other members of their families to be functional and some of them must be phosphorylated as well, the roles of the EGF receptor transactivation in their dimalization partner and their phosphorylation state also remain to be tested.

Recently, a cell type dependent diversity of the angiotensin AT_1 receptor signals has been recognized (Sadoshima, 1998). However, during the preparation of the present manuscript, Murasawa et al. (1998) have reported that c-*fos* gene expression and DNA synthesis induced by the AT_1 receptor in cardiac fibroblasts are mediated through the EGF receptor transactivation. In the present study, we have expanded their findings that the c-Fos protein expression was similarly operated through the transactivation in vascular smooth muscle cells and reported for the first time that the transactivation also plays a critical roles in angiotensin II-induced protein synthesis in these cells through ERK activation. Along with its involvement in gene transcription, ERK contributes to the translational machinery possibly through the translational initiation factor eIF4E (Lawrence and Abraham, 1997). Angiotensin II was showed to phosphorylate eIF4E in vascular smooth muscle cells (Rao et al., 1994). Furthermore, the present observation of inhibition of protein synthesis by PD98059 is in agreement with Servant et al. (1996). Thus, it is reasonable to speculate that angiotensin II stimulates protein synthesis through ERK/eIF4E that is located downstream of the EGF receptor transactivation.

Since the doses of AG1478 and PD98059 used in the present study are sufficient to block EGF- and angiotensin II-induced ERK activation, respectively (Servant et al., 1996; Eguchi et al., 1998), incomplete inhibition of angiotensin II-induced c-Fos expression and protein synthesis by AG1478 as well as PD98059 indicates that the hypertrophic signal of angiotensin II uses an additional mechanism(s) other than EGF receptor and/or ERK. The alternative hypertrophic signals may involve rapamycin-sensitive effectors such as mTOR, 4E-BP1, and p70 S6 kinase (Giasson and Meloche, 1995; Fleurent et al., 1997) or reactive oxygen intermediates and p38 MAP kinase (Ushio-Fukai et al., 1998). In addition, although we have assessed the global protein synthesis as a marker of vascular hypertrophy, other specific markers related to cell proliferation and hypertrophy may be useful to demonstrate specific modes of cell growth. Further studies are warranted to determine the relative contribution and the

possible crosstalks of these mechanisms leading to the global hypertrophic response to angiotensin II.

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