Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells

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Mitogen-activated protein (MAP) kinases are regarded as switch kinases in the phosphorylation cascade initiated by various agonists. We have investigated whether endothelins (ET), which are constrictor and mitogenic isopeptides, can increase MAP kinase activity in rat mesangial cells, using bovine myelin basic protein (MBP) as a substrate for an *in vitro* kinase assay. Treatment of quiescent mesangial cells with ET-1 rapidly stimulated a kinase activity which phosphorylated exogenous MBP. This stimulation was dose-dependent, with threshold responses at 1 nM-ET-1. Epidermal growth factor and thrombin also activated this kinase in mesangial cells. We also examined the ET signal transduction pathways leading to activation of MBP kinase. Pertussis toxin had no effect on ET-stimulated MBP kinase activity. Stimulation of protein kinase C by phorbol ester increased MBP kinase activity, and down-regulation of PKC partially inhibited ET-stimulated MBP kinase as well as phorbol ester-stimulated MBP kinase activity. Interestingly, genestein, an inhibitor of protein tyrosine kinases, partially inhibited MBP kinase stimulated by ET but not by phorbol esters. These results suggest that ET stimulates MBP kinase activity in rat mesangial cells via at least two pathways: one which is protein kinase C-dependent and a second one that involves a protein tyrosine kinase. Finally, by raising rabbit antibodies against the two forms of MAP kinase, p44^{mapk} and p42^{mapk}, we demonstrated that both isoforms are expressed in mesangial cells. Antibody α 1 Cp42 specifically immunoprecipitated p42^{mapk} and allowed us to demonstrate that ET stimulates MBP kinase activity in the p42^{mapk} immunocomplex. In conclusion, we have provided evidence that, in rat mesangial cells, MAP kinases are rapidly activated by ET-1, a regulatory process that involves at least protein kinase C activation and also a contribution of a tyrosine kinase not yet characterized.

INTRODUCTION

Endothelins (ETs) are 21-amino-acid isopeptides which are synthesized and secreted by endothelial cells as well as by the central nervous system and by diverse epithelial cells, including pulmonary, gastrointestinal and renal epithelia (Yanagisawa *et al.*, 1988; Simonson & Dunn, 1990). The receptors for these peptides are G-protein-coupled receptors with seven transmembrane domains (Arai *et al.*, 1990; Sakurai *et al.*, 1990). These peptides bind to their distinct receptors and trigger a common set of early signalling events in rat mesangial cells, including rapid production of inositol 1,4,5-trisphosphate, sustained release of arachidonic acid with synthesis of prostaglandins, mobilization of Ca²⁺ from intracellular and extracellular sources, activation of protein kinase C (PKC) and rapid expression of the *c-jun* and *c-fos* genes (Simonson *et al.*, 1989, 1992). ET has also been shown to be a mitogen for mesangial cells (Simonson *et al.*, 1989).

The signal transduction pathways activated by ET in rat mesangial cells have been only partially characterized. A novel group of serine/threonine kinases, comprising p44^{mapk} and p42^{mapk} which phosphorylate microtubule-associated protein-2 and myelin basic protein (MBP), has been identified as an important intermediary step in converting extracellular signals into intracellular responses (Ray & Sturgill, 1987; Sturgill & Wu, 1991; Cobb *et al.*, 1991; Thomas, 1992). These kinases are activated through the phosphorylation on both tyrosine and threonine residues of the kinase by diverse stimuli, including

most if not all mitogens (Ray & Sturgill, 1987; Hoshi *et al.*, 1988; Sturgill *et al.*, 1988; Erickson *et al.*, 1990; Ahn *et al.*, 1990; Sanghera *et al.*, 1990; Tsao *et al.*, 1990; L'Allemain *et al.*, 1991a; Meloche *et al.*, 1992a). Therefore these kinases were also named mitogen-activated protein (MAP) kinases. MAP kinases can phosphorylate and activate many substrates, including the 90 kDa ribosomal protein S6 kinase (Sturgill *et al.*, 1988), transactivating factors such as *c-jun* (Pulverer *et al.*, 1991) and *myc* (Alvarez *et al.*, 1991), and *raf* kinase (Anderson *et al.*, 1991b). Failure to activate MAP kinase in TNR 9 cells, which do not respond mitogenically to phorbol esters (Butler-Gralla & Herschman, 1981), correlates with impaired S6 phosphorylation and mitogenesis in response to phorbol esters (L'Allemain *et al.*, 1991b). The cDNA sequences of MAP kinases, also called extracellular signal-regulated protein kinases (ERK1 and ERK2), are very similar to those of KSS1 and FUS3, protein kinases involved in pheromone action and cell cycle control in yeast (Boulton *et al.*, 1991). Therefore MAP kinase may play a significant role in the process of signal transduction and growth control.

We hypothesized that signal transduction through the MAP kinase cascade may be an important mechanism in mediating ET post-receptor signalling in rat mesangial cells. Our results show that the treatment of quiescent mesangial cells with ET-1 rapidly activates MAP kinases and that this activation occurs via PKC-dependent and protein tyrosine kinase pathways. Therefore the activation of MAP kinases may be critical for the transduction of ET signals into cellular responses.

Abbreviations used: ET, endothelin; PKC, protein kinase C; MBP, myelin basic protein; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; HBSS, Hanks' buffered saline solution; FBS, fetal bovine serum; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

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MATERIALS AND METHODS

Mesangial cell culture

Mesangial cells were isolated from Sprague-Dawley rat glomeruli using previously described methods (Mené & Dunn, 1986). Briefly, minced cortex was sequentially sieved through a 106 μm metal sieve and a 74 μm nylon sieve (Nitex, Elmsford, NY, U.S.A.) to capture the isolated glomeruli in Hank's balanced salt solution (HBSS). The glomeruli were then digested with bacterial collagenase (Worthington type I) in Ca^{2+} - and Mg^{2+} -free HBSS. Mesangial cells were cultured in RPMI-1640 medium containing 17% fetal bovine serum (FBS), 100 units of penicillin/ml, 100 μg of streptomycin/ml, 5 μg of each of insulin and transferrin/ml and 5 ng of selenium/ml. The cultures were maintained in an incubator at 37 °C in a humidified atmosphere of 95% air/5% CO_2 . Cells were used in passages 10–30.

Cell stimulation and preparation of cell extract

Rat mesangial cells grown to a density of $(0.8\text{--}1.0) \times 10^6/\text{ml}$ in 9.6 cm^2 area were incubated for another 48 h in RPMI-1640 medium containing 0.5% FBS. Prior to treatment with ET-1 or other agents, quiescent mesangial cells were rinsed once with warmed RPMI-1640. The cell cultures were replenished with RPMI-1640 containing ET, epidermal growth factor (EGF), thrombin, phorbol 12-myristate 13-acetate (PMA) or genestein for continued incubation periods. Fluoroaluminate (AlF_4^-) was prepared fresh daily by mixing 10 vol. of NaF (300 mM) with 1 vol. of AlCl_3 (1 mM). This solution was added directly to the cell medium to give final concentrations of 30 mM-NaF and 10 μM - AlCl_3 (Anderson *et al.*, 1991a). For PKC down-regulation experiments, 0.1 μM -PMA was added into the medium 24 h before the subsequent experiments, and for pertussis toxin experiments, 100 ng of pertussis toxin/ml was added 12 h before the subsequent treatments.

After these incubations at 37 °C, all subsequent manipulations were done at 4 °C. The monolayers of cells were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM-NaCl, 2.7 mM-KCl, 8 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4 , pH 7.5) containing 10 mM-NaF and 1 mM-phenylmethanesulphonyl fluoride (PMSF), and detached using a rubber policeman into an Eppendorf tube. After centrifugation, the cells were resuspended in the kinase assay buffer containing 20 mM-Tris/HCl (pH 7.4), 1.0 mM-EGTA, 2 mM- MnCl_2 , 0.1 mM-sodium orthovanadate, 10 mM- β -nitrophosphate and 1 mM-PMSF (Ray & Sturgill, 1987). The cells were twice disrupted by an ultrasonic probe with a relative output of 50 for 15 s. The cellular homogenates were then centrifuged at 10000 g for 5 min and the supernatants were prepared for kinase assay after the determination of protein concentration.

MBP kinase assay

MBP kinase activity was measured using bovine MBP as substrate. The different MBP kinase preparations were incubated at 30 °C in the kinase assay buffer containing 20 μg of protein from the cell extract in the presence of 10 μg of MBP, 10 μM -ATP and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in a final volume of 40 μl . The reactions were terminated by addition of 20 μl of Laemmli's buffer (Laemmli, 1970) and boiling for 3 min. The proteins were then separated by SDS/12%-PAGE followed by autoradiography to visualize the phosphorylation of MBP. The activity of the kinase was determined both by scanning the autoradiograph bands using a densitometer and by cutting the band and measuring radioactivity using liquid scintillation counting.

Antibodies and immunoblotting

Antibodies against p44^{mapk} and p42^{mapk} were generated using

synthetic peptides as immunogens. The peptides were synthesized by Dr. Guy Drapeau, University of Laval, Quebec City, Quebec, Canada. The sequences of the peptides were IFQETARFQPGAPEAP for p44^{mapk} and IFETARFQPGYRS for p42^{mapk}, according to the reported sequences (Boulton *et al.*, 1991). The sequence and purity (> 90%) of the peptides were determined by g.c.-m.s. analysis. The peptides, conjugated to keyhole limpet haemocyanin (KLH), were used to immunize rabbits. Aliquots of the cellular supernatant fractions containing 50 μg of protein were electrophoresed on 10% polyacrylamide gel in the presence of SDS, followed by transfer to the nitrocellulose sheet. The sheets were then incubated with anti-p44^{mapk} or anti-p42^{mapk}. Immunoreactive protein bands were then detected using horseradish peroxidase-conjugated goat anti-rabbit IgG visualized by the Amersham ECL system after intensive washing of the sheets. The immunospecificity was determined both by pre-absorbing the antiserum of 100 μg of either peptide, and by pre-immune serum controls.

Immunoprecipitation and immunocomplex kinase assay

For immunoprecipitation of p42^{mapk}, quiescent cells were labelled with phosphate-free medium containing 200 μCi of ^{32}P /ml for 5 h, followed by addition of different agonists. The preparation of cell extract was the same as for the MBP kinase assay. Protein A-Sepharose (25 μl) and 2 μl of the pre-immune serum were first incubated at 4 °C for 1 h, followed by centrifugation to collect the Sepharose beads. The beads were then resuspended in 25 μl of lysis buffer and 2 μl of the anti-MAP kinase antibodies and incubated at 4 °C for 2 h, followed by addition of the cellular extracts containing 50 μg of protein. The incubation was continued overnight. The immunocomplex was washed five times and boiled for 3 min before being separated on SDS/10%-PAGE. The separated proteins were then visualized by autoradiography. For the immunocomplex kinase assay, quiescent mesangial cells were stimulated by ET-1 at 37 °C for the indicated times. The cells were then rinsed and broken as described above. The cellular extracts were incubated with the complex of Protein A-Sepharose-anti-(MAP kinase) antibody. Immunocomplexes were obtained by centrifugation and washed five times in the lysis buffer and once in kinase assay buffer as described above. The MAP kinase activity in the immunocomplex was measured by resuspending the pellet in 40 μl of kinase assay buffer containing 10 μg of MBP, 10 μM -ATP and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reactions were incubated at 30 °C for 15 min and were terminated by addition of 20 μl of Laemmli buffer and boiling for 3 min prior to separation on SDS/10%-PAGE.

Materials

ET-1 was purchased from Peptides International (Louisville, KY, U.S.A.), genestein from Biomol Research (Philadelphia, PA, U.S.A.), pertussis toxin from List Biological Laboratories (Campbell, CA, U.S.A.), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5500 Ci/mmol) and $^{32}\text{P}]\text{P}_i$ from ICN Biomedicals (Irvine, CA, U.S.A.). MBP, thrombin and all other reagents were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Sangivamycin came from the National Cancer Institute of the NIH.

RESULTS

ET activates a kinase which phosphorylates exogenous MBP

Treatment of the quiescent rat mesangial cells with ET-1 for 5 min stimulated a kinase that phosphorylated exogenous MBP. This stimulation was dependent on the concentration of ET-1, with threshold responses at 1 nM-ET and maximal stimulation (4-fold) at 0.1 μM (Fig. 1). In the time-course experiments the

Fig. 1. Dose-dependence of ET- and thrombin-stimulated MBP kinase activity

Quiescent rat mesangial cells were treated with various concentrations of ET-1 and thrombin for 5 min and harvested in kinase assay buffer for the MBP kinase activity assay as described in the Materials and methods section.

Fig. 2. Time course of induction of MBP kinase activity by ET-1, thrombin and EGF

Quiescent rat mesangial cells were incubated with 0.1 μ M-ET-1 (Δ), 10 nM-thrombin ($\blacktriangle$) or 200 ng of EGF/ml ($\blacksquare$) for the times indicated. The soluble cellular extracts were assayed for MBP kinase activity as described in the Materials and methods section. The relative kinase activity was quantified by densitometry of the autoradiographs and is expressed in arbitrary units of absorbance as the means $\pm$ S.E.M. ($n = 3$).

Fig. 3. Time course of AlF_4^- activations of MBP kinase

Quiescent mesangial cells were incubated with AlF_4^- for the times indicated. The cell extracts were then assayed for kinase activity towards MBP as described in the Materials and methods section.

increased kinase activity could be seen 2 min after the addition of an agonist (ET-1, thrombin, EGF) to the cell culture. Maximal activity, a 4–5-fold increase over basal levels, occurred between 5 and 10 min after the addition of 0.1 μ M-ET-1. Thereafter there was a rapid decline in ET-stimulated kinase activity, so that by 20 min it had returned to the original level (Fig. 2). The EGF receptor has intrinsic tyrosine kinase activity, whereas the thrombin and ET receptors are G-protein-coupled to phospholipase C without intrinsic tyrosine kinase activity. Both EGF

Fig. 4. Inhibition of ET-stimulated MBP kinase activity by genestein

Quiescent mesangial cell monolayers were treated with ET for 5 min in the presence or absence of 40 μ M-genestein. MBP kinase activity in the cell extracts was measured as described in the Materials and methods section. The relative kinase activity was quantified by densitometry of the autoradiographs. Results represent means $\pm$ S.E.M. for three determinations in triplicate.

Table 1. Effect of different treatments on MBP kinase activity

Quiescent rat mesangial cells were treated with: 1, 0.1 μ M-ET-1 or 100 nM-PMA for 5 min (PKC depletion, $-$); 2, 100 nM-PMA for 24 h then stimulated with 0.1 μ M-ET-1 or 100 nM-PMA (PKC depletion, $+$); or 3, 0.1 μ M-ET-1 + genestein (40 μ M) or 100 nM-PMA + genestein for 5 min. The cells were collected at the end of incubation and the soluble cell extracts were assayed for MBP kinase activity as described in the Materials and methods section.

Treatment	Relative activity (absorbance units)		
	PKC depletion		Genestein
	$-$	$+$	
Control	190.5 $\pm$ 12.4	181.2 $\pm$ 7.1	172.7 $\pm$ 15.2
ET	678.9 $\pm$ 39.4	342.7 $\pm$ 21.9	359.4 $\pm$ 38.1
PMA	1280.1 $\pm$ 81.9	752.7 $\pm$ 42.1	1187.9 $\pm$ 96.8

and thrombin increased the MBP kinase activity, with a threshold at 1 nM for thrombin (Figs. 1 and 2). EGF- and thrombin-stimulated MBP kinase activity was detectable 2 min after addition to the cell culture, was maximal after 5–10 min and returned towards the baseline after 20 min (Fig. 2). The results showed that ET-1, thrombin and EGF can increase a kinase activity which phosphorylates MBP in rat mesangial cells, suggesting that agonists with receptors either not having (ETs and thrombin) or having (EGF) intrinsic tyrosine kinase activity can transmit their signals through the activation of a kinase which may phosphorylate multiple substrates in the integrated signal transduction network.

Effect of pertussis toxin and fluoroaluminate on ET-stimulated MBP kinase activity

Since G-proteins have been implicated in the ET signal transduction process in mesangial cells, and since pertussis toxin inhibits approx. 50 % of ET-1-stimulated phospholipase C responses (increments of intracellular Ca^{2+} and inositol trisphosphate) (Thomas *et al.*, 1991), we examined the effect of both pertussis toxin and AlF_4^- on MBP kinase activity. Treatment of the mesangial cells with 100 ng of pertussis toxin/ml for 12 h had no inhibitory effect on the subsequent ET-1-augmented MBP kinase activity over an ET dose range of 10^{-10} to 10^{-6} M (results not shown), indicating the involvement of a pertussis toxin-insensitive signal transduction process in ET-1-augmented

Fig. 5. Immunoblot analysis of antibodies against p42^{mapk} and p44^{mapk}

Antisera were elicited in rabbits immunized against C-terminal 14- or 16-amino-acid peptides corresponding to p42^{mapk} (α I,Cp42 or α II,Cp42) and p44^{mapk} (α I,Cp44 or α II,Cp44). Pre-immune sera (PSI or PSII Cp42 or Cp44) were run as controls. Solubilized mesangial cell extracts were immunoblotted as described in the Materials and methods section. (a) Typical immunoblot with the four antisera; (b) effects of pre-absorption of the antibodies with either Cp42 or Cp44.

Fig. 6. Immunoprecipitation of phosphorylated p42^{mapk}

α I,Cp42 was used to immunoprecipitate p42^{mapk} from ³²P-loaded cells and the proteins were separated by SDS/10%-PAGE as described in the Materials and methods section. The cells were stimulated with 0.1 μ M-ET-1 (lane 2) or 10% FBS (lane 3) for 5 min (lane 1, control).

Fig. 7. Kinetics of activation of p42^{mapk} by ET-1 in mesangial cells

Quiescent rat mesangial cells, stimulated with 10 μ M-ET-1 for the times indicated, were broken in lysis buffer and the soluble extracts were immunoprecipitated with antibody α I,Cp42 as described in the Materials and methods section. MBP kinase activity in immunocomplexes was analysed by SDS/10%-PAGE and quantified by cutting the MBP bands and measuring the activity.

MBP kinase activity. However, treatment of the cells with AlF_4^- , a direct activator of G-proteins, increased MBP kinase activity (Fig. 3). The time-course experiments showed that AlF_4^- increased MBP kinase activity 5 min after its addition, was maximal at 10 min, and persisted until 20 min. The activity returned to control levels 60 min after the addition of AlF_4^- (Fig. 3). We did not find a synergistic effect on AlF_4^- of ET-stimulated MBP kinase activity.

Regulation of ET-augmented MBP kinase activity

To evaluate the importance of protein tyrosine kinase activity *in vivo* in ET-augmented MBP kinase activity, we studied mesangial cells incubated with genestein, an inhibitor of tyrosine kinase (Akiyama *et al.*, 1987), together with ET-1. As shown in Fig. 4, incubation of the cells with ET-1 for 5 min in the presence of 40 μ M-genestein resulted in the inhibition of ET-stimulated MBP kinase activity. Next we investigated the role of PKC, which may regulate the ET enhancement of MBP kinase activity in the signal transduction pathways. Sangivamycin (0.1 μ M), an inhibitor of PKC, completely inhibited ET-augmented MBP kinase activity (results not shown). The PKC activator PMA at 0.1 mM dramatically increased MBP kinase activity, showing that PKC activation is sufficient to activate MBP kinase (Table 1). Furthermore, in cells in which PKC was down-regulated through treatment with 0.1 μ M-PMA for 24 h, the subsequent MBP kinase activation by PMA stimulation was reduced by about 40% and the ET-mediated MBP kinase activity was inhibited by 50% (Table 1). Genestein inhibited the responses to ET by approx. 40%, whereas the PMA-stimulated MBP kinase was unaffected (Table 1). These results indicate that both tyrosine kinase and PKC activities were involved in ET-stimulated MBP kinase in rat mesangial cells.

Identification of ET-stimulated MBP kinase

In order to confirm the identity of the mesangial cell MBP kinase as p42^{mapk} or p44^{mapk} (Boulton & Cobb, 1991), we generated polyclonal antibodies in rabbits using KLH-conjugated synthetic peptides as immunogens. Fig. 5(a) shows the immunoblots with the four antisera as well as the pre-immune sera. α I,Cp42, raised against the C-terminal 14 amino acids of p42^{mapk}, identified only a 42 kDa protein, whereas α II,Cp42 reacted with both 42 and 44 kDa proteins. α II,Cp44, raised against the C-terminal 16 amino acids of p44^{mapk}, recognized predominantly a 44 kDa protein, whereas α I,Cp44 immunoblotted 42 and 44 kDa proteins equivalently. Pre-immune sera (PSI or PSII) were negative. Fig. 5(b) summarizes the results of antibody pre-absorption with either Cp42 or Cp44 peptides. Both antisera against Cp42 were selectively absorbed by only Cp42 and not by Cp44, whereas both antisera against Cp44 were absorbed by either Cp44 or Cp42. Subsequent experiments used α I,Cp42 to characterize the ET-stimulated MBP kinase as p42^{mapk}.

We immunoprecipitated p42^{mapk} in ³²P-loaded cells using α I,Cp42. Both ET-1 at 0.1 μ M and 10% FBS significantly enhanced phosphorylation of mesangial p42^{mapk} (Fig. 6). Kinetic analysis of immunoprecipitated p42^{mapk} activity after exposure of mesangial cells to 0.1 μ M-ET-1 showed rapid activation, with peak activity after 5–10 min, and declined to control levels after 20 min (Fig. 7).

DISCUSSION

The vasoactive peptide ET exerts multiple biological effects on rat mesangial cells, including activation of phospholipase C and induction of expression of *c-fos* and *c-jun* (Simonson *et al.*, 1989, 1992). The intermediate steps between these responses have been

only partially explored. MAP kinases are serine/threonine kinases which are believed to occupy a pivotal position in mediating intracellular signals for many agonists, especially growth factors (Ray & Sturgill, 1987; Sturgill *et al.*, 1988; Hoshi *et al.*, 1988; Erickson *et al.*, 1990; Ahn *et al.*, 1990; Sanghera *et al.*, 1990; Tsao *et al.*, 1990; L'Allemain *et al.*, 1991a; Boulton *et al.*, 1991; Meloche *et al.*, 1992a). We therefore hypothesized that activation of MAP kinases may also be an important mechanism for ET post-receptor signalling. We assessed the effect of ET on MAP kinase activity in rat mesangial cells, and evaluated the role of G-proteins, PKC and tyrosine kinases in the coupling of the ET receptor to MAP kinase. We now report that ET activates MAP kinase, and that both PKC and tyrosine kinase are involved in the process of MAP kinase activation in rat mesangial cells.

Addition of ET to the cell culture rapidly stimulated a kinase activity which phosphorylated exogenous MBP, and this increase in activity was dose-dependent (Fig. 1). The time course of ET-augmented MBP kinase activity was similar to that reported for the phosphorylation of MAP kinase in insulin-stimulated 3T3-L1 adipocytes (Ray & Sturgill, 1987). We also compared the effects of ET-1 with those of thrombin and EGF on MBP kinase activity, and found that all of these agonists stimulated MBP kinase activity with a similar time course (Fig. 2). These profiles were reminiscent of the growing family of MAP/MBP kinases that have been reported from diverse sources.

We therefore attempted to determine if ET augmented an MBP kinase which was identical to one of the MAP kinases. The mesangial cell extracts were Western blotted using four different antibodies raised against synthetic peptides from the C-terminus of ERK1 and ERK2/MAP kinases (Boulton *et al.*, 1991) (Fig. 5a and 5b). Antibodies α I, Cp44 and α ICp42, raised against p44^{mapk} (ERK1), recognized both 44 and 42 kDa proteins, whereas antibody α I,CP42, directed against p42^{mapk} (ERK2), only detected the 42 kDa protein. Both antibodies were specific, as indicated by blockade with homologous peptide and pre-serum blotting results. The direct evidence that MAP kinase was responsible for the ET-augmented MBP kinase activity came from both immunoprecipitation and immunocomplex kinase assay experiments. Because antibody α I,CP42 was specific for the 42 kDa protein, we used it to immunoprecipitate ³²P-labelled cell extracts followed by autoradiography. As shown in Fig. 6, ET-1 and 10% FBS enhanced the phosphorylation of a 42 kDa protein which was immunoprecipitated by α I,CP42. Immunocomplex kinase assays using the same antibody showed that the immunocomplex contained MBP kinase activity after ET stimulation. The time course of activation of immunoprecipitated MBP kinase was similar to that of the whole cell extract kinase assay (compare Figs. 2 and 7). In subsequent experiments we have found that ET-1 induces immunocomplex p42^{mapk} activity 3–5 h after addition, although this second surge of kinase stimulation is always less than that observed at 5–10 min (Y. Wang, J. Pouyssegur & M. J. Dunn, unpublished work). Similar observations were reported for thrombin-stimulated MAP kinase activity in CCL39 cells (Meloche *et al.*, 1992a). We have thus concluded that ET stimulation can increase p42^{mapk} activity in rat mesangial cells. ET stimulation may also increase p44^{mapk} activity, but our experiments did not address this question.

We also studied the question of what links the ET receptor to MAP kinase activation. ET receptors are G-protein-linked, and several G-protein-linked agonists such as thrombin (L'Allemain *et al.*, 1991a) and 5-hydroxytryptamine (Meloche *et al.*, 1992b) have been found to activate MAP kinases. We thus investigated the potential involvement of G-proteins in ET signal transduction pathways leading to activation of MAP kinase, by using both pertussis toxin and AIF₄⁻. The stimulatory effect of ET-1 on

MAP kinase activity was not inhibited by pre-treatment of the mesangial cells with PT, compatible with a lack of involvement of the G_i or G_o families of pertussis toxin-sensitive G-proteins. Pertussis toxin has been proven to block the transfer of ADP-ribose to a 41–43 kDa protein as well as to decrease by 50% ET-stimulated phospholipase C activity in rat mesangial cells (Thomas *et al.*, 1991), substantiating that the protocol used to inactivate G_i/G_o was effective. AIF₄⁻ activates the α subunit of G-proteins independent of receptor stimulation or type of G-protein. AIF₄⁻ stimulated MBP kinase in rat mesangial cells (Fig. 3), thereby pointing to some coupling between G-proteins and MAP kinase. These results are in agreement with those of Anderson *et al.* (1991a), who showed that AIF₄⁻ stimulated MAP kinase in BC₃H1 myocytes.

It has been claimed that PKC is a principal mediator of the mitogen-stimulated tyrosine phosphorylation of pp42 (Cooper, 1989). Since PKC is also an important serine/threonine kinase in ET post-receptor signalling (Simonson & Dunn, 1990), we explored the role of PKC in ET stimulation of MAP kinase. Treatment of rat mesangial cells with PMA alone increased MAP kinase activity. A similar effect of phorbol esters has been reported in fibroblasts and myocytes (Hoshi *et al.*, 1988; Anderson *et al.*, 1991a). Furthermore, pre-treatment of the mesangial cells with PMA for 24 h, to deplete PKC, decreased by 40–50% the stimulation of MAP kinase activated by ET-1 or by PMA. The decrease in ligand-stimulated cellular responses following prolonged phorbol ester pre-treatment is regarded as evidence for the involvement of PKC in the signalling pathway of the ligand. These results thus indicate that PKC is one pathway (kinase–kinase) involved in the stimulation by ET of MAP kinase activity. This notion was supported by the fact that sangivamycin, an inhibitor of PKC, blocked ET-augmented MAP kinase activity *in vivo*. The partial inhibitory effect (40%) of PKC down-regulation on PMA-stimulated MBP kinase activity (Table 1) may be due to incomplete inhibition of different PKC isoforms by PMA pre-exposure in mesangial cells (Huwiler *et al.*, 1991). The partial reduction (50%) of ET-stimulated MBP kinase by PKC down-regulation seems to be attributable to the parallel activation of tyrosine kinases by ET (see below).

One invariable feature in the stimulation of MAP kinase is the requirement for phosphorylation on both tyrosine and threonine residues for full activation of the enzyme (Anderson *et al.*, 1990). The recent demonstrations that MAP kinase can undergo autophosphorylation on threonine residues, and surprisingly also on tyrosine residues, complicate the model of its activation, and the question of whether a specific tyrosine kinase is required to phosphorylate MAP kinase is open to debate (Seeger *et al.*, 1991; Wu *et al.*, 1991; Gomez & Cohen, 1991). Our results with the tyrosine kinase inhibitor genestein suggest that a tyrosine kinase upstream of MAP kinase is involved in ET-mediated MAP kinase activation. Indeed, genestein significantly reduced the degree of stimulation of MAP kinase by ET-1, but not by PMA, showing that genestein does not interfere at the level of MAP kinase activity, but rather upstream of it, in the ET signalling cascade. Another possibility is the existence of a genestein-sensitive pathway parallel to PKC in the ET-stimulated MAP kinase pathway. Recently, Force *et al.* (1991) reported that ET rapidly enhanced tyrosine phosphorylation of five cytosolic or membrane-associated proteins in rat mesangial cells, all of which were larger than 70 kDa and hence were not MAP kinases. It is therefore likely that both tyrosine kinase and PKC (serine/threonine kinase) activities are necessary for ET to activate MAP kinase. It is noteworthy that ET also augments S6 kinase activity in human vascular smooth muscle cells (Resink *et al.*, 1989). Since MAP kinase stimulation enhances S6 kinase in diverse cell types (Sturgill *et al.*, 1988; Ahn *et al.*, 1990), perhaps

ET stimulation of p42^{mapk} is followed by phosphorylation and activation of S6 kinase (p90^{rsk}) in the sequence of mitogenic events.

In summary, our results show that rat glomerular mesangial cells express both p42^{mapk} and p44^{mapk}. ET activates an MBP kinase which is, at least partially, p42^{mapk}. The signals, upstream from MBP kinase and responsible for phosphorylation and activation of the kinase in response to ET, include both PKC and tyrosine kinases. The co-ordinate actions of ET to enhance mesangial expression of *fos* and *jun* AP-1 transactivating factors (Simonson *et al.*, 1992) and to increase MAP kinase may be inter-related, as p42/p44^{mapk} phosphorylates and activates *c-jun* (Pulverer *et al.*, 1991).

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