Platelet-derived growth factor and angiotensin II stimulate the mitogen-activated protein kinase cascade in renal mesangial cells: comparison of hypertrophic and hyperplastic agonists

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Exposure of mesangial cells to platelet-derived growth factor (PDGF) BB caused a significant stimulation of cell proliferation and protein synthesis, as measured by [³H]thymidine incorporation and [³H]leucine incorporation respectively. In contrast, cells treated with angiotensin II had no significant increase in [³H]thymidine incorporation, but demonstrated a marked increase in [³H]leucine incorporation. Furthermore, angiotensin II significantly increased total protein content per cell. These data show that, whereas PDGF-BB is a mitogen and stimulates mesangial-cell hyperplasia, angiotensin II causes hypertrophy of the cells without hyperplasia. Treatment of mesangial cells with PDGF and angiotensin II rapidly and dose-dependently stimulated mitogen-activated protein (MAP) kinase activity, as shown by an assay for activity *in vitro* using myelin basic protein as a

substrate, and by immunoprecipitation of ³²P-labelled cells with specific antibodies against the 42 kDa and 44 kDa mitogen-activated protein kinases p42^{mapk} and p44^{mapk}, respectively. Whereas stimulation with PDGF-BB caused a potent and sustained (for more than 30 min) phosphorylation and activation of p42^{mapk} and p44^{mapk}, as well as of the upstream activators MAP kinase kinase and c-Raf, the effect of angiotensin II was less potent, reaching a peak at 5–10 min and thereafter declining rapidly. In summary, these results suggest that PDGF-BB and angiotensin II differ in their potency and duration of activation of the MAP kinase cascade, which may explain why PDGF-BB is a potent mitogen for mesangial cells, whereas angiotensin II only triggers mesangial-cell hypertrophy.

INTRODUCTION

Mitogen-activated protein (MAP) kinases, also known as extracellular-signal-regulated protein kinases (ERK), are important intermediates in signal-transduction pathways that are stimulated by a variety of agents, such as growth factors, hormones, neurotransmitters and mitogenic agents. However, the detailed mechanisms by which diverse signals initiated at the plasma membrane are able to converge on cytosolic kinases, which are essential for cell growth and differentiation, are still unclear.

To date, three distinct MAP kinases have been isolated. ERK1 (or p44^{mapk}) and ERK2 (or p42^{mapk}) are closely related to each other, whereas ERK3 encodes a more distantly related kinase that is only 42% identical with ERK1. MAP kinases appear to be highly conserved across species, which may attest their important roles in signal transduction. MAP kinases require for full activation phosphorylation of both threonine and tyrosine residues, and the enzyme activity is abolished when either type of residue is dephosphorylated. Several reports suggested that phosphorylation of both tyrosine and threonine residues in the MAP kinases can result from autophosphorylation, although no full activation by autophosphorylation is achieved. Possible physiological targets of MAP kinase include the S6 ribosomal protein kinase p90^{rsk} and transcription factors c-Jun and c-Myc. Thus MAP kinase is thought to be directly involved in transmitting signals from growth-factor receptors to the nucleus in

order to regulate gene transcription and protein synthesis (for reviews see [1–4]).

The direct upstream activator of MAP kinase is a dual-specificity protein kinase, MAP kinase kinase or MEK (MAP kinase or ERK kinase). Recent data have shown that at least two different signalling pathways converge to activate MEK; one is activated by G-protein-linked receptor agonists and involves MEK kinase, whereas the other pathway is triggered by receptor tyrosine kinase agonists and utilizes the two proto-oncogene products Raf and Ras [2–4].

Glomerular mesangial cells resemble vascular smooth-muscle cells, and are able to contract upon addition of vasoconstrictive hormones such as angiotensin II or vasopressin, thus contributing to the regulation of the glomerular filtration rate [5]. In addition to this physiological function, mesangial cells also play a prominent role in the pathogenesis of inflammatory glomerular processes. Proliferation of mesangial cells is a hallmark of many types of chronic glomerular diseases, thus underlining the great interest in understanding the regulation of mesangial-cell proliferation and a possible contribution of the MAP kinase cascade in this process [6].

Platelet-derived growth factor (PDGF) is a dimeric peptide composed of two closely related, but not identical, chains, denoted A and B, which are linked by disulphide bonds. All three possible dimeric molecules of PDGF have been isolated from natural sources. PDGF is a potent mitogen for mesenchymal cells, including mesangial cells. It possesses diverse biological

Abbreviations used: PDGF, platelet-derived growth factor; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; ERK, extracellular-signal-regulated kinase; TCA, trichloroacetic acid; MBP, myelin basic protein; DMEM, Dulbecco's modified Eagle's medium.

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activities, including chemoattraction and activation of inflammatory cells. Mesangial cells are potential targets for PDGF, since deposition of platelets within the glomerulus with a subsequent release of platelet constituents is a common feature of inflammatory glomerular diseases [7,8].

The octapeptide angiotensin II functions as a regulator of vascular tone, glomerular haemodynamics, tubular transport, and also of chemoattraction of cells, involvement in mesangial-cell processing of macromolecules, immunomodulation, angiogenesis and growth regulation [9]. In cultured mesangial cells, angiotensin II stimulates cellular hypertrophy, with increases in cell size, total protein content and synthesis in the absence of proliferation [10]. Contradictory results have been reported with respect to angiotensin II-induced expression of the immediate early genes *c-fos*, *c-jun* and *c-myc* [9,11].

In the present paper, we compared the activation of the MAP kinase signalling cascade by a hyperplastic agonist (PDGF) and by a hypertrophic agonist (angiotensin II), which each trigger distinct signalling pathways at the cell membrane. Importantly, our data demonstrate that PDGF, but not angiotensin II, is capable of maintaining sustained *c-Raf*, *MEK* and *MAP kinase* activation, which correlates with the mitogenic potency of these agonists, and may explain why PDGF is a potent mitogen for mesangial cells, whereas angiotensin II initiates a hypertrophic response of the cells.

MATERIALS AND METHODS

Materials

Angiotensin II was purchased from Calbiochem, Lucerne, Switzerland; [32 P]P_i and Hyperfilm MP were from Amersham International, Amersham, Bucks., U.K.; Protein A-Sepharose 4B-CL was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; recombinant PDGF-BB was kindly provided by Dr. M. Pech, F. Hoffman-La Roche, Basel, Switzerland; recombinant ERK2 and glutathione S-transferase-coupled MEK were prepared by Ciba-Geigy, Basel, Switzerland; all cell-culture nutrients were from Boehringer, Mannheim, Germany; all other chemicals were from either Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

Cell culture

Rat renal mesangial cells were cultivated as described previously [12]. In a second step, single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial-cell morphology were used for further processing. The cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal-calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and bovine insulin (0.66 unit/ml). Mesangial cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, and negative staining for factor-VIII-related antigen and cytokeratin, which excludes endothelial and epithelial contaminations. Generation of InsP₃ upon activation of angiotensin II AT₁-type receptor [13] was used as a functional criterion for characterizing the cloned cell line. For the experiments reported, passages 8–19 of mesangial cells were used. For cell growth measurements, total protein content was determined spectrophotometrically, and cell numbers were counted in a Neubauer chamber.

[3 H]Thymidine incorporation

Confluent mesangial cells in 24-well plates were made quiescent

by incubation for 3 days in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mg/ml fatty-acid-free BSA, and then stimulated for 24 h with 1 µCi/ml [3 H]thymidine in the presence or absence of the different agonists. To stop the incubation, the medium was withdrawn and the cells were washed twice with PBS and incubated for 30 min at 4 °C with 5% trichloroacetic acid (TCA). Cells were then washed twice with 5% TCA and incubated for 30 min at 37 °C in 1 M NaOH to solubilize the DNA, and the radioactivity incorporated into DNA was counted in a β -scintillation counter.

[3 H]Leucine incorporation in mesangial cells

Subconfluent mesangial cells in 24-well plates were incubated for 24 h in leucine-free DMEM in the absence or presence of the stimuli. During the last 6 h of incubation, 2 µCi/well [3 H]leucine was added. Thereafter cells were washed twice with ice-cold PBS and incubated in 1 ml of 5% TCA for 30 min at 4 °C. Cells were washed twice with 5% TCA and solubilized in 1 M NaOH for 30 min at 37 °C. After neutralization, solubilized proteins were counted for radioactivity in a β -counter.

Peptide synthesis and generation of antibodies

For p44^{mapk} and p42^{mapk}, synthetic peptides based on the C-terminal sequences (IFQETARFQPGAPEAP for p44^{mapk} and IFQETARFQPGYRS for p42^{mapk}) were synthesized, coupled to keyhole-limpet haemocyanin, and used to immunize rabbits. Whereas the anti-p44^{mapk} antiserum recognized specially the p44 isoform, the anti-p42^{mapk} antiserum recognized both p42 and p44 isoforms.

An antiserum which specifically recognized the p42 isoform was kindly provided by Dr. M. J. Dunn, Cleveland, OH, U.S.A. (α I, Cp42) [14].

For MEK, a synthetic peptide based on the N-terminal sequence of murine MEK-1 was synthesized and coupled to keyhole-limpet haemocyanin and used to immunize rabbits. The sequence of the peptide was CPKKKTPIQLNPAPDG. Anti-MARCKS antiserum was generated by immunizing rabbits with a synthetic peptide of the sequence EAAEPEQPEQPEQPAA, which was coupled to keyhole-limpet haemocyanin. The affinity-purified anti-phosphotyrosine antibodies were generously provided by Dr. Paul Burn, Basel, Switzerland.

Cell labelling and immunoprecipitation

Confluent mesangial cells in 100 mm-diameter dishes were washed with PBS and incubated for 2 days in DMEM containing 0.1 mg of fatty-acid-free BSA/ml, and then washed three times with phosphate-free DMEM in order to remove all phosphate. Then the cells were metabolically labelled for 4 h at 37 °C with [32 P]P_i (1 mCi/plate) in phosphate-free DMEM. After labelling, cells were stimulated for the indicated time periods at 37 °C with various agents. To stop the reaction, the medium was removed and the cells were washed twice with ice-cold buffer containing 50 mM Tris, pH 7.5, and 150 mM NaCl. Cells were then scraped directly into 1 ml of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β -glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 µM sodium orthovanadate, 10 µg of leupeptin/ml, 200 units of aprotinin/ml, 1 µM pepstatin A, 1 mM phenylmethanesulphonyl fluoride, 100 nM okadaic acid) and homogenized with 10 passes through a 26-gauge needle fitted to a 1-ml syringe. The homogenate was centrifuged for 10 min at 14000 *g* and the supernatant was taken for immunoprecipitation.

Samples (1 ml), containing 150×10^6 c.p.m. of labelled proteins, 5% fetal-calf serum and 1.5 mM iodoacetamide in lysis buffer, were incubated with different antisera for 3 h at room temperature, or overnight at 4 °C. Then 100 μ l of a 50% (v/v) slurry of Protein A-Sepharose 4B-CL in PBS was added, and the mixture was incubated for 1.5 h at room temperature with mild shaking. After centrifugation for 5 min at 3000 g, immuno-complexes were washed with 3×1 ml of a low-salt buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS), then with 3×1 ml of a high-salt buffer (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS) and once with 1 ml of 10 mM Tris. Pellets were boiled for 5 min in 50 μ l of Laemmli dissociation buffer and subjected to SDS/PAGE (10%-acrylamide gel). After fixing in 25% propan-2-ol/10% acetic acid, the gels were dried and exposed to Hyerfilm MP at -70 °C.

MBP kinase assay

Quiescent mesangial cells in 60 mm-diameter dishes were stimulated with the indicated concentrations of agonists. To stop the reaction, the cells were washed with PBS and scraped into 0.5 ml of kinase buffer (20 mM Tris/HCl, pH 7.5, 1 mM EGTA, 2 mM $MnCl_2$, 0.1 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl fluoride, 25 μ g/ml leupeptin). Cells were then homogenized by ten passes through a 26-gauge needle, centrifuged for 5 min at 14000 g, and the supernatant was taken for determination of protein concentrations. Samples (50 μ g) of cell extracts were incubated for 15 min at 30 °C in the presence of 20 μ g of MBP, 10 μ M ATP and 4 μ Ci of [γ - 32 P]ATP. The reaction was terminated by adding 40 μ l of Laemmli buffer and heated for 5 min at 95 °C. The proteins were then separated by SDS/PAGE (14%-acrylamide gel), followed by autoradiography to reveal the phosphorylation of MBP.

c-Raf activity assay

Confluent mesangial cells in 100 mm-diameter dishes were stimulated with PDGF or angiotensin II for the indicated time periods. To stop the reaction, the medium was removed and the cells were washed with ice-cold PBS. Cells were then scraped directly into 1 ml of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β -glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μ M sodium orthovanadate, 25 μ g of leupeptin/ml, 200 units of aprotinin/ml, 1 μ M pepstatin A, 1 mM phenylmethanesulphonyl fluoride and 100 nM okadaic acid) and homogenized by ten passes through a 26-gauge needle fitted to a 1-ml syringe, and the homogenate was centrifuged for 10 min at 14000 g. Raf proteins were immunoprecipitated with a C-terminal-specific polyclonal antibody as described [15] and incubated for 10 min at 37 °C in a 50 μ l kinase reaction containing 10 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 150 mM NaCl, 2 mM dithiothreitol, 1% Triton X-100, 1 μ M ATP, 0.2 μ Ci of [32 P]ATP and 0.8 μ g of purified recombinant GST-MEK. The kinase reaction was stopped by addition of SDS-sample buffer and subjected to SDS/PAGE (8%-acrylamide gel). After fixing in 25% propan-2-ol/10% acetic acid, the gels were dried and analysed by Phosphor Imaging.

MEK activity assay

MEK proteins were immunoprecipitated from stimulated cells with a specific antiserum as described above. For activity measurements, Protein A-beads were incubated for 10 min at

37 °C in a 50 μ l kinase reaction containing 10 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 4 mM $MgCl_2$, 150 mM NaCl, 2 mM dithiothreitol, 1% Triton X-100, 100 μ M ATP and 1 μ g of purified recombinant ERK2. The assays were centrifuged at 10000 g for 1 min at 4 °C, and 5 μ l of the supernatant was assayed for MBP phosphorylation at 30 °C for 30 min in a 50 μ l assay volume containing 30 mM Tris/HCl, pH 8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 15 μ g of MBP and 10 μ M ATP containing 1 μ Ci of [32 P]ATP. The reactions were stopped with SDS-sample buffer and subjected to SDS/PAGE (13%-acrylamide gel). After fixing in 25% propan-2-ol/10% acetic acid, the gels were dried and analysed by Phosphor Imaging.

Western-blot analysis

Portions (100 μ g) of cell extracts were subjected to SDS/PAGE (10%-acrylamide gel), and proteins were transferred on to nitrocellulose paper for 1 h at 250 mA by using a Bio-Rad Transblot apparatus. The blotting buffer used was 25 mM Tris/HCl (pH 7.4)/190 mM glycine in 20% (v/v) methanol. After the transfer, nitrocellulose filters were washed extensively in distilled water and blocked in blocking buffer [50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 0.2% (v/v) Triton X-100, 3% (w/v) BSA, 10% (v/v) horse serum] for 1 h at 25 °C. Filters were then incubated for 4 h at 25 °C with an antiserum reactive with p42^{mapk} and p44^{mapk}, or an anti-phosphotyrosine antiserum (diluted in blocking buffer as indicated in the legend of Figure 1). After washing in buffer A (50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 0.2% Triton X-100; 4 $\times$ 5 min), the filters were incubated for 1 h with horseradish-peroxidases-conjugated anti-rabbit IgG antibodies in blocking buffer. Thereafter, filters were washed again (4 $\times$ 5 min) in buffer A, and finally, for the colour reaction, the filters were incubated in PBS containing 0.5 mg of 3,3'-diaminobenzidine/ml and 0.03% H_2O_2 for 10 min and then washed extensively in distilled water.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

RESULTS

Effects of PDGF and angiotensin on [3 H]thymidine and [3 H]leucine incorporation

Quiescent mesangial cells were assayed for their ability to incorporate [3 H]thymidine into DNA after exposure to PDGF-BB or angiotensin II for a total of 24 h. As shown in Table 1, exposure of the cells to PDGF-BB caused a concentration-dependent increase in [3 H]thymidine incorporation; in contrast, angiotensin II did not significantly stimulate [3 H]thymidine uptake.

In order to assess protein synthesis in mesangial cells, we measured [3 H]leucine incorporation. As shown in Table 1, both agonists, angiotensin II and PDGF-BB, stimulated a concentration-dependent increase in [3 H]leucine incorporation. As leucine incorporation reflects not only protein synthesis but also protein turnover, we additionally determined total protein content per cell. The data in Table 1 demonstrate that only angiotensin II significantly increased cell mass. These results indicate that PDGF is a potent mitogen for mesangial cells, whereas angiotensin II triggers mesangial-cell hypertrophy.

Table 1 [^3H]Thymidine and [^3H]leucine incorporation in PDGF- and angiotensin II-stimulated mesangial cells

Mesangial cells were made quiescent by incubation for 3 days in serum-free medium and then stimulated for 24 h with the indicated concentrations of PDGF-BB or angiotensin II in the presence of [^3H]thymidine or [^3H]leucine, respectively. [^3H]Thymidine incorporation into DNA, [^3H]leucine incorporation into proteins and total protein content per cell were quantified as described in the Materials and methods section. Results are expressed as percentage of control (in the absence of agonists) and are means $\pm$ S.D. of 4 or 5 experiments. [^3H]Thymidine uptake in control cells was 14472 ± 1015 c.p.m./well, [^3H]leucine uptake in control cells was 311000 ± 3394 c.p.m./well, and total protein content in control cells was 30 ± 4.78 $\mu\text{g}/10^5$ cells. Significant differences from corresponding control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (ANOVA). Abbreviation: n.d., not determined.

Addition	[^3H]Thymidine incorporation	[^3H]Leucine incorporation	Total protein per cell
Control	100 ± 7	100 ± 1	100 ± 16
PDGF-BB			
1 ng/ml	121 ± 10	n.d.	90 ± 12
10 ng/ml	$146 \pm 12^*$	$128 \pm 4^{**}$	132 ± 37
50 ng/ml	$238 \pm 18^{***}$	$146 \pm 11^{***}$	147 ± 30
Angiotensin II			
1 nM	121 ± 11	$119 \pm 3^*$	n.d.
10 nM	112 ± 29	$136 \pm 1^{***}$	101 ± 8
100 nM	119 ± 8	$148 \pm 5^{***}$	137 ± 24
1 μM	120 ± 16	$146 \pm 9^{***}$	$164 \pm 8^*$

PDGF and angiotensin II stimulate MAP kinase activity

Western-blot analysis of mesangial-cell lysates with a polyclonal antiserum reactive with phosphotyrosine residues reveals that PDGF-BB and angiotensin II both cause a rapid increase in tyrosine phosphorylation of a 42 kDa protein within 5 min (Figure 1a, lower panel). This increase is not due to alteration in protein level, since the amount of MAP kinase remained constant over the whole time period tested, as shown in Figure 1 (a, upper panel). In addition, there is a faint band visible at 44 kDa, which may correspond to p44^{mapk}. Densitometric analysis of the data is shown in Figure 1(b).

Figure 1 Immunodetection of MAP kinase and of tyrosine-phosphorylated proteins in PDGF- and angiotensin II-stimulated mesangial cells

Mesangial cells were treated for the indicated time periods with PDGF-BB (25 ng/ml) and angiotensin II (All) (500 nM), and the cell homogenates (100 μg of protein) were subjected to SDS/PAGE (10%-acrylamide gel) and transferred to nitrocellulose. (a) Western-blot analysis was performed using an anti-phosphotyrosine (anti-P-Tyr) antibody at a dilution of 1:1000 (lower panel) or using an antiserum reactive with p42^{mapk} at a dilution of 1:500 (upper panel). Bands were revealed by horseradish peroxidase. Abbreviation: co, control. Positions of molecular-size markers (in kDa) are shown to the left, in this and other Figures. (b) Densitometric evaluation of tyrosine phosphorylation in PDGF-BB (□)- and angiotensin II (△)-stimulated cells. Results are expressed as percentage of maximal phosphorylation of p42^{mapk} and are means of three experiments. Significant differences from corresponding stimulation with PDGF-BB or angiotensin II: * $P < 0.05$; ns, not significant (ANOVA).

Figure 2 Time course of PDGF-BB-stimulated p42^{mapk} and p44^{mapk} phosphorylation in mesangial cells

Mesangial cells labelled with [^{32}P]P, were stimulated with vehicle (co) or PDGF-BB (25 ng/ml) for the indicated time periods. Cells were then lysed, and p42^{mapk} (a) or p44^{mapk} (b) was immunoprecipitated with specific antisera at a dilution of 1:200 for p42^{mapk} and 1:100 for p44^{mapk}. The immunoprecipitates were separated on SDS/PAGE (10%-acrylamide gel) and exposed to Hyperfilm MP at -70°C for 1–3 days.

To assess more directly the phosphorylation of MAP kinases, we metabolically labelled mesangial cells with [^{32}P]P_i, and, after stimulation, p42^{mapk} and p44^{mapk} were immunoprecipitated with polyclonal antibodies specific for each isoform. The phosphorylation was quantified after SDS/PAGE separation and autoradiography. Whereas unstimulated mesangial cells show only a minor amount of phosphorylated p42^{mapk}, PDGF-BB stimulated a marked increase in the levels of p42^{mapk} phosphorylation within 5 min (Figure 2a). Maximal levels of phosphorylation were observed 5 min after stimulation and then slowly declined again, reaching almost control levels after 90 min. Similar kinetics were obtained for the p44^{mapk} isoform (Figure 2b).

Figure 3 Time course of angiotensin II-stimulated $p42^{\text{mapk}}$ and $p44^{\text{mapk}}$ phosphorylation in mesangial cells

Mesangial cells labelled with [^{32}P]P were stimulated with vehicle (co) or angiotensin II (All) (500 nM) for the indicated time periods. Cells were then lysed, and $p42^{\text{mapk}}$ (a) or $p44^{\text{mapk}}$ (b) was immunoprecipitated with specific antisera at a dilution of 1:200 for $p42^{\text{mapk}}$ and 1:100 for $p44^{\text{mapk}}$. The immunoprecipitates were separated on SDS/PAGE (10% acrylamide gel) and exposed to Hyperfilm MP at -70°C for 1–3 days.

Figure 4 Comparison of kinetics of PDGF- and angiotensin II-stimulated $p42^{\text{mapk}}$ and $p44^{\text{mapk}}$ phosphorylation in mesangial cells

Densitometric evaluation of the data from Figures 2 and 3 and two further independent experiments on PDGF-BB ($\square$)- and angiotensin II ($\triangle$)-stimulated cells were performed. Results are expressed as percentage of maximal phosphorylation of $p42^{\text{mapk}}$ (a) and $p44^{\text{mapk}}$ (b) and are means of three independent experiments. Significant differences from corresponding stimulation with PDGF-BB or angiotensin II: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant (ANOVA).

In contrast, stimulation with angiotensin II, which acts through a G-protein-coupled receptor, revealed a more transient increase in $p42^{\text{mapk}}$ phosphorylation with maximal effects at 5 min, and then declined rapidly to control level within 30–60 min (Figure

Figure 5 Time course of PDGF- and angiotensin II-stimulated MBP kinase activity in mesangial cells

Mesangial cells were treated with vehicle (co), PDGF-BB (25 ng/ml) or angiotensin II (All) (500 nM) for the indicated time periods. Thereafter, 50 μg of cell protein was harvested in kinase assay buffer for the MBP kinase activity assay as described in the Materials and methods section. One representative experiment is shown in (a). (b) Densitometric evaluation of MBP kinase activity in PDGF-BB ($\square$)- and angiotensin II ($\triangle$)-stimulated cells. Results are expressed as percentage of maximal activity and are means of three independent experiments. Significant differences from corresponding stimulation with PDGF-BB or angiotensin II: ** $P < 0.01$; ns, not significant (ANOVA).

Figure 6 Dose-dependence of PDGF-BB- and angiotensin II-stimulated MBP kinase activity in mesangial cells

Mesangial cells were treated for 10 min with the indicated concentrations of PDGF-BB, angiotensin II (All) or vehicle (Co). Thereafter, 50 μg of cell protein was harvested in kinase assay buffer, and MBP kinase activity was measured as described in the Materials and methods section. Similar results were obtained in three independent experiments.

3a). Again, identical kinetics were observed for $p44^{\text{mapk}}$ as shown in Figure 3(b). Densitometric analysis and comparison of PDGF- and angiotensin II-stimulated $p42^{\text{mapk}}$ and $p44^{\text{mapk}}$ are shown in Figure 4.

Next we assessed MAP kinase activity after stimulation by PDGF-BB and angiotensin II by an assay *in vitro* using MBP as a substrate. The data in Figure 5 show that PDGF and angiotensin II both stimulate MBP kinase activity, with kinetics comparable with the time courses of phosphorylation (Figures 2 and 3). Again, PDGF-BB causes a much more sustained increase in MAP kinase activity, compared with angiotensin II (Figures 5a and 5b). Figure 6 shows the dose-dependence of MAP kinase activation by PDGF and angiotensin II. Maximal stimulation is obtained at a concentration of 10 ng/ml for PDGF-BB and 100 nM for angiotensin II.

Figure 7 Time course of PDGF-BB- and angiotensin II-stimulated MEK phosphorylation in mesangial cells

Mesangial cells labelled with [32 P]P_i were stimulated with PDGF-BB (25 ng/ml) (a) and angiotensin II (All, 500 nM) (b) for the indicated time periods. Cells were then lysed, and MEK was immunoprecipitated with a MEK-specific antiserum at a dilution of 1:100. The immunoprecipitates were separated on SDS/PAGE (10%-acrylamide gel) and exposed to Hyperfilm MP at -70°C for 1 day. (c) Display of the densitometric evaluation of the data from (a) and (b).

PDGF and angiotensin II stimulate MEK and c-Raf phosphorylation and activation

The MAP-kinase-activating kinase, MEK, has recently been cloned from mouse, human and *Xenopus* [16–20]. Three different human MEK cDNAs (MEK1, MEK2 and MEK3) have been isolated [20,21]. In order to investigate the effects of PDGF and angiotensin II on the phosphorylation of this direct upstream activator of MAP kinase, we have generated an anti-peptide antibody against the N-terminus of murine MEK1.

Figure 7(a) shows that in unstimulated mesangial cells, MEK already exists in a slightly phosphorylated form. Upon PDGF stimulation this phosphorylation rapidly increases and remains at an elevated level for at least 90 min, the latest time point measured. In contrast, angiotensin II caused a transient and less potent stimulation of MEK phosphorylation (Figures 7b and 7c).

Figure 8 Time course of PDGF- and angiotensin II-stimulated phosphorylation of c-Raf

Mesangial cells labelled with [32 P]P_i were stimulated with PDGF-BB (25 ng/ml) (a) and angiotensin II (All, 500 nM) (b) for the indicated time periods. Cells were then lysed and c-Raf was immunoprecipitated with a polyclonal antibody reactive with c-Raf at a dilution of 1:250. The immunoprecipitates were separated on SDS/PAGE (8%-acrylamide gel) and exposed to Hyperfilm MP at -70°C for 7–10 days. (c) Display of the densitometric evaluation of the data from (a) and (b).

Since several groups have suggested that MEK functions as a convergence point for the receptor tyrosine kinase pathway and G-protein-linked receptor pathway, that mediates subsequent MAP kinase activation, we wanted to investigate the involvement of the upstream activator of MEK used by receptor tyrosine kinase agonists, the proto-oncogene product c-Raf. Immunoprecipitation of 32 P-labelled cells with polyclonal antibodies reactive with c-Raf revealed an increase in phosphorylated c-Raf after PDGF stimulation, and unexpectedly also after angiotensin II stimulation (Figures 8a and 8b). Again, densitometric evaluation of the bands shows that PDGF causes a more potent and sustained phosphorylation of c-Raf than does angiotensin II (Figure 8c). Analysis of MEK and c-Raf phosphorylation is difficult to interpret, as it cannot be excluded that feedback phosphorylation of MEK and c-Raf by MAP kinase accounts for the sustained phosphorylated appearance of the enzymes [22]. Therefore we have determined the activities of c-Raf and

Figure 9 Time course of PDGF-BB- and angiotensin II-stimulated c-Raf and MEK activity in mesangial cells

Mesangial cells were treated with vehicle, PDGF-BB (25 ng/ml) (□) or angiotensin II (500 nM) (△) for the indicated time periods. Thereafter, activities of c-Raf (a) or MEK (b) in mesangial-cell homogenates were determined by sequential phosphorylation reactions *in vitro* using recombinant GST-MEK and ERK-2 as substrates as described in the Materials and methods section. Results are expressed as percentage of control (c-Raf and MEK activities in unstimulated cells) and are means of three independent experiments. Significant differences from corresponding stimulation with PDGF-BB or angiotensin II: ** $P < 0.01$; * $P < 0.05$; ns, not significant (ANOVA).

MEK by sequential phosphorylation reactions *in vitro* using recombinant GST-MEK and ERK-2 as substrates. The data in Figures 9(a) and 9(b) demonstrate that PDGF-BB triggers a sustained activation of c-Raf and MEK activity, whereas angiotensin II causes a transient activation of both enzymes.

DISCUSSION

Excessive proliferation of mesangial cells is a hallmark of many forms of glomerulonephritis, finally leading to glomerulosclerosis. However, to date little is known about the regulation of mesangial-cell growth *in vivo* and *in vitro*. Recently a novel group of serine/threonine kinases, the MAP kinases, have been described that are activated by a variety of agents. They are now considered to function as key enzymes in the signalling process leading to growth control of mammalian cells [2,4,21]. Evidence has been obtained that at least two independent pathways exist that lead to MAP kinase activation: one that is initiated by activation of tyrosine kinase receptors and involves the proto-oncogene products Ras and Raf, and another pathway which is triggered by receptors coupled to heterotrimeric G-proteins and involves protein kinase C [2,4,21].

We therefore decided to investigate the contribution of these two pathways in the activation of the MAP kinase cascade in mesangial cells, and selected PDGF and angiotensin II as important and representative agonists triggering these two signalling cascades.

PDGF is a potent mitogen for mesangial cells and represents a classical activator of the tyrosine kinase receptor pathway

[7,23,24]. Release of PDGF by infiltrating platelets and inflammatory cells as well as by mesangial cells themselves is assumed to play a key role in the development of glomerular hypercellularity [6,8]. Angiotensin II is a potent vasoconstrictor that activates phosphoinositide turnover in mesangial cells via a G-protein-coupled phospholipase C, and is a prominent regulator of glomerular haemodynamics [25–27]. However, in contrast with PDGF, angiotensin II does not induce proliferation of mesangial cells, but stimulates cellular hypertrophy [10,28]. Mesangial hypertrophy and matrix accumulation are characteristic features of glomerulosclerosis [6].

Our data demonstrate, for the first time, that both PDGF and angiotensin II stimulate the MAP kinase cascade in mesangial cells. By comparing the phosphorylation of p42^{mapk} with the phosphorylation of p44^{mapk}, we found that both agonists stimulate the activation of both MAP kinase isotypes with comparable kinetics and potencies. Thus, PDGF- and angiotensin II-induced MBP kinase activity measured may equally be attributed to both isoforms.

To elucidate further the mechanisms of PDGF- and angiotensin II-induced MAP kinase activation, we investigated the effects of PDGF and angiotensin II on the upstream activator, MEK, which acts as a MAP kinase kinase. By immunoprecipitating MEK from ³²P-loaded cells we observed a rapid and sustained increase (for more than 90 min) in phosphorylated MEK upon PDGF stimulation, whereas angiotensin II causes a transient phosphorylation of MEK. The potencies and time courses of MEK phosphorylation correspond well to the potencies and time courses of PDGF- and angiotensin II-induced MAP kinase phosphorylation. Parallel changes in MEK activity were observed in PDGF- and angiotensin II-stimulated mesangial cells, thus demonstrating that the sustained activation of MEK by PDGF and its transient activation by angiotensin II underlie the corresponding changes of MAP kinase activity. Our data are reminiscent of the observations reported by Gotoh et al. [29] and Traverse et al. [30]. These authors showed that stimulation of PC12 cells with nerve growth factor, a differentiating factor, stimulates a sustained activation of MAP kinase and MEK and causes a translocation of MAP kinase from the cytosol to the nucleus. In contrast, epidermal growth factor, a mitogen, induces a transient activation of both enzymes that is not accompanied by a nuclear translocation of MAP kinase [30].

In further experiments, we traced the cascade one step upstream and examined the potential activator of MEK, the proto-oncogene product c-Raf, after stimulation of the cells with PDGF and angiotensin II. Lange-Carter et al. [31] have suggested that c-Raf may regulate the MAP kinase cascade primarily in response to agonists that activate receptor tyrosine kinases, whereas a MEK kinase is responsible for mediating signals originating from G-protein-coupled receptors and PKC. With regard to these proposals by Lange-Carter et al. [31], we expected an increased phosphorylation of c-Raf in response to stimulation with PDGF and an unaltered phosphorylation state after angiotensin II treatment. As expected, we found that PDGF induces a rapid and sustained increase in c-Raf phosphorylation and activation. Most notable was the fact that angiotensin II also shows a stimulatory effect on c-Raf phosphorylation and activation; however, the effect was weak and more transient as compared with the PDGF-induced responses. Obviously, c-Raf activation can also be triggered by agonists through G-protein-coupled receptors. This is consistent with previous reports showing that c-Raf is activated by thrombin and endothelin in 3T3 cells [32], by carbachol in Rat 1a fibroblasts [33] and by phorbol esters in a variety of other cell types [15,32,34]. In contrast with these reports, others [35] demonstrated that ex-

pression of a dominant negative mutant of Ras in PC12 cells does not inhibit activation of MAP kinase in response to stimulation of G-protein-coupled receptors or activation of PKC. In addition, over-expression of MEK kinase, the other upstream activator of MEK, does not result in c-Raf activation [31], suggesting that these two pathways are independent of each other. Obviously different modes of regulation exist for MEK activation. Whereas receptor tyrosine kinase agonists activate MEK via the proto-oncogenes c-Ras and c-Raf, the flow of signals from G-protein-linked receptor agonists is via PKC to either c-Raf or MEK kinase, and these signals subsequently converge again to activate MEK. Further work will be required to unravel fully this complex network of signalling.

Further support of the involvement of G-protein-coupled receptors c-Raf activation is provided by our observation that extracellular nucleotides such as ATP and UTP, which trigger the G-protein-PKC pathway in mesangial cells [36], exert a comparable stimulatory effect on c-Raf phosphorylation as angiotensin II (A. Huwiler and J. Pfeilschifter, unpublished work).

In summary, the present studies demonstrate that PDGF, which is a potent mitogen in mesangial cells, causes a potent and sustained phosphorylation and activation of several enzymes in the MAP kinase cascade, such as p42^{mapk}, p44^{mapk}, MEK and c-Raf, whereas angiotensin II, lacking any mitogenic activity in mesangial cells, mediates a transient activation of the MAP kinases MEK and c-Raf. From these results it is tempting to speculate that not the activation of a specific enzyme, but rather the time period of the activation, may be essential for triggering entry into the cell cycle.

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