Involvement of the platelet-derived growth factor receptor in angiotensin II-induced activation of extracellular regulated kinases 1 and 2 in human mesangial cells

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Abstract In mesangial cells angiotensin II (Ang II) has been shown to activate extracellular regulated kinases 1 and 2 (ERK1/2). Here, we studied the role of the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) in Ang II-induced ERK1/2 activation in human mesangial cells. Ang II induced activation of ERK1/2 via the AT_1 receptor, and this response was blocked by the PDGFR-selective tyrosine kinase inhibitor AG1295, but not by AG1478, an EGFR-selective tyrosine kinase inhibitor, indicating participation of the PDGFR, but not of the EGFR in Ang II-induced ERK1/2 activation. In agreement with this assumption, Ang II caused tyrosine phosphorylation of the PDGFR and the adapter protein Shc in an AG1295-sensitive fashion. In conclusion, our data show that Ang II-induced activation of mitogenic signalling cascade in human mesangial cells involves ligand-independent activation of the PDGFR, but not of the coexpressed EGFR.

Key words: Angiotensin II; Platelet-derived growth factor receptor; Tyrosine kinase; Extracellular regulated protein kinase; Grb2; Shc

1. Introduction

Mesangial cells are mesenchymal derived specialized pericytes, that possess contractile fibers and exhibit smooth muscle cell-like properties [1]. In renal disease mesangial cells may proliferate and are subjected towards locally produced vasoactive and proliferative peptides such as angiotensin II (Ang II). Mesangial cell proliferation is regulated mainly by receptor tyrosine kinase such as receptors for the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and by G-protein-coupled receptors (GPCR) for vasoactive peptides such as Ang II and endothelins [2]. Two distinct subtypes of Ang II receptors (AT_1, AT_2) have been characterized [3]. In mesangial cells the AT_1 receptor with its subtypes AT_{1A} and AT_{1B} predominates [4]. In rat mesangial cells activation of AT_1 receptor is coupled to ERK activation [5]. Activation of extracellular regulated protein kinase (ERK) pathway has been considered to play a key role in mitogenesis and hypertrophy of cells and acts as a convergence point of receptor tyrosine kinase and GPCR activation [6].

The mechanism of receptor tyrosine kinase-stimulated ERK activation is well understood [7]. In contrast, heterogeneity exists in the mechanism of MAPK activation by GPCRs. Depending upon receptor and cell type, MAPK activation may be mediated by G-protein α or $\beta\gamma$ subunits, protein kinase C-, Src-, phosphatidylinositol 3-kinase-, and Ras-dependent or -independent pathways [8–10]. Ang II-induced signaling towards ERK activation is not entirely clear and appears to be cell type specific. In fact, recent work suggests that Ang II-induced ERK activation requires complex formation between Shc, Grb2, and Sos and subsequent activation of Ras as well as several candidate tyrosine kinases such as proline-rich tyrosine kinase (PYK2) [11], PDGFR-β [12], EGFR [13,14], and Src family tyrosine kinases [15,16]. Moreover, Ang II has been described to activate ERK either by a PKC-dependent, Ras-independent pathway, or by a Ras- and Src-dependent pathway after down-modulation of PKC by phorbol ester [17]. In this report we demonstrate that in human mesangial cells which express both the EGFR and the PDGFR, AT_1 receptor-induced ERK activation requires PDGFR tyrosine kinase, and that Ang II does not utilize the EGFR for ERK activation even when the PDGFR was blocked.

2. Materials and methods

2.1. Materials

Ang II, PDGF-BB, human recombinant EGF, PD12319 and agarose-conjugated anti-phosphotyrosine antibody were from Sigma (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) reagents, X-ray films and horseradish peroxidase (HRP)-conjugated IgGs were obtained from Amersham (Little Chalfont, UK). Anti-ERK2 antibody, AG1478 [18], AG1295 [19] and PP1 were from Calbiochem (La Jolla, CA, USA). The antibody raised against dually phosphorylated activated ERK1/2 and the ERK1/2 assay kit were from New England Biolabs (Beverly, MA, USA). Anti-PDGFR antibody was from Upstate Biochech Inc. (Lake placid, NY, USA). Antibodies against Shc, Grb2, and the HRP-labelled anti-phosphotyrosine antibody were from Transduction Laboratories (San Diego, CA, USA). RPMI 1640 medium and fetal calf serum (FCS) were from Gibco (Eggenstein, Germany). Polyvinylidene difluoride transfer membranes were from Millipore (Eschborn, Germany). Losartan was from MSD (München, Germany).

2.2. Cell culture

Human mesangial cells were isolated by sequential sieving technique as described from patients undergoing tumor nephrectomy [20]. All experiments were performed from passages 3–6. Mesangial cell cultures were identified by morphological and immunological characteristics such as positive staining for actin and negative staining for Factor VIII and cytokeratin. To exclude fibroblast contamination

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cells were grown for 2 weeks in L-valine-deficient RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin. Culture media were replaced three times per week.

2.3. Cell stimulation and preparation of cell extract

Cells were grown until subconfluence in media supplemented with 10% FCS. Cells were washed once and made quiescent by a 48 h preincubation in serum-free medium. After stimulation, incubations were terminated by washing cells twice with ice-cold phosphate-buffered saline (PBS). After scraping the cells were pelleted by centrifugation and lysed in a buffer containing 1% Triton X-100, 5 mM EDTA, 10 mM Tris–HCl, pH 8.0, 1.0 mM PMSF, 2 mM dithiothreitol, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate.

2.4. Immunoprecipitation of the PDGFR and tyrosine-phosphorylated proteins

The PDGFR types α and β or tyrosine-phosphorylated proteins were immunoprecipitated with anti-PDGFR antibody recognizing α and β isoforms and protein G-Sepharose or agarose-conjugated anti-phosphotyrosine antibody by incubation at 4°C overnight with gentle agitation. Immunoprecipitates were washed twice in lysis buffer, resuspended in SDS–PAGE sample buffer, and boiled for 5 min. Following SDS–PAGE, proteins were transferred to polyvinylidene difluoride membranes and processed for immunoblot analysis.

2.5. Immunoblotting

Western blotting was carried out as described recently [21]. Nonspecific binding sites were blocked in PBS containing 0.1% Tween 20 (PBST) and 3% bovine serum albumin or PBST containing 5% skim milk. Thereafter, the immunoblots were washed to remove excess primary antibody and incubated with appropriate HRP-conjugated secondary antibodies. Antigen–antibody complexes were visualized using the ECL system. In some experiments filters were reprobed after stripping in a solution containing 0.1 M Tris–HCl, pH 8.2, 2% SDS and 0.1 M β-mercaptoethanol for 30 min at 52°C.

2.6. ERK1/2 activity assay

To determine ERK1/2 activity we used a commercially available assay kit (New England Biolabs, Beverly, MA, USA). In brief, active phosphorylated ERK1/2 was immunoprecipitated from cell lysates. The immunoprecipitates were incubated in the presence of Elk-1 and ATP in kinase buffer provided by the manufacturer. Phosphorylated Elk-1 was detected by Western blotting using a phospho-Elk-1-(Ser-383) specific antibody and chemiluminescent detection with an HRP-conjugated secondary antibody.

2.7. Data presentation

All experiments were performed at least three times with a similar result and representative immunoblots are shown.

3. Results and discussion

In renal disease mesangial cells may proliferate and are subjected towards locally produced vasoactive peptides such as angiotensin II. The MAPK pathway has been considered to play a pivotal role in mitogenesis and hypertrophy of cells and acts as a convergence point in G-protein-coupled receptor activation and growth hormone receptor activation. Recently, it has been shown that Ang II activates ERK1/2 via activation of the AT_1 receptor in rat mesangial cells [5]. We confirmed these results in human mesangial cells by specific detection of the dually phosphorylated, activated form of ERK1/2 by Western blotting. Ang II (1 μM) stimulated phosphorylation of ERK1 and ERK2. The AT_1 receptor blocker Losartan (1 μM) completely inhibited Ang II-induced ERK1/2 phosphorylation, whereas the AT_2 receptor blocker PD123319 (1 μM) had no effect (data not shown).

The convergence of GPCR and RTK signaling pathways is reported for PDGF [11], EGF [13,22] and IGF [23] receptors. To investigate possible involvement of the EGFR or the

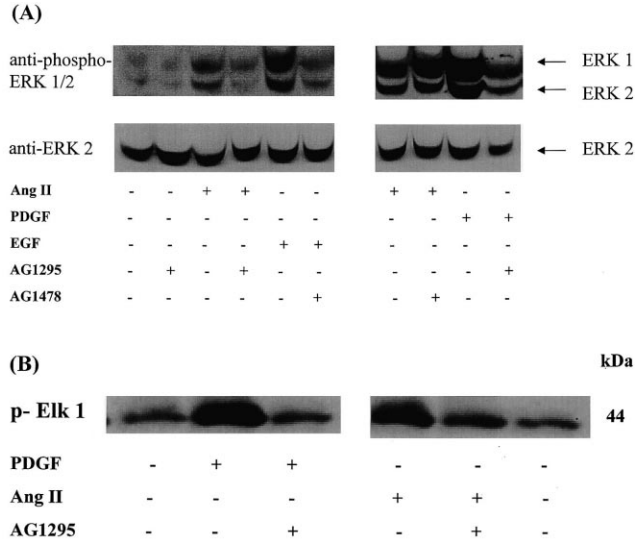

Fig. 1. Effect of Ang II, PDGF and EGF alone and in combination with the PDGFR AG1295 or EGFR AG1478 specific tyrosine kinase inhibitors on phosphorylation (A) and activity (B) of ERK1/2 in human mesangial cells. Cells were incubated with Ang II (1 μM), PDGF (100 ng/ml) or EGF (100 ng/ml) with and without AG1295 (5 μM) or AG1478 (0.5 μM) for 10 min. A: After lysis of the cells equal amounts of protein were immunoblotted with an anti-phospho-ERK1/2 antibody (upper part). The blot was stripped and reprobed with anti-ERK2 antibody (lower part). B: Activity was measured by immunoprecipitation of activated ERK1/2 and in vitro phosphorylation of the ERK1/2 substrate Elk-1.

PDGFR in Ang II-induced ERK activation in human mesangial cells, growth-arrested cells were stimulated with Ang II, PDGF (100 ng/ml), or EGF (100 ng/ml) alone or in combination with the PDGFR-specific tyrosine kinase inhibitor AG1295 (5 μM) or the EGFR-specific tyrosine kinase inhibitor AG1478 (0.5 μM). As shown in Fig. 1A, AG1295 abolished both PDGF- and Ang II-induced phosphorylation of ERK1/2. In contrast, the EGFR-specific tyrosine kinase inhibitor AG1478 had no effect, indicating that the PDGFR, but not the EGFR, is involved in Ang II-induced MAPK phosphorylation. Similarly, when ERK1/2 activity was assayed by detection of Elk-1 phosphorylation as substrate for ERK1 and ERK2, AG1295 inhibited both Ang II- and PDGF-induced ERK1/2 activation (Fig. 1B). To investigate if Ang II stimulates tyrosine phosphorylation of the EGFR or PDGFR, serum-starved cells were incubated with Ang II for the indicated time and subjected to immunoprecipitation with agarose-conjugated anti-phosphotyrosine antibodies. Immunoprecipitates were analyzed by anti-EGFR and anti-PDGFR Western blotting. As shown in Fig. 2A, Ang II caused tyrosine phosphorylation of the PDGFR, whereas Ang II did not alter EGFR tyrosine phosphorylation. A similar result was obtained when cells were stimulated with Ang II, and the PDGFR was immunoprecipitated and the immunoprecipitates were analyzed by anti-phosphotyrosine Western blotting (Fig. 2B). AG1295 abolished Ang II-induced tyrosine phosphorylation of the PDGFR. Downstream signaling of both the PDGFR and the EGFR has been reported to involve recruitment of both Shc and Grb2 to the receptors. Immunoprecipitation of cell lysates from Ang II- or PDGF-stimulated cells with anti-phosphotyrosine antibody and analysis of the immunoprecipitates by anti-Shc Western blotting revealed that Ang II caused tyrosine phosphorylation of Shc isoforms, although

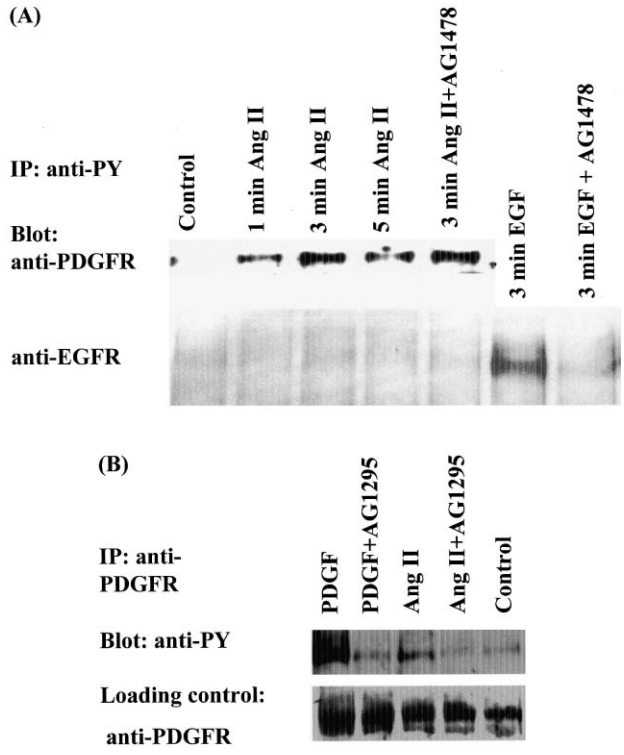

Fig. 2. Effect of Ang II on PDGFR and EGFR tyrosine phosphorylation. A: Quiescent mesangial cells were stimulated with Ang II (1 μM) or EGF (100 ng/ml) for the indicated time. Anti-phosphotyrosine immunoprecipitates were analyzed by anti-PDGFR and anti-EGFR Western blotting. B: Serum-deprived cells were stimulated with Ang II (500 nM) or PDGF (100 ng/ml) alone or in the presence of AG1295 (5 μM). Anti-PDGFR immunoprecipitates were analyzed by anti-phosphotyrosine and anti-PDGFR Western blotting.

to a smaller extent than PDGF. AG1295, but not AG1478 blocked Ang II-induced tyrosine phosphorylation of Shc, indicating that Ang II-induced tyrosine phosphorylation of Shc is mediated by the PDGFR, but not by the EGFR (Fig. 3A). Accordingly, Ang II increased the amount of Shc binding to exogenous Grb2 (Fig. 3B). However, we did not find significant differences in the amounts of Shc and Grb2 in PDGFR immunoprecipitates from Ang II-stimulated cells, whereas PDGF clearly induced these responses (Fig. 3C). Precedent for this phenomenon comes from vascular smooth muscle cells, in which stretch-stress leads to tyrosine phosphorylation of the PDGFR without significant increase in the amount of Grb2 coprecipitating with the PDGFR [24]. Thus, one can speculate that an increased complex formation of the PDGFR with Shc and Grb2 is not necessary for activation of the ERK pathway by Ang II. Another possibility is that the recruitment of other adapter proteins such as Gab1 or Cbl [25–28] are involved in Ras and ERK activation by Ang II in mesangial cells. AT_1 receptor-induced ERK activation has been extensively studied in vascular smooth muscle cells. In these cells Ang II has been reported to induce tyrosine phosphorylation of the PDGFR [12]. A more recent study indicates that Ang II induces tyrosine phosphorylation of the EGFR and Shc, complex formation of Shc, Src, and Grb2 with the EGFR, and that AG1478, but not AG1295, inhibited Ang II-induced ERK activation [13], suggesting that tyrosine phosphorylation of the PDGFR is not involved in ERK activation in vascular smooth muscle cells. It has recently been reported that Src mediates lysophosphatidic acid-induced EGFR tyrosine phosphorylation [9], whereas in another study PP1, an Src family tyrosine kinase inhibitor [29], had no effect [30]. To examine if Src family tyrosine kinase is involved in Ang II-induced tyrosine phosphorylation of the PDGFR, cells were stimulated in the absence or presence of PP1. As shown in Fig. 3D, PP1 (10 μM) did not inhibit Ang II-induced PDGFR tyrosine phosphorylation, suggesting that Src family tyrosine kinases are not involved in this process in mesangial cells. In cardiac

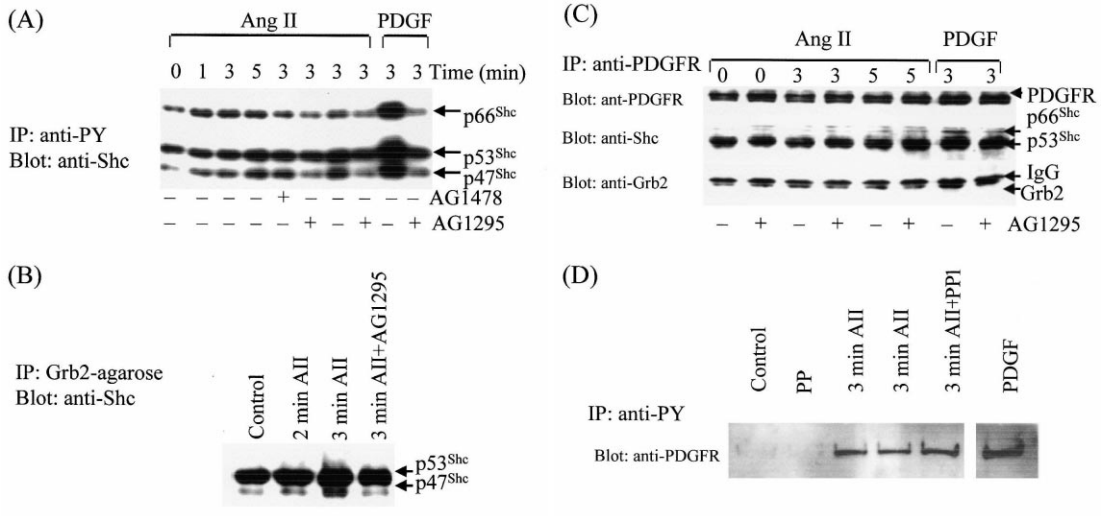

Fig. 3. Effect of Ang II and PDGF on tyrosine phosphorylation of Shc. Serum-deprived mesangial cells were stimulated with Ang II (500 nM) or PDGF (100 ng/ml) in the presence or absence of AG1295/AG1478 or PP1 (10 μM) for the indicated time. In A, lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by anti-Shc Western blotting. B: Lysates were incubated with Grb2 agarose followed by anti-Shc Western blotting. C: Lysates were immunoprecipitated with anti-PDGFR antibody followed by anti-PDGFR, anti-Shc and anti-Grb2 Western blotting. D: Lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by anti-PDGFR Western blotting.

myocytes [14] and GN4 rat liver epithelial cells Ang II stimulates EGFR activation [17]. Similar to the present study showing that the AT_1 receptor, a G_q-coupled receptor [4], stimulates ERKs by a PDGFR-dependent mechanism.

Recent studies have shown that the receptor for lysophosphatidic acid, a G_i-coupled receptor, induces ERK activation through a PDGFR-dependent mechanism [31,32]. Thus, it is reasonable to assume that GPCR can utilize both the EGFR and the PDGFR for activation of ERK depending on the cell type investigated. A recent investigation showed that EGFR transactivation by GPCR requires cleavage of pro-heparin-binding EGF by a metalloproteinase [33]. Whether a similar mechanism is involved in PDGF receptor transactivation requires further investigation. Although numerous studies have shown that AG1478 and AG1295 are highly specific for inhibition of the EGFR and the PDGFR tyrosine kinase, respectively [13,22,34], we cannot exclude that it affects other kinases or signaling intermediates non-specifically. However, our data indicate that the effects of AG1295 and AG1478 are specific in our cells, since AG1295 did not affect EGF-induced EGFR autophosphorylation, tyrosine phosphorylation of Shc and ERK1/2 activation, whereas AG1478 completely inhibited EGFR autophosphorylation without inhibiting PDGF- or Ang II-induced signaling.

In conclusion, the present study using human mesangial cells shows that Ang II utilizes only the PDGFR to induce tyrosine phosphorylation of Shc and to stimulate ERK1/2, although these cells express both the PDGFR and the EGFR. It remains to be determined why GPCR utilizes either the PDGFR or the EGFR for activation of the ERK pathway.

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