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Transforming growth factor β_2 stimulates acute and chronic activation of the mitogen-activated protein kinase cascade in rat renal mesangial cells

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Abstract Exposure of rat glomerular mesangial cells to transforming growth factor β_2 (TGFβ_2) stimulates a biphasic mitogen-activated protein kinase (MAP kinase) activation. A rapid increase in activity (maximal at 10 min) is followed by a second persistent level of activity which steadily increases over 24 h. The second peak of MAP kinase activity is markedly attenuated by the protein synthesis inhibitor cycloheximide and consequently is paralleled by a pronounced de-novo synthesis of p42 and p44 MAP kinases as measured by immunoprecipitation of [^{35}S]methionine-labeled mesangial cells. In addition, an increased de-novo synthesis of MAP kinase kinase (MEK), the upstream activator of MAP kinase, is observed in response to TGFβ_2 stimulation. We propose that TGFβ-induced activation and de-novo synthesis of MAP kinases and MEK is important for the multifunctional actions of this cytokine in mesangial cells and its role in disease states characterized by excessive fibrosis.

Key words: Transforming growth factor β; Mitogen-activated protein kinase; Mitogen-activated protein kinase kinase; Mesangial cell; Cell proliferation

1. Introduction

The transforming growth factor β (TGFβ) family of molecules are small homo- and heterodimeric polypeptides originally purified from platelets [1], placenta [2] and kidney [3]. These multifunctional molecules are produced by neoplastic and normal cells, including macrophages and lymphocytes (for review see [4,5]). In the kidney, besides the presence of TGFβ, the existence of high-affinity TGFβ receptors on glomerular endothelial, epithelial and mesangial cells has been reported [6]. TGFβ increases the production of extracellular matrix components, especially of collagen and fibronectin by mesangial cells [6] and blunts mechanisms responsible for extracellular matrix degradation [7]. In addition, TGFβ suppresses interleukin 1- and tumor necrosis factor α-triggered expression of secretory phospholipase A_2 [8,9] and inducible nitric oxide synthase [10] in mesangial cells. Furthermore, TGFβ was shown to modulate mesangial cell proliferation in response to growth factor stimulation [6,11,12]. In murine mesangial cells, TGFβ acts as a growth promotor at higher cell densities and inhibits cell proliferation at lower cell densities [6]. However, in-vivo the proliferative effects of TGFβ dominates as shown recently by an in vivo transfection technique that allows to selectively overexpress TGFβ in the kidney [13].

Very little is known about the mechanisms of signal transduction by the different receptors for the TGFβ family. Mitogen-activated protein kinases (MAP kinases) are important intermediates in signal transduction pathways that are stimulated by many agents, including most if not all mitogens [14–16]. Activation of MAP kinases results from phosphorylation of adjacent tyrosine and threonine residues within MAP kinase by a dual specificity kinase termed MAP kinase kinase (MEK). We therefore set out to investigate whether TGFβ activates this important class of signaling enzymes thought to mediate mitogenic events within many cell types [14–16]. We report here for the first time that TGFβ induces a biphasic activation of MAP kinase activity. Most importantly, our data demonstrate the TGFβ causes a delayed de-novo synthesis of MAP kinases and MEK which may guarantee prolonged activity of this signaling cascade for cell proliferation.

2. Materials and methods

2.1. Chemicals

Protein A-Sepharose 4B-CL was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; [^{35}S]methionine (specific activity 55.5 Bq/mM) was from Amersham International, Zürich, Switzerland; recombinant human TGFβ_2 was kindly provided by Drs. Nico Cerletti, David Cox and Garry McMaster, Ciba-Geigy Ltd., Switzerland; all cell culture nutrients were from Gibco-BRL, Basel, Switzerland; all other chemicals were from either Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

2.2. Cell culture

Rat renal mesangial cells were cultivated as described previously [10]. 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 units/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 $InsP_3$ upon activation of angiotensin II AT_1-type receptor [17] was used as a functional criterion for characterizing the cloned cell line. For the reported experiments, passages 8–19 of mesangial cells were used.

2.3. 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 IFEETARFQPGYRS for p42^{mapk}) were synthesized, coupled to keyhole-limpet haemocyanin, and used to immunize rabbits. The anti-p44^{mapk} antiserum and the anti-p42^{mapk} antiserum recognized specially the p44-isoform and the p42-isoform, respectively. A second antiserum which

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specifically recognized the p42-isoform was kindly provided by Dr. M.J. Dunn, Cleveland [18]. For MEK a synthetic peptide based on the N-terminal sequence of murine MEK-1 was synthesized and coupled to keyhole-limpet haemocyanine and used to immunize rabbits. The sequence of the peptide was CPKKKPTPIQLNPFAPDG.

2.4. Cell labeling and immunoprecipitation

Confluent mesangial cells in 100 mm-diameter dishes were washed with PBS and incubated in methionine-free DMEM in the absence or presence of the stimulators for the indicated time periods. For the last 4 h of incubation [^{35}S]methionine was added (100 μCi/plate). After labeling, cells were washed twice 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 sodium fluoride/10 mM sodium pyrophosphate/200 μM sodium orthovanadate/10 μg of leupeptin/ml/200 U of aprotinin/ml/1 μM pepstatin A/1 mM phenylmethanesulphonyl fluoride) and homogenized with 10 passes through a 26-gauge needle fitted to a 1-ml syringe. The homogenate was centrifuged for 10 min at 14,000 × g and the supernatant taken for immunoprecipitation. Samples of 1 ml volume, containing 100 × 10^6 cpm of labelled proteins, 5% fetal calf serum and 1.5 mM iodoacetamide in lysis buffer, were incubated for 4 h at 4°C. Then 100 μl of a 50% slurry of protein A-Sepharose 4B-CL in PBS was added and the mixture was incubated for 1.5 h at room temperature under mild shaking. After centrifugation for 5 min at 3,000 × g immunocomplexes were washed 3 times with 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), 3 times with 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 μl Laemmli dissociation buffer and subjected to SDS-PAGE (10% acrylamide gel). After fixing in 25% isopropanol, 10% acetic acid, the gels were soaked for 30 min in 16% sodium salicylate solution and then dried and exposed to Hyperfilm MP at −70°C using two intensifying screens.

2.5. 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 homogenized in 1 ml of icecold lysis buffer. For immunoprecipitation 50 μg of cell extracts were incubated overnight at 4°C with an antiserum reactive with $p42^{mapk}$ in a dilution of 1:200. All further steps were performed following the immunoprecipitation protocoll described above. Finally the beads were incubated for 15 min at 30°C in 50 μl of kinase buffer including 20 μg myelin basic protein, 20 μM ATP and 4 μCi [γ-^{32}P]ATP. The reaction was terminated by adding 40 μl Laemmli dissociation buffer and subjected to SDS-PAGE (13% acrylamide gel). After fixing in 25% isopropanol, 10% acetic acid, the gels were exposed to Hyperfilm MP for 3–5 h or evaluated by Phosphor imaging.

3. Results

TGFβ_2 is a potent stimulator of MAP kinase activity in mesangial cells and induces a concentration-dependent activation of the enzyme as shown in Fig. 1. Maximal stimulation is observed at 10 ng/ml TGFβ_2 and half-maximal activity is obtained at 0.42 ng/ml TGFβ_2. Treatment of quiescent mesangial cells with 25 ng/ml TGFβ_2 triggers a biphasic activation of the enzyme as shown in Fig. 2. An early peak of activation is observed at 10 min after exposure of cells to TGFβ_2 and is followed by a second increase in MAP kinase activity which steadily increases over 24 h (Fig. 2A and B). The degree of activation of MAP kinase is paralleled by an increased phosphorylation of p42 and p44 MAP kinases as determined by immunoprecipitation of p42 and p44 MAP kinases from [^{32}P$_i$]-labelled mesangial cells (data not shown). Inhibition of protein synthesis by cycloheximide (10 μM) has no effect on the rapid first phase of MAP kinase activation but it markedly attenuates

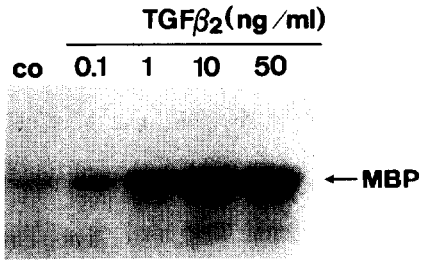

Fig. 1. Concentration dependence of TGFβ_2-stimulated p42 MAP kinase activity in mesangial cells. Mesangial cells were treated for 10 min with the indicated concentrations of TGFβ_2. Cells were then lysed and cell extracts were immunoprecipitated with a specific anti-p42 MAP kinase antiserum at a dilution of 1:200. Myelin basic protein kinase activity in immunocomplexes was analyzed by SDS-PAGE (13% acrylamide gel) and autoradiography. Bands were quantified by Phosphor Imaging.

the second peak of kinase activity as shown in Fig. 2B, thus suggesting that protein synthesis is required for sustained MAP kinase activity. To study whether TGFβ_2 has an effect on de-novo synthesis of MAP kinase, mesangial cells were stimulated with TGFβ_2 (25 ng/ml) for different time periods and

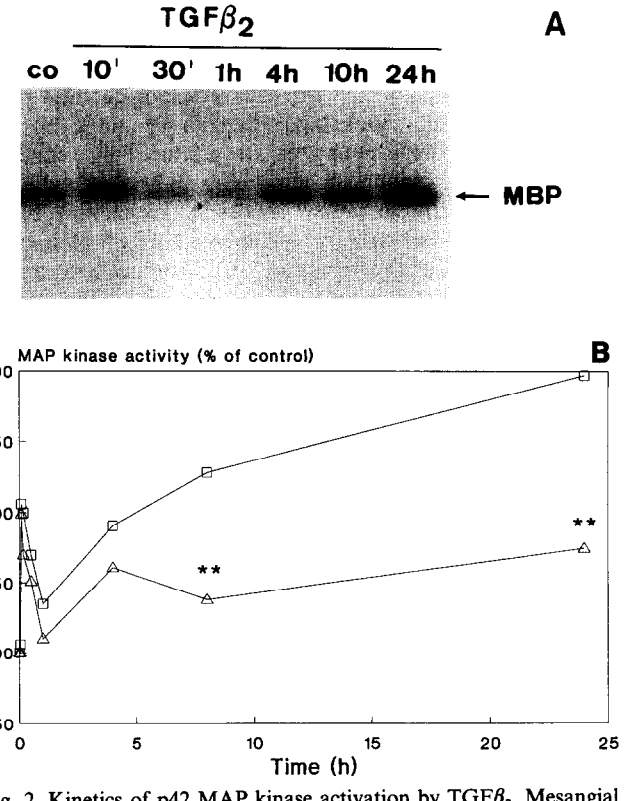

Fig. 2. Kinetics of p42 MAP kinase activation by TGFβ_2. Mesangial cells were treated with TGFβ_2 (25 ng/ml) either in the presence (△) or absence (□) of cycloheximide (10 μM) for the indicated time periods. Cells were then lysed and cell extracts were immunoprecipitated with a specific anti-p42 MAP kinase antiserum at a dilution of 1:200. Myelin basic protein kinase activity in immunocomplexes was analysed by SDS-PAGE (13% acrylamide gel) and autoradiography. A typical experiment (without cycloheximide) is shown in (A). Bands were quantified by Phosphor Imaging and the data in (B) are means of three independent experiments. Significant differences from corresponding stimulation with TGFβ in the absence of cycloheximide: $^{**}P < 0.01$; ANOVA.

[^{35}S]methionine was added for the last 4 h of the stimulation period. p42 MAP kinase was immunoprecipitated with a specific polyclonal antibody and subject to SDS-PAGE. As shown in Fig. 3 only a faint band of p42 MAP kinase labelling is detected in unstimulated cells. Addition of TGFβ_2 dramatically upregulates biosynthesis of the enzyme, an effect that is maximal at 6 h after exposure of the cells to the cytokine and remains at high levels for at least 24 h (Fig. 3). Comparable data were obtained for p44 MAP kinase (data not shown). A pronounced effect of TGFβ_2 is also detected for MEK (Fig. 4). Already under control conditions a strong incorporation of label into MEK is observed, suggesting a higher turn-over rate of MEK as compared to p42 MAP kinase. Addition of TGFβ_2 results in a potent increase in MEK biosynthesis after 4–6 h (Fig. 4).

4. Discussion

TGFβ is a multifunctional cytokine regulating quite diverse cellular activities including differentiation, extracellular matrix production and cell proliferation. Three mammalian isoforms TGFβ_1, TGFβ_2 and TGFβ_3 initiate these responses by binding with high affinity to several distinct receptors [4,5]. A heteromeric receptor complex composed of both type I and type II receptors is essential for TGFβ signal transduction. Both the type I as well as the type II receptor for TGFβ have intracellular serine/threonine protein kinase domains. However, aport from these kinase domains of the TGFβ receptors very little is known about the signalling events that mediate gene expression in cells exposed to TGFβ. TGFβ does not activate phosphoinositide turnover, Ca^{2+} mobilization, protein kinase C activation or Na^+/H^+ antiport stimulation [5]. In mink lung epithelial cells the antiproliferative action of TGFβ is overcome by microinjection of oncogenic Ha-ras [19]. In contrast, under different conditions, TGFβ results in a rapid activation of ras proteins in rat intestinal epithelial cells [20] and a colon carcinoma cell line [21]. Ras is a common signalling element that is known to activate the MAP signalling cascade and thereby triggers cell proliferation in many cell types [14–16]. We therefore hypothesized that TGFβ might activate MAP kinase activity in mesangial cells. Our data are the first report of p42 MAP kinase activation in the signal transduction pathway of TGFβ_2. Moreover, we demonstrate for the first time an increased de-novo synthesis of p42 MAP kinase and MEK in response to TGFβ_2 stimulation. A coordinate, biphasic stimulation of p44 MAP kinase has also been observed in hamster fibroblasts exposed to thrombin and is paralleled by a biphasic increase in ribosomal S6 kinase activity [22]. The authors, however, did not check whether the second peak of MAP kinase activity is due to increased de-novo synthesis of the enzymes as shown here for mesangial cells. Recently Wang et al. [23] reported that endothelin 1 biphasically stimulates p42 MAP kinase activity in mesangial cells. In contrast to our data with TGFβ_2, cycloheximide does not inhibit endothelin 1 activation of p42 MAP kinase neither at early nor at late points [23]. Moreover, we have reported that interleukin 1 stimulates MAP kinase activation and de-novo synthesis of p42 MAP kinase in a manner comparable to TGFβ_2 [24]. Taken together, these data suggest that whereas cytokines such as TGFβ_2 or interleukin 1 are able to trigger translational induction of MAP kinase, G-protein coupled receptor agonists such as endothelin 1 cause long-term activation of MAP kinase by phosphorylation rather than by gene expression and protein synthesis. The increased de-novo synthesis of MAP kinases and MEK reported in this and another report [24] may be of crucial importance for cytokine-induced activation of MAP kinase cascade responsible for initiating G_0 to S-phase transition or other long-term actions of cytokines in renal mesangial cells. Additional studies are required to unravel the mechanism of increased biosynthesis of MAP kinases and MEK in mesangial cells and to evaluate its pathophysiological relevance.

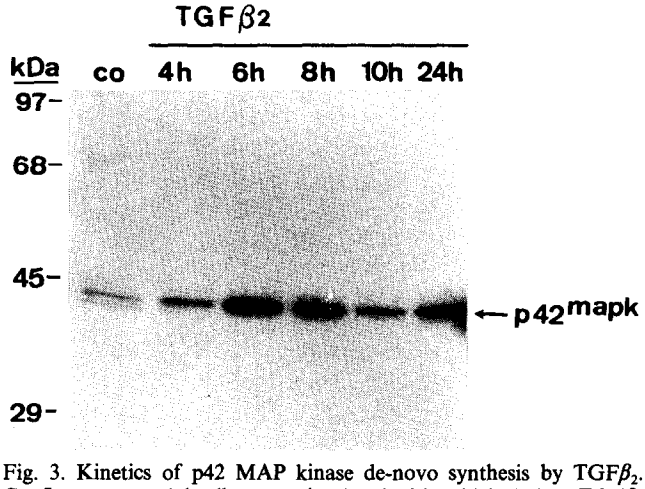

Fig. 3. Kinetics of p42 MAP kinase de-novo synthesis by TGFβ_2. Confluent mesangial cells were stimulated with vehicle (co) or TGFβ_2 (25 ng/ml) for the indicated time periods and incorporatation of [^{35}S]methionine was measured by immunoprecipitation of p42 MAP kinase (p42^{mapk}) and subsequent SDS-PAGE analysis as described in section 2. Similar results were obtained in three independent experiments.

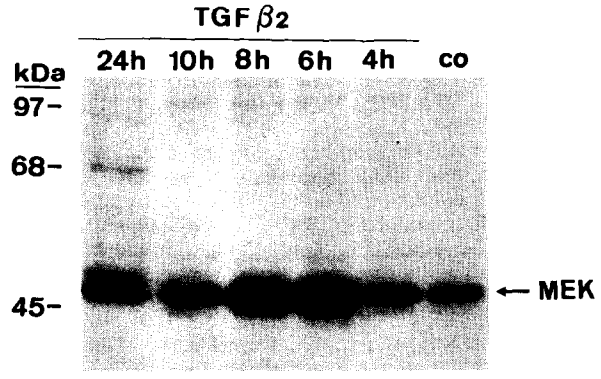

Fig. 4. Kinetics of MEK de-novo synthesis by TGFβ_2. Confluent mesangial cells were stimulated with vehicle (co) or TGFβ_2 (25 ng/ml) for the indicated time periods and incorporation of [^{35}S]methionine was measured by immunoprecipitation of MEK and subsequent SDS-PAGE analysis as described in section 2. Similar results were obtained in three independent experiments.

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