Lysophosphatidic acid-mediated signal-transduction pathways involved in the induction of the early-response genes prostaglandin G/H synthase-2 and Egr-1: a critical role for the mitogen-activated protein kinase p38 and for Rho proteins

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During inflammatory processes of the kidney, lesions of the glomerulus lead to aggregation of thrombocytes and infiltration of macrophages, which can release bioactive mediators. One of these important signalling molecules is lysophosphatidic acid (LPA). Incubation of rat mesangial cells with LPA induced mRNA and protein expression of the early-response genes *pghs-2* (for prostaglandin G/H synthase-2/cyclo-oxygenase-2) and *egr-1*. As shown by antisense experiments, induction of *egr-1* was related to the strong mitogenic effect of LPA. LPA-mediated gene expression was inhibited by pertussis toxin, indicating coupling to G-proteins of the G_i family. Specific inhibition of proteins of the small G-protein subfamily Rho with toxin B from *Clostridium difficile* led to changes in mesangial cell morphology without induction of apoptosis. LPA-mediated expression of *pghs-2* and *egr-1* was reduced to base-line levels by toxin B,

indicating a role for Rho proteins in LPA-mediated gene induction. Of the two mitogen-activated protein kinase (MAPK) pathways investigated, the MAPK kinase-extracellular signal-regulated kinase pathway was involved in the induction of both *pghs-2* and *egr-1* mRNA expression, as shown by the inhibitory effect of PD98059. Activation of the MAPK p38, however, was only related to *pghs-2* expression, whereas *egr-1* expression was not affected by treatment of mesangial cells with the specific inhibitor SB203580. Taken together our data provide evidence that LPA-mediated activation of MAPK kinase and Rho proteins leads to the induction of the functionally distinct early-response genes *pghs-2* and *egr-1*, whereas activation of MAPK p38 revealed considerable differences between the regulation of these two genes.

INTRODUCTION

The cellular messenger molecule lysophosphatidic acid (LPA, 1-acyl-*sn*-glycerol 3-phosphate) is a naturally occurring glycerophospholipid that elicits growth-factor-like cellular responses. LPA is rapidly released from activated human platelets [1]. In mammalian serum, LPA concentrations are in the range of 2–20 μ M, with oleoyl- and palmitoyl-LPA being the predominant species [1,2]. LPA evokes a diverse range of physiological responses, including mitogenesis, smooth-muscle contraction, cell adhesion and changes in the actin cytoskeleton. Most of these data have been obtained in fibroblasts (for reviews see [3,4]). There are indications of an LPA plasma-membrane receptor [5], and cross-linking studies revealed LPA-binding membrane proteins [6]. Two groups reported the isolation of a cDNA clone encoding a functional LPA receptor [7,8], but the predicted amino acid sequences were remarkably different. Several studies indicate that the putative LPA receptor couples to G-proteins of the subfamilies G_i , G_q and $G_{12/13}$ (review in [4]). Downstream of G_i proteins there is the Ras-mitogen-activated protein kinase (MAPK) pathway, and both G_q and $G_{12/13}$ appear

to be related to the GTPase Rho [3,9]. These two branches of LPA signal transduction have been implicated in cell proliferation and gene transcription of the immediate-early gene *c-fos* [10–13]. Regulation of other early-response genes that might be involved in the physiological responses induced by LPA has not been investigated in detail. Therefore, we studied the effect of LPA on the expression of prostaglandin G/H synthase-2 (*pghs-2*; cyclo-oxygenase-2) and of the early growth response gene-1 (*egr-1*).

pghs-2 is classified as an immediate-response gene due to its protein-synthesis-independent rapid inducibility by various stimuli [14]. It is functionally related to pathophysiological conditions that are characterized by an enhanced prostanoid synthesis, especially in inflammatory processes [15]. *egr-1* is rapidly and transiently induced in response to multiple mitogenic signals [16,17]. In renal mesangial cells *Egr-1* is an essential component of the mitogenic signal-transduction cascade [18].

In order to characterize further the signalling pathways that induce immediate-response genes in the context of inflammatory processes of the kidney, we investigated the LPA-mediated induction of *pghs-2* and *egr-1* mRNA expression in cultured rat mesangial cells. We report that components of the Ras pathway

Abbreviations used: Egr-1, early growth response gene-1; FCS, fetal-calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PA, phosphatidic acid; LPA, lysoPA; MAPK, mitogen-activated protein kinase; MEK1, MAPK/extracellular signal-regulated kinase kinase; PGHS-2, prostaglandin G/H synthase-2; PI 3-K, phosphatidylinositol 3'-kinase; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PTX, pertussis toxin; 5-HT, serotonin; DMEM, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor.

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and the GTPase Rho are necessary for *pghs-2* and *egr-1* induction in renal mesangial cells, whereas the MAPK p38 is only essential for *pghs-2* induction, revealing a considerable difference between the regulation of these early-response genes.

MATERIALS AND METHODS

Materials

LPA, dimyristoylphosphatidic acid, phosphatidic acid (PA) from egg yolk, fura 2-acetoxymethyl ester and serotonin (5-HT) were from Sigma (Deisenhofen, Germany), pertussis toxin (PTX) and wortmannin were from Biomol (Hamburg, Germany). PD 98059, SB203580, U73122 and U73343 were purchased from Calbiochem (Bad Soden, Germany). Cell culture media consisted of Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin and streptomycin (Biochrom, Berlin, Germany), fetal-calf serum (FCS) (Gibco, Eggenstein, Germany) and bovine insulin (Sigma). All other chemicals were purchased from Merck (Darmstadt, Germany). Toxin B from *Clostridium difficile* was isolated as described previously [19].

Cell culture

Rat mesangial cells were isolated according to the method of Lovett and Sterzel [20] and were grown in DMEM supplemented with 2 mM L-glutamine, 5 µg/ml insulin, 4.5 g/l glucose, 100 units/ml penicillin and 100 µg/ml streptomycin containing 10% (v/v) FCS. Mesangial cells [(0.5–1.0) × 10⁶ cells/10 ml] were plated in 100-mm-diameter petri dishes in medium with 10% (v/v) FCS. At subconfluency (after 3–4 days) cells were serum starved in DMEM containing 0.5% (v/v) FCS for 3 days.

Cell viability

Viable cell number was determined by Trypan Blue staining. Trypsinized cells were treated with 1 vol. of 0.05% (w/v) Trypan Blue/PBS and checked for blue staining, which is a marker for non-viable cells. Determination of apoptosis was done by Hoechst staining with bisbenzimidazole H 33258 (Hoechst 33258) according to the method of Labarca and Paigen [21].

DNA synthesis

For determination of [³H]thymidine uptake, mesangial cells were serum deprived for 2 days in serum-free DMEM. Cells were then stimulated with LPA for 24 h. [³H]Thymidine (2 µCi/ml; 2 Ci/mmol; ICN, Meckenheim, Germany) was added for the last 4 h of the incubation time. Then the cells were washed with PBS, treated with 0.05% (w/v) trypsin/0.02% (w/v) EDTA and harvested by a cell harvester. Incorporation of [³H]thymidine was quantified by liquid-scintillation counting for radioactivity. For proliferation-inhibition experiments, phosphothioate-modified *egr-1*-specific antisense and sense oligodeoxynucleotides (MWG Biotech, Ebersberg, Germany) dissolved in H₂O were used. Oligonucleotides (final concentration 1 µM) were supplied 16 h before stimulation of mesangial cells. The sequences of the oligonucleotides were as follows [18]: antisense AS: 5'-CGG CCT TGG CCG CTG CCA T-3'; and sense S: 5'-ATG GCA GCG GCC AAG GCC G-3'.

Northern-blot analysis

Northern-blot analysis was performed as described previously [22]. After stimulation for the indicated times, total RNA was extracted according to the protocol of Chomczynski and Sacchi [23], with minor alterations. Usually, RNA yield was about 30–40 µg/10-cm-diameter petri dish. Separation of total RNA

(10 µg/lane) was achieved by the use of 1.2% agarose gels containing 1.9% (v/v) formaldehyde with 1 × Mops as gel running buffer. Separated RNA was transferred to nylon membranes by capillary blotting and fixed by baking at 80 °C for 2 h.

Hybridization was performed with cDNA probes labelled with [³²P]dCTP, using the Megaprime random prime kit (Amersham, Braunschweig, Germany) as described previously [22]. The specific *pghs-2* probe was a 1.156 kb *EcoRI* fragment from the 5' end of mouse cDNA [22]. The length of the *pghs-2* mRNA recognized was 5.0 kb. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was obtained with a 500 bp reverse-transcribed fragment [22]. The probe specific for *egr-1* was a 3.1 kb full-length cDNA [17]. Blots were prehybridized for at least 2 h at 40 °C. The probes were allowed to bind overnight at the same temperature. Washing at 40 °C was carried out 2 × 15 min in high salt [2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% (w/v) SDS] and 1 × 15 min in low salt (0.2 × SSC/0.1% SDS) conditions. DNA–RNA hybrids were detected by autoradiography using Kodak X-Omat AR film. Quantitative analysis was performed by densitometric scanning of the autoradiographs (Froebel, Wasserburg, Germany). All values were corrected for differences in RNA loading by calculating the ratio of *pghs-2* or *egr-1* to GAPDH expression, unless otherwise indicated.

Western-blot analysis

Mesangial cells were grown in 3.5-cm-diameter dishes until subconfluency, growth arrested for 3 days in DMEM containing 0.5% (v/v) FCS, stimulated for the indicated times, rinsed twice with ice-cold PBS, and then incubated for 20 min at 4 °C in 120 µl of lysis buffer [50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium desoxycholate, 0.1% (w/v) SDS, 1 mM PMSF and 14 µg/ml aprotinin]. Lysates were incubated for 15 min on ice with vortexing and then centrifuged at 15000 g for 20 min to collect supernatants. Total protein (15–20 µg) was separated by SDS/PAGE (8% polyacrylamide) and electroblotted on to fluorotransfer membrane (Pall Biosupport Division Dreieich, Germany). Blots were incubated in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 3% (w/v) BSA to block non-specific binding, washed in Tris-buffered saline containing 0.1% Tween 20, and incubated with the primary anti-Cox-2 (Santa Cruz, Heidelberg, Germany) or anti-Egr 1 antibody described elsewhere [18]. PGHS-2 and Egr-1 proteins were revealed using the enhanced-chemiluminescence system (Amersham).

Determination of intracellular Ca²⁺ levels

Intracellular Ca²⁺ levels were determined as described previously [24]. Serum-deprived cells were washed twice with PBS and trypsinized with 0.05% (w/v) trypsin/0.02% (w/v) EDTA for 10 min. Trypsination was stopped by addition of an equal volume of medium supplemented with 10% (v/v) FCS. After centrifugation cells were resuspended in medium containing 10% FCS (10⁷ cells/ml) and incubated in a water bath (37 °C, 5 min). Fura 2-acetoxymethyl ester was added (final concentration 4 µM), and incubation was carried on for 15 min. Then the cell suspension was diluted fivefold with medium complemented with 10% FCS, and incubation was continued for another 15 min. Afterwards the cells were washed once with assay buffer (10 mM Hepes, pH 7.4, 1 mM NaH₂PO₄, 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂ and 5.5 mM glucose), and resuspended in assay buffer at 10⁶ cells/ml. During the experiments cells were kept at room temperature. Fluorescence measurements were performed using a Kontron

SFM25 fluorimeter. Cells were placed in a cuvette holder equipped with a magnetic stirrer. The excitation wavelength was set to 340 nm, and emission was monitored at 510 nm. Maximum and minimum fluorescence of fura-2 were determined by lysing the cells with Triton X-100 (0.1 %, v/v) and by adding EGTA (60 mM). The k_d of fura 2 for Ca^{2+} was assumed to be 225 nM [25].

RESULTS

Mitogenic effect of lysophosphatidic acid

The mitogenic effect of LPA was assessed by [^3H]thymidine incorporation during DNA synthesis. The dose-response curve for LPA in rat mesangial cells, shown in Figure 1, demonstrates that LPA is a potent stimulus for DNA synthesis. Half-maximal stimulation was observed at a concentration of 10 μM , whereas saturation of the mitogenic effect was seen between 25 and 50 μM LPA. At 10 μM stimulation of [^3H]thymidine incorporation was about 6-fold; at 50 μM it was 18-fold over control values. Comparable stimulation could be detected at 100 μM LPA in contrast with an inhibitory effect described by Gaits et al. [26]. Knauss et al. [27] emphasized that LPA and PA are similarly effective as endogenous mitogens in rat mesangial cells. Unlike these results, we detected only a small stimulation with dimyristoyl-PA or PA from egg yolk (40 μM , about 2.5–3.0-fold stimulation; results not shown).

LPA-stimulated Ca^{2+} mobilization

In a great variety of cell types LPA activates phosphoinositide-specific phospholipase C (PI-PLC) with subsequent mobilization of intracellularly stored Ca^{2+} . To characterize the LPA-mediated signal transduction in mesangial cells, we examined the effect of LPA on Ca^{2+} mobilization in suspended cells loaded with the fluorescent Ca^{2+} chelator fura 2 (Figure 2). Comparable results were obtained during single-cell measurements with adherent mesangial cells (results not shown). In unstimulated mesangial cells, the average basal value of the intracellular Ca^{2+} concentration was estimated to be 98 ± 18 nM ($n = 15$). LPA (1 μM)

Figure 1 LPA dose dependently stimulates mesangial cell growth

Subconfluent cultured rat mesangial cells were growth arrested and stimulated with increasing concentrations of LPA (1–100 μM) for 24 h. [^3H]thymidine was added for the last 4 h of the incubation time. Error bars indicate S.D. of four independent experiments.

Figure 2 Ca^{2+} signalling in fura 2-loaded mesangial cells

Traces shown are representative for the effect of LPA on the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in fura 2-loaded mesangial cells. Upper trace, cells were preincubated for 18 h in the presence or absence of PTX (100 ng/ml) and then stimulated with 100 nM LPA. Middle trace, mesangial cells were preincubated with the PI-PLC inhibitor U73122 (10 μM) and the inactive analogue U73343 (10 μM) for 15 min and then treated with 100 nM LPA. Lower trace, mesangial cells were incubated with 100 nM LPA and 1 μM 5-HT as indicated.

Figure 3 LPA-mediated induction of *pghs-2* and *egr-1* mRNA

Mesangial cells were incubated with 10 μM or 25 μM LPA for 15, 30, 60, 120 and 240 min; control, medium control without LPA; co, $t = 0$. The Northern blot was probed for *pghs-2*, *egr-1* and GAPDH and is representative of five independent experiments.

Table 1 Inhibition of mesangial cell growth by an antisense oligonucleotide (Oligo) directed against *egr-1*

Serum-starved mesangial cells were incubated with a phosphothioate-modified antisense oligonucleotide (AS) or the respective sense (S) control oligonucleotide directed against *egr-1* (both 1 μ M) for 16 h before LPA stimulation (10 μ M, 24 h). [3 H]Thymidine uptake was determined for the last 4 h of the incubation. Data shown are means $\pm$ S.D. of three independent experiments. —, not done.

Stimulus	Oligo ...	Incorporated [3 H]thymidine (c.p.m.)		
		None	AS	S
Control		650 $\pm$ 160	—	—
LPA		3037 $\pm$ 405	1068 $\pm$ 250	2500 $\pm$ 350

resulted in a rapid increase of fluorescence within 20–30 s. Baseline values were reached after 1.5–2 min. Only in a few experiments was a biphasic Ca^{2+} response with a slight plateau detected. A homologous desensitization of LPA-induced Ca^{2+} release was observed, similar to that described by Inoue et al. [28]. Desensitization was not seen when the different G-protein-coupled agonists 5-HT and LPA were used for stimulation independent of the order of addition (Figure 2, lower panel), indicating independent signal pathways. Preincubating mesangial cells with the PI-PLC inhibitor U73122 completely abolished the LPA-mediated Ca^{2+} signal, whereas the inactive analogue U73343 had only marginal effects (Figure 2, middle panel). It has been reported that G-proteins, which couple to the LPA receptor, are of the G_q , $\text{G}_{12/13}$ and G_i types [9]. Ca^{2+} signalling was completely suppressed by preincubating mesangial cells for 18 h with 100 ng/ml PTX, suggesting the involvement of G_i proteins (Figure 2, upper panel). The effect of LPA on peak Ca^{2+} responses was concentration dependent. The lowest concentration of LPA to elicit a calcium signal was 500 pM. A maximal increase in intracellular Ca^{2+} was obtained at 100 nM–1 μ M LPA; at higher concentrations a saturation effect was observed (results not shown).

Induction of *egr-1* by LPA

We and others could demonstrate a critical involvement of *egr-1* in the regulation of mesangial cell proliferation [16,17] and a strong correlation between the induction of *egr-1* and the proliferative response to platelet-derived growth factor (PDGF), serotonin, arginine-vasopressin and endothelin-1 [17,18]. Incubation of mesangial cells with LPA transiently induced *egr-1* mRNA expression. Maximum stimulation was reached between 30 and 60 min and dropped to the basal value after 120 min (Figure 3). We have shown recently that antisense oligonucleotides directed against *egr-1* effectively inhibited serum-induced *egr-1* mRNA and protein expression. Sense, scrambled and mismatched oligonucleotides served as controls for non-specific effects and had no significant impact on *egr-1* mRNA and protein levels [18]. To clarify the significance of *egr-1* expression for LPA-induced proliferation, we preincubated serum-deprived mesangial cells for 16 h in the presence of 1 μ M antisense or sense oligonucleotides directed against *egr-1* and measured the LPA-mediated stimulation (10 μ M) of DNA synthesis by [3 H]thymidine incorporation. Incubation with *egr-1* antisense oligonucleotides resulted in a significant inhibition (65%) of LPA-mediated proliferation in comparison with sense *egr-1* oligonucleotides (Table 1).

Figure 4 Inhibition of LPA-mediated induction of *pghs-2* and *egr-1* mRNA by PTX

Mesangial cells were preincubated with PTX (100 ng/ml) for 18 h and then stimulated with LPA (L, 10 μ M), 5-HT (S, 1 μ M) or co-stimulated with LPA and 5-HT (L/S) for 1 h. As a control, RNAs from mesangial cells not treated with PTX are shown. Abbreviation: C, control, $t = 0$. A representative Northern blot is shown.

Induction of *pghs-2* by LPA

Activated mesangial cells have been shown to contribute to glomerular inflammation by liberation of multiple mediators, e.g. prostaglandins. We investigated the effect of LPA on the expression of one of the key enzymes of prostaglandin synthesis, PGHS-2. Incubation of mesangial cells with LPA resulted in a transient induction of *pghs-2* mRNA with a maximum between 60 and 120 min (Figure 3). The first signal was seen after 30 min; at 4 h *pghs-2* mRNA was no longer detectable. We observed no correlation between the induction of *pghs-2* and the proliferation of mesangial cells, since inhibition of *pghs-2* with the specific cyclo-oxygenase-2 inhibitor NS-398 (up to 10 μ M) did not influence LPA-stimulated DNA synthesis monitored by [3 H]thymidine incorporation (results not shown).

Characterization of LPA-induced *egr-1* and *pghs-2* mRNA induction

Preincubation of mesangial cells with PTX (100 ng/ml, 18 h) completely blocked LPA-mediated *egr-1* and *pghs-2* mRNA induction (Figure 4). This result indicates a role for a G_i -protein in LPA-mediated gene expression. 5-HT-induced *pghs-2* and *egr-1* mRNA induction is mediated via 5-HT $_2A$ receptors, which are coupled to PTX-insensitive G-proteins of the G_q family [29]. For this reason we used 5-HT as control for a G_q -coupled receptor (Figure 4). Co-stimulation of mesangial cells with 5-HT (1 μ M) and LPA (10 μ M) resulted in additive signals of *pghs-2* and *egr-1* mRNA compared with incubation with one component alone. When cells were preincubated with PTX before co-stimulation, only the serotonin-mediated signal remained.

In addition to other kinases, one possible G_i -Ras signalling pathway implies involvement of phosphatidylinositol 3-kinase (PI 3-K), which seems to be an early intermediate in $\text{G}\beta\gamma$ -mediated MAPK signalling [30]. Since wortmannin, a specific PI 3-K inhibitor, did not alter LPA-mediated *pghs-2* or *egr-1* mRNA induction over a concentration range of 1 nM–10 μ M (incubation time 1 h), PI 3-K is probably not involved in this pathway (results not shown).

MAPKs have been described to be involved in LPA signalling. The flavone derivative PD 98059 selectively inhibits MAPK/

Figure 5 Involvement of MEK in LPA-mediated induction of *pghs-2* and *egr-1* mRNA

Mesangial cells were preincubated with PD 98059 (PD, 10 μ M) for 1 h and then stimulated with 10 μ M LPA for the indicated times in the presence of DMSO, used as solvent for PD 98059. Data are representative of two independent experiments.

Figure 6 Toxin B (Tox B) inhibits LPA-mediated induction of *pghs-2* and *egr-1* mRNA expression

Mesangial cells were preincubated for 3 h with increasing concentrations of toxin B (0.1–10 ng/ml) and then stimulated with 10 μ M LPA for 1 h. Abbreviations: co (first lane), $t = 0$, co (second lane), $t = 60$ min without LPA. The Northern blot shown is representative of four experiments.

Figure 7 Toxin B (Tox B) inhibits LPA-mediated induction of PGHS-2 and Egr-1 protein expression

Mesangial cells were preincubated for 3 h with 1 or 5 ng/ml toxin B and then stimulated with 10 or 25 μ M LPA, respectively, for 3 h. Expression of PGHS-2 and Egr-1 protein was detected by Western-blot analysis. Protein (15 μ g) from each sample was resolved by SDS/PAGE (8% polyacrylamide) and immunoblotted with PGHS-2- or Egr-1-specific antibodies. As a control, protein from mesangial cells incubated only with toxin B is shown. Similar results were obtained in three experiments.

Figure 8 The MAPK p38 is involved in LPA-mediated induction of PGHS-2 but not in the induction of Egr-1 protein expression

Mesangial cells were preincubated for 30 min with 1 or 5 μ M of the p38 inhibitor SB203580 and stimulated with 10 μ M LPA for 3 h subsequently. PGHS-2 and Egr-1 proteins were detected by Western-blot analysis (see the legend to Figure 7). As a control, protein from mesangial cells not incubated with LPA is shown. Abbreviations: D, DMSO (solvent for SB203580) control.

extracellular signal-regulated kinase kinase (MEK1) by binding to the inactive form of MEK1 and preventing activation by upstream proteins such as c-Raf [31]. PD 98059 (10 μ M, 1 h) nearly completely abolished *egr-1* mRNA expression (about 90% inhibition). *pghs-2* mRNA induction was clearly decreased (about 50% inhibition), but did not reach basal values (Figure 5).

Different models for LPA signalling propose an important role of a coupled PI-PLC–Rho pathway [3,32]. We investigated whether these components were also involved in LPA-mediated gene expression of *pghs-2* and *egr-1*. Mesangial cells were preincubated with the PI-PLC inhibitor U73122 and the inactive analogue U73343 (1 h, 10 μ M). Neither *pghs-2* nor *egr-1* expression was significantly influenced by these inhibitors, showing that activation of PI-PLC is not necessary for LPA-mediated stimulation of these early-response genes (results not shown).

The Ras homologue Rho, a member of the family of small GTPases, has been suggested to be involved in LPA signalling. To investigate the role of Rho in LPA-mediated gene expression of *pghs-2* and *egr-1*, mesangial cells were preincubated for 3 h with toxin B from *C. difficile*, which specifically inactivates the GTPases Rho, Rac and Cdc42 by monoglucosylation [33]. This treatment of mesangial cells with toxin B resulted in substantial changes in cell morphology. Determination of the number of viable cells by Trypan Blue staining and of apoptosis with Hoechst staining revealed no significant changes before and after toxin B treatment (results not shown). Within the concentration range from 0.1 to 10 ng/ml, toxin B reduced both *pghs-2* and *egr-1* mRNA induction stimulated by LPA (Figure 6). Half-maximal inhibition was observed at 5 ng/ml. At 7.5 ng/ml, toxin B completely abolished LPA-mediated *pghs-2* and *egr-1* mRNA induction.

Inhibition of LPA-mediated induction of PGHS-2 and Egr-1 protein expression by toxin B

LPA-induced *pghs-2* and *egr-1* mRNA expression was verified at the protein level. Both PGHS-2 and Egr-1, barely detectable in control cells, were found to be induced in a dose-dependent manner (LPA concentrations between 1 and 100 μ M; results not shown). At 10 and 25 μ M, a strong stimulation of both proteins was observed (Figure 7) compared with basal values of unstimulated mesangial cells (Figure 8). Preincubation of mesangial

cells with toxin B (1 and 5 ng/ml, 3 h) nearly completely inhibited LPA-mediated PGHS-2 and Egr-1 protein expression at 5 ng/ml toxin B, reflecting the mRNA changes shown in Figure 6.

The MAPK p38 is critically involved in LPA-mediated induction of PGHS-2 but not in the induction of Egr-1 protein

We could demonstrate at the mRNA level that MEK (Figure 5) and the Rho protein family (Figure 6) are involved in LPA-mediated induction of PGHS-2 and Egr-1. In order to characterize further basic cascades that are necessary for LPA signalling, we tested the possibility that the MAPK p38 is implicated in PGHS-2 and Egr-1 induction. It has been shown that signalling via Rho proteins may include p38 [34,35]. For this purpose we preincubated growth-arrested mesangial cells with the specific p38 inhibitor SB203580 [36,37] (1 and 5 μ M) for 30 min and stimulated with 10 μ M LPA for 3 h subsequently. After protein isolation, PGHS-2 and Egr-1 protein expression was determined by Western-blot analysis. SB203580 inhibited LPA-mediated induction of PGHS-2 protein in a concentration-dependent manner. At 5 μ M SB203580, PGHS-2 protein was barely detectable. SB203580 alone did not change PGHS-2 protein synthesis. In contrast with these results, inhibition of p38 by SB203580 did not change LPA-mediated Egr-1 protein induction. Neither 1 μ M nor 5 μ M SB203580 inhibited Egr-1 expression (Figure 8). Even at 10 μ M SB203580, no effect could be detected (results not shown). However, in some experiments 5 μ M SB203580 alone resulted in a low stimulation of Egr-1 protein (Figure 8).

DISCUSSION

Glomerular lesions during inflammatory processes of the kidney lead to aggregation of thrombocytes and infiltration of macrophages, which release bioactive mediators. These substances can contribute to activation and proliferation of renal mesangial cells. The roles of PDGF, basic fibroblast growth factor and TGF- β during mesangial cell proliferation and matrix expansion have been examined in detail [38,39]. LPA is an important signalling molecule, which is secreted from activated thrombocytes [1]. Whereas effects of LPA on other cell types, especially fibroblasts, are well characterized, there is little information available regarding LPA signal transduction in renal mesangial cells. To our knowledge, up to now two papers have described the effect of LPA on cultured mesangial cells. One focused on the increase in intracellular Ca^{2+} and a transient mesangial cell contraction [28], and the other concentrated on a mitogenic effect of LPA [26]. In our present work we further characterize the strong mitogenic effect of LPA and, for the first time, the LPA-mediated induction of the early-response genes *egr-1* and *pghs-2* at the mRNA and protein levels and characterize signalling pathways involved in this induction. We demonstrate that LPA is a potent stimulus for proliferation of cultured rat mesangial cells. This is one of the prerequisites for LPA to function as a mediator of mesangial hypercellularity during inflammatory processes of the kidney *in vivo*. In contrast with Knauss et al. [27], we were not able to detect a similar stimulation of proliferation by PA, a product of phospholipase D (PLD). PA is also a precursor of LPA and has been discussed in correlation with PLD activity. A role of PLD in control of mitogenesis is still under discussion [40,41]. In our previous work we were not able to detect a correlation between PLD activity and proliferation in rat mesangial cells [42].

LPA induced a rapid and transient increase of intracellular Ca^{2+} concentration in mesangial cells that was dependent on PI-

PLC activation, since the specific inhibitor U73122 completely abrogated Ca^{2+} mobilization. It has been discussed whether PI-PLC could be involved in LPA-mediated mitogenesis and gene transcription [3,32]. However, we demonstrate that the concentration ranges of Ca^{2+} mobilization and the mitogenic effect of LPA differ significantly and that no effects on proliferation and gene expression of *pghs-2* and *egr-1* were detectable at LPA concentrations that were in the saturation range of the LPA/ Ca^{2+} -dose-response curve. Further evidence that PI-PLC is not involved in mRNA induction is the observation that preincubation of mesangial cells with the PI-PLC inhibitor U73122 abrogates Ca^{2+} mobilization completely, but did not change *pghs-2* and *egr-1* mRNA induction. G-proteins, which mediate LPA signals, seem to be distinct in different cell types. In Rat-1 cells, pretreatment with PTX showed no effect on Ca^{2+} rise [43], whereas human skin fibroblasts were PTX sensitive [44]. Our results reveal that PTX-sensitive G-proteins are essential for LPA-mediated induction of the early-response genes *pghs-2* and *egr-1* (see below), but stimulation of PI-PLC does not seem to be important for transcription of these genes. In mesangial cells the PI-PLC-mediated Ca^{2+} signal as well as the induction of gene expression are PTX sensitive and thus mediated by different G_i proteins.

The immediate-early gene *egr-1* is rapidly and transiently induced in response to multiple mitogenic signals [16,17]. Additionally, a strong correlation between the induction of *egr-1* mRNA and the proliferative response of mesangial cells to PDGF, arginine-vasopressin, 5-HT and endothelin was observed [17]. We could now demonstrate that *egr-1* expression is also critically involved in LPA-mediated proliferation. We detected a rapid and strong LPA-mediated stimulation of *egr-1* mRNA in cultured rat mesangial cells. The involvement of *egr-1* in the proliferative response to LPA was shown by antisense experiments. We have previously demonstrated in cultured rat mesangial cells that oligonucleotides directed against *egr-1* potentially inhibited PDGF- and serum-induced proliferation and *egr-1* mRNA and protein increase with high specificity compared with sense, scrambled and mismatched control oligonucleotides [18]. Here we show that *egr-1* antisense oligonucleotides reduced LPA-mediated proliferation by about 65%, indicating involvement of *egr-1* in this mitogenic signal pathway.

In addition to *egr-1* mRNA, LPA transiently induced *pghs-2* mRNA in rat mesangial cells. The kinetics were similar to those of *egr-1*, but in most cases the maximum of the peak was about 30 min later compared with that of *egr-1* mRNA. *pghs-2* was classified as an immediate-response gene due to its protein-synthesis-independent rapid inducibility by various stimuli [14]. It is functionally related to pathophysiological conditions that are characterized by an enhanced prostanoid synthesis, especially during inflammation. Prostaglandins possess both pro- and anti-inflammatory properties and can modulate inflammatory processes in glomeruli and mesangial cells. LPA is thus not only related to structural and mitogenic cellular responses but seems to be involved in the regulation of inflammation.

In order to characterize further the signalling pathways for *egr-1* and *pghs-2* mRNA induction, we used different toxins and inhibitors. The small GTPases Rho and Ras may both be involved in LPA-mediated gene transcription. In fibroblasts, the PTX-sensitive G-protein G_i is an element of the Ras pathway [45], whereas G_q and $\text{G}_{12/13}$ are elements of the Rho-dependent cytoskeletal reorganization in 3T3 cells [9]. However, in the case of rat mesangial cells, it is unclear whether G_i or G_q pathways are both essential for LPA-mediated *egr-1* and *pghs-2* induction. In the present report, the G_i -protein coupling of LPA-mediated *egr-1* and *pghs-2* mRNA induction was demonstrated by PTX-

induced inhibition. One candidate could be the α subunit of G_{12} , which mediates LPA-stimulated *c-fos* induction in fibroblasts [13].

In our experiments with mesangial cells, inhibition of MEK1 with PD 98059 completely abolished *egr-1* expression and clearly decreased *pghs-2* mRNA induction. We assume that different IC_{50} values of PD 98059 for MEK1 (5 μ M) and for MEK2 (50 μ M) may contribute to these differences and that therefore MEK2 is more likely to be involved in *pghs-2* as in *egr-1* induction. In transfected COS-7 and CHO cells, PI 3-K is required for LPA-mediated MAPK signal transduction and activation downstream of PTX-sensitive G-proteins and upstream of Sos, Ras and MEK [30]. Since MEK was required for *egr-1* and *pghs-2* induction, we studied the involvement of PI 3-K in this signalling pathway. However, since wortmannin, a potent inhibitor of PI 3-K, had no influence on mRNA induction, PI 3-K does not seem to be necessary for *egr-1* and *pghs-2* induction in mesangial cells.

Besides the Ras pathway, the Rho pathway appears to be an important part of LPA-mediated signalling. The small GTPase Rho (review in [46]) acts as a switch for cytoskeletal reorganization, cell adhesion and motility. To test a role of Rho proteins for LPA-mediated *pghs-2* and *egr-1* mRNA induction, we used toxin B from *C. difficile*, which specifically inhibits Rho, Rac and Cdc42 [33]. Both *pghs-2* and *egr-1* mRNA induction were strongly inhibited by toxin B, showing for the first time a crucial role of these GTPases in the induction of the immediate-response genes *pghs-2* and *egr-1* in rat mesangial cells.

There is evidence for the existence of three distinct mammalian MAPK-mediated protein-phosphorylation cascades that are able to cross-talk and to interact, resulting in a fine-tuned signal output, which is dependent on the stimulus and the degree of activation of these signalling pathways (for a review see [47]). Besides the Ras, Raf, MEK and extracellular signal-regulated kinase pathway, the stress-activated protein kinase/Jun N-terminal kinase and the p38 kinase pathways are known. p38 has been related to Rho protein-mediated signalling [34,35]. Therefore, we used the highly specific p38 pyridinylimidazole inhibitor SB 203580, which binds to the ATP-binding site of p38 [36,37]. Inhibition of p38 resulted in an almost complete blocking of LPA-mediated PGHS-2 protein synthesis. In contrast with these results, LPA-induced Egr-1 protein expression was not influenced or even showed a slight stimulation after SB 203580 treatment of mesangial cells.

These results strongly suggest that the p38 MAPK cascade plays a substantial part in LPA-mediated induction of the early-response gene *pghs-2*. It has been discussed that Rho family GTPases regulated p38 MAP kinase through the downstream mediator p21-activated kinase 1 [35,48] and that p38 plays a key role in regulating inflammatory processes. As we could show that Rho proteins are necessary for PGHS-2 induction, these GTPases might be upstream of p38 and could transmit LPA signalling via cross-talk from the Rho signal pathway to the p38 cascade. Nevertheless, p38 is not an essential component of the Rho cascade itself, because LPA-mediated stimulation of Egr-1 is dependent on Rho proteins but independent of p38. The difference between PGHS-2 and Egr-1 expression suggests that activation of p38 is necessary for activation of a downstream effector, e.g. a transcription factor, that is essential for PGHS-2 induction, but not for Egr-1 expression.

Our data provide evidence for the first time that the MAPK p38 and Rho proteins are involved in LPA-mediated expression of the immediate-response genes *pghs-2* and *egr-1*. The differences in *pghs-2* and *egr-1* induction require the identification of further factors in this network.

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