# Role of Rac and Cdc42 in lysophosphatidic acid-mediated cyclo-oxygenase-2 gene expression

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The role of Rho proteins in lysophosphatidic acid (LPA)-mediated induction of cyclo-oxygenase-2 (Cox-2) was investigated in renal mesangial cells. Previous studies had shown that toxin B, an inhibitor of Rho, Rac and Cdc42, suppressed Cox-2 induction. A role for RhoA in pertussis toxin-sensitive LPA signalling was excluded with C3 transferase from *Clostridium limosum*, used as the fusion toxin C2IN–C3 (where C2IN is part of the C2I toxin of *C. botulinum*). Incubation of the cells with C2IN–C3 disrupted cytosolic actin stress fibres, but had no effect on Cox-2 induction. Similarly, activation of p42/44 mitogen-activated protein kinase (MAP kinase), an upstream step in Cox-2 induction, was inhibited by toxin B, but not affected by C2IN–C3. Upon treatment with toxin B, focal adhesion kinase and paxillin were dephosphorylated at tyrosine residues and the actin cytoskeleton was completely destroyed. An intact cytoskeleton, however, was not required for p42/44 MAP-kinase activation or Cox-2 induction, as shown by the actin-depolymerizing agent cytochalasin D. Toxin B did not influence functionality of LPA receptors, because $G_i$-mediated $Ca^{2+}$ release from intracellular stores remained unchanged. Within 1 h, toxin B inactivated and translocated RhoA and Cdc42 to the cellular membranes. Within the same time frame, monoglucosylated Rac1 was degraded. Direct stimulation of Rho proteins by cytotoxic necrotizing factor type 1 (CNF1) induced Cox-2 expression, which was sensitive to inhibition of the MAP-kinase pathway by PD98059, but not to an inhibitor of RhoA kinase. By exclusion of RhoA and non-specific cytoskeletal effects, the results in the present study indicate an important role for Rac and/or Cdc42 in pertussis toxin-sensitive LPA-mediated Cox-2 induction.

Key words: C3 transferase, cytoskeleton, MAP kinases, toxin B.

## INTRODUCTION

The small GTPases of the Rho protein family, Rho, Rac and Cdc42, participate in the assembly and organization of the actin cytoskeleton [1,2]. Thus they are involved in the regulation of multiple cellular processes that relate to the cytoskeleton, such as cell polarity, adhesion, motility, but also are involved in apoptosis and gene transcription. Rho GTPases are part of a complex signalling network. Various exchange factors link them to membrane receptors, and their diverse effects on signalling pathways and cytoskeletal structures are mediated by multiple downstream effectors (reviewed in [3,4]).

Lysophosphatidic acid (LPA) is an activator of the seven-transmembrane receptors of the edg family [5], the signalling of which has been linked to RhoA activation via different signalling molecules, among them the trimeric GTPase $G_{12/13}$ and the guanine nucleotide exchange factor p115 RhoGEF (summarized in [6]). Activation of RhoA by LPA leads to typical changes in the actin cytoskeleton, which have been related to LPA-mediated cellular effects, such as chemotaxis, adhesion, contraction or aggregation (reviewed in [7]). Signalling of LPA via pertussis toxin-insensitive G-proteins of the $G_{12/13}$ type may also result in gene expression. In fibroblasts, LPA induced the immediate early response gene c-*fos* via $G_{12}$ [8]. Furthermore, a constitutive active mutant of $G_{13}$ induced c-*fos* and early growth response gene-1 (Egr-1) expression in NIH 3T3 fibroblasts [9]. In a recent study, overexpression of $G_{13}$ in the same cells has been shown to induce the promoter activity of another early response gene, cyclo-oxygenase-2 (Cox-2) [10], suggesting that LPA might also induce Cox-2 via this signalling pathway. In these cells, RhoA acted downstream of $G_{13}$ and stimulated the transfected Cox-2 promoter construct. The overexpression of another member of the Rho family, RhoB, increased the constitutively expressed Cox-2 levels in colon carcinoma cells [11].

These results obtained in fibroblasts are in contrast with results in mesangial cells, where LPA-mediated expression of Cox-2 and Egr-1 was inhibited by pertussis toxin and was, therefore not mediated by $G_{12/13}$ proteins [12]. Inhibition of the Rho GTPases, Rho, Rac and Cdc42, by toxin B interfered with the induction of the early response genes, but the type of Rho protein responsible for this effect was not further investigated.

Mesangial cells are of mesenchymal origin and have many properties in common with vascular smooth muscle cells. In the kidney, they provide structural support for capillary loops and are involved in the regulation of glomerular contraction and renal haemodynamics [13]. Signalling pathways in fibroblasts and mesangial cells or vascular smooth muscle cells may differ markedly, not only regarding the involvement of different types of G proteins, but also other signalling modules, such as Src kinases or protein kinase A; v-*src* promotes Cox-2 expression in fibroblasts and prevents induction in mesangial cells [14,15]. Agonists, which signal through cAMP, induce Cox-2 in fibroblasts, but inhibit induction in vascular smooth muscle cells or mesangial cells [16,17]. Therefore, signalling pathways elaborated

Abbreviations used: CNF1, cytotoxic necrotizing factor type 1; Cox-2, cyclo-oxygenase-2, CTGF, connective tissue growth factor; Egr-1, early growth response gene-1; FAK, focal adhesion kinase; FCS, foetal calf serum; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; MEK-1, MAP kinase/extracellular signal-regulated kinase kinase; P-Tyr, phosphorylated tyrosine.

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in fibroblasts cannot be transferred to vascular smooth muscle cells or mesangial cells.

In a recent study [18], we showed that induction of connective tissue growth factor (CTGF) by LPA in mesangial cells was pertussis toxin insensitive and mediated by RhoA. The aim of the present study was to further characterize the role of GTPases of the Rho family in the pertussis toxin-sensitive LPA-mediated induction of Cox-2 in mesangial cells. Toxin B from *Clostridium difficile* [19] and C3 transferase from *C. limosum* [20] were used to inhibit the three members of the Rho family, Rho, Rac and Cdc42, and to interfere specifically with RhoA signalling respectively. In particular, we employed the C3-fusion toxin C2IN–C3 (where C2IN is part of the C2I toxin of *C. botulinum*), which is able to enter target cells readily [21,22]. Using this approach, we excluded RhoA as mediator of induction of the endogenous Cox-2 by LPA, and provide evidence that Rac and/or Cdc42 are involved in Cox-2 induction.

## EXPERIMENTAL

### Materials

LPA (1-oleoyl-lysophosphatidic acid) and cytochalasin D were from Sigma (Deisenhofen, Germany). PD98059 was from Calbiochem (Bad Soden, Germany). Antibodies against phospho-p38 MAP kinase and phospho-p42/44 MAP kinase were purchased from New England BioLabs. Antibodies against RhoA (26C4), Cdc42 (P1), phosphorylated tyrosine (P-Tyr) (PY99) and Cox-2 (M-19) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against Rac1 (R56220), paxillin (P13520) and focal adhesion kinase (FAK) (F15020) were from Transduction Laboratories (San Diego, CA, U.S.A.), and the anti-($\beta$-tubulin) antibody was from Chemicon International (Temecula, CA, U.S.A.). Cell culture medium comprised RPMI 1640 medium, penicillin and streptomycin from Biochrom (Berlin, Germany), and foetal calf serum (FCS) from Gibco Laboratories (Eggenstein, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). Toxin B from *C. difficile* was isolated as described previously [19]. For delivery of C3 transferase into cells, the C2IN–C3 fusion toxin was used together with the activated C2II-binding component of *C. botulinum* C2 toxin [21]. Cytotoxic necrotizing factor type 1 (CNF1) from *Escherichia coli* was provided kindly by Dr G. Schmidt and Dr K. Aktories (Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany). Y-27632 was provided kindly by Yoshitomi Pharmaceutical Industries, Osaka, Japan.

### Cell culture

Rat mesangial cells were grown in RPMI medium supplemented with 100 units/ml penicillin, 100 $\mu$g/ml streptomycin and 5% FCS. Mesangial cells were either plated in 10-cm diameter ($6 \times 10^4$ cells/10 ml of medium) or in 6-cm diameter ($2 \times 10^4$ cells/5 ml of medium) Petri dishes in medium supplemented with 5% FCS. At subconfluency (after 3–4 days growth) cells were serum starved in RPMI containing 0.5% FCS for 3 days.

### Cell viability

Necrosis was determined by the measurement of lactate dehydrogenase release as described previously [23]. Determination of apoptosis was done by Hoechst staining with bisbenzimide H 33258 (Hoechst 33258) as described previously [24].

### Northern-blot analysis

Northern-blot analysis was performed as described previously [25]. After stimulation for the times indicated below, total RNA was extracted according to the protocol of Chomczynski and Sacchi [26] with minor alterations. Usually, the yield was about 30–40 $\mu$g of RNA/10-cm diameter Petri dish. Separation of total RNA (10 $\mu$g/lane) was achieved by the use of 1.2% agarose gels containing 1.9% formaldehyde with 1 × MOPS (3-[*N*-morpholino]propanesulphonic acid) 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 [$^{32}$P]dCTP using the Megaprime random prime kit (Amersham International, Braunschweig, Germany). The Cox-2-specific probe was a 1.156-kb *Eco*RI fragment from the 5′ end of mouse cDNA [27]. Blots were pre-hybridized for at least 1 h at 40 °C. The probes were allowed to bind overnight at the same temperature. Washing at 40 °C was carried out for 2 × 15 min in high salt conditions (2 × SSC/0.2% SDS, where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and for 2 × 15 min in low salt conditions (0.2 × SSC/0.2% SDS). DNA/RNA hybrids were detected by autoradiography using Kodak X-Omat AR film. As a control for equal loading of the gels, the 18 S rRNA was stained with Methylene Blue (0.04% in 500 mM sodium acetate, pH 5.2), and the DNA/RNA was quantitated directly by densitometry. Quantitative analysis of the autoradiographs was performed by densitometric scanning (Froebel, Wasserburg, Germany). All values were corrected for differences in RNA loading by calculating the ratio of the specific bands to 18 S rRNA expression.

### Determination of intracellular calcium levels

Mesangial cells were grown on glass cover slips and then loaded with 3 $\mu$M fura 2 acetoxymethyl ester (fura 2/AM). Single cell calcium measurements were performed as described previously [28].

### Western-blot analysis

Mesangial cells were grown in 6-cm diameter dishes until they reached subconfluency, growth arrested for 3 days in RPMI medium containing 0.5% FCS, stimulated for the indicated times, rinsed twice with ice-cold PBS and then scraped in 90 $\mu$l of lysis buffer [50 mM Hepes (pH 7.4)/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X-100/10% (w/v) glycerol/1 mM PMSF/10 $\mu$g/ml aprotinin/10 $\mu$g/ml leupeptin/10 mM sodium orthovanadate/25 mM NaF]. Lysates were incubated for 30 min on ice, and then centrifuged at 15000 ***g*** for 15 min to collect the supernatants. Total protein (30 $\mu$g) were separated by SDS/PAGE (10% gel) and electroblotted on to Fluorotrans transfer membrane (Pall Biosupport Division, Dreieich, Germany). Blots were incubated in Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) non-fat dried milk or 5% horse serum to block non-specific binding, washed in Tris-buffered saline containing 0.1% Tween 20, and incubated with the respective primary antibody. Proteins were revealed using enhanced chemiluminescence (ECL®; Amersham International).

### Immunoprecipitation

Cell lysates (400–600 $\mu$g of protein) were pre-cleared by incubation with protein G+ agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The pre-cleared supernatants were further

incubated overnight with a combination of specific anti-FAK and anti-paxillin antibodies (2 $\mu$g of each antibody). Immune complexes were precipitated by incubation with protein G+ agarose for 1 h. Precipitated proteins were separated by SDS/PAGE. Antibodies against P-Tyr, FAK or paxillin were used to detect proteins in the Western blots.

### Fractionation of cell lysates

Cells, grown on 10-cm diameter Petri dishes, were rinsed twice with ice-cold PBS, before they were scraped in 600 $\mu$l of lysis buffer [250 mM sucrose/10 mM EGTA/2 mM EDTA/20 mM Tris/HCl (pH 7.5)/1 mM PMSF/0.1 % leupeptin/0.1 % aprotinin]. For preparation of the cytosolic fraction samples were sonicated (three times for 10 s for each sample) and then centrifuged for 45 min at 100000 ***g*** at 4 °C. The high speed pellet, which comprises all the membrane subfractions, was resuspended in 100 $\mu$l of lysis buffer. Proteins were separated on SDS/PAGE (15 % gels), and the RhoA, Cdc42 and Rac1 proteins were detected by Western-blot analysis.

### Staining of actin filaments

Cells were cultured and growth was arrested on 8-well multi-test glass slides placed in a Petri dish. Further treatment with different toxins was carried out in wet chambers. After toxin treatment, cells were fixed with 3 % paraformaldehyde in PBS for 10 min and then permeabilized with 0.2 % (v/v) Triton X-100 in PBS for 7 min at approx. 23 °C. To examine DNA fragmentation, cells were incubated with Hoechst 33258 (8 $\mu$g/ml) for 5 min. Rhodamine–phalloidin (Molecular Probes, Eugene, OR, U.S.A.) was used for the staining of the actin cytoskeleton at a dilution of 1:80 in PBS for 20 min.

### ADP ribosylation of all lysates

Cells were lysed in ice-cold buffer [50 mM Tris/HCl (pH 7.2)/150 mM NaCl/1 % (v/v) Triton X-100/1 % (w/v) sodium desoxycholate/0.1 % SDS/1 mM PMSF/14 $\mu$g/ml aprotinin/0.2 mM sodium orthovanadate]. Lysates were incubated with [$^{32}$P]NAD and 50 ng of C2IN–C3 at 37 °C for 30 min. Samples were subjected to SDS/PAGE (12.5 % gel), and the radiolabelled proteins were detected by autoradiography with a PhosphorImager (Molecular Dynamics).

## RESULTS

### Rho GTPases, but not RhoA, mediate LPA-induced Cox-2 expression

As a model system to investigate the role of Rho proteins in the induction of endogenous Cox-2, a rat renal mesangial cell line was used, which was obtained from primary mesangial cell isolates by long-term culture *in vitro* [29]. In accordance with previous results [12], Cox-2 mRNA was induced by LPA within 1 h (Figure 1A). Pre-incubation of the cells with toxin B for 3 h reduced LPA-mediated Cox-2 mRNA expression to baseline levels. A role for Rho GTPases in the induction of Cox-2 mRNA was further confirmed by the use of CNF1 from *E. coli*, which activates members of the Rho family [30]. Cox-2 mRNA levels were markedly increased by incubation with CNF1 (400 ng/ml) for 4–7 h (Figure 1B).

To differentiate between the GTPases Rho and Rac/Cdc42, the specific inhibition of Rho was achieved by incubation with C3 transferase. The latter is an ADP-ribosyltransferase that modifies RhoA, RhoB and RhoC at Asp$^{41}$ and, therefore

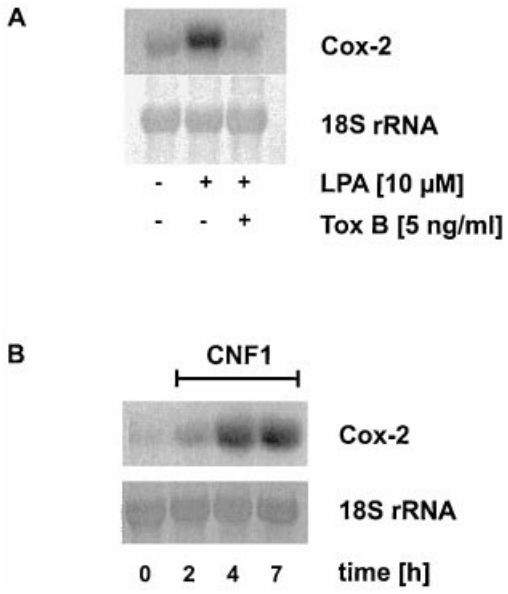


**Figure 1 LPA-mediated Cox-2 induction is transmitted by Rho GTPases**

Cellular mRNA was extracted and analysed by Northern blotting. Blots were probed with a cDNA specific for Cox-2, whereas 18 S rRNA was stained by Methylene Blue. (**A**) Mesangial cells were stimulated with 10 $\mu$M LPA for 1 h and pre-incubated with toxin B (Tox B; 5 ng/ml, 3 h) as indicated. (**B**) Stimulation of mesangial cells with CNF1 (400 ng/ml) was carried out for 0 h, 2 h, 4 h or 7 h.

selectively inactivates these proteins, whereas Rac and Cdc42 are not targeted [22]. As there are no cellular receptors for C3 transferase, its uptake into the cytoplasm is insufficient. Therefore, we used the fusion toxin C2IN–C3, comprising the catalytic domain of C3 transferase of *C. limosum* and part of the C2I toxin of *C. botulinum*, which together with the activated binding component C2II of the C2 toxin allows efficient uptake into the cells [21].

Effective inactivation of RhoA by C2IN–C3 was controlled by an ADP-ribosylation assay *in vitro*. After 3 h incubation with 200 ng/ml C2IN–C3, RhoA was partially ADP ribosylated (Figure 2A). Approx. 80 % of the cellular RhoA was modified and, thus, inactivated after 6 h. This percentage remained unchanged with longer incubation periods (16.5 h).

Efficient inhibition of RhoA activity was also obvious by modification of the actin cytoskeleton. Resting mesangial cells show a high density of actin stress fibres, which was revealed by rhodamine–phalloidin, a complex that binds to F-actin, but not to depolymerized actin (Figure 2B). Incubation with C2IN–C3 for 3 h or 6 h led to the resolution of the stress fibres spanning the cytosol, whereas actin filaments below the plasma membrane were not affected by C2IN–C3.

Pre-incubation of mesangial cells with C2IN–C3 did not inhibit LPA-induced Cox-2 expression (Figures 2C and 2D). LPA-stimulated Cox-2 protein levels were slightly increased after pre-incubation with C2IN–C3 for 6 h, but this effect was not statistically significant. These findings indicated that RhoA was not involved in the LPA-dependent signalling, which is associated with Cox-2 expression.

### Cox-2 is induced independently of an intact cytoskeleton

Modification and inactivation of Rho proteins by toxin B occurred more rapidly when compared with C3 transferase.

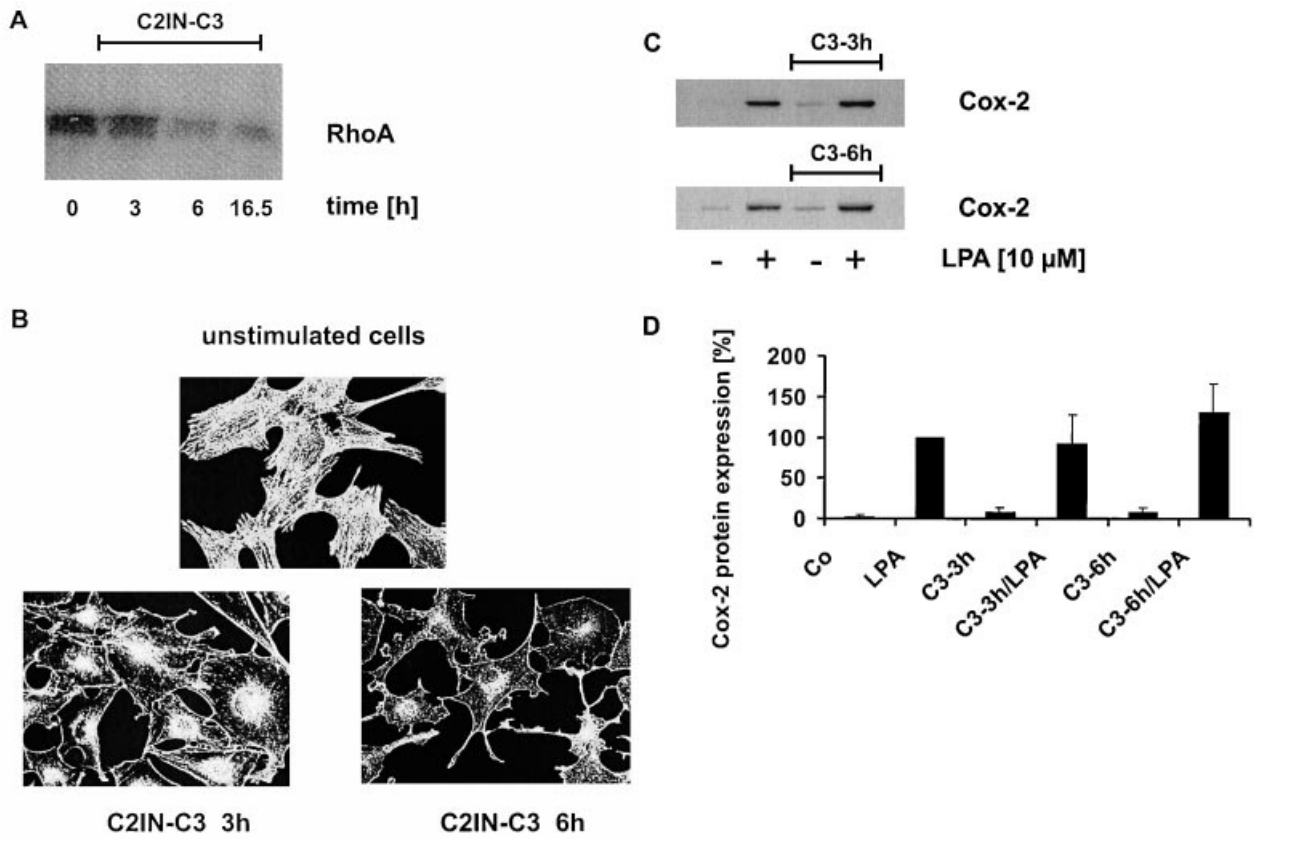


**Figure 2 RhoA regulates cytoskeletal structures, but not Cox-2 protein expression**

(**A**) Mesangial cells were treated with 200 ng/ml C2IN–C3 for 3 h, 6 h, 16.5 h or were left untreated (0 h). Cell lysates were ADP-ribosylated and separated by SDS/PAGE. [$^{32}$P]ADP-ribosylated RhoA was detected by PhosphorImager analysis. (**B**) Mesangial cells were unstimulated, or were incubated with 200 ng/ml C2IN–C3 for 3 h or 6 h. A rhodamine–phalloidin complex was used to reveal the actin filaments. (**C**) Mesangial cells were pre-incubated with C2IN–C3 for 3 h or 6 h, and then stimulated with LPA for an additional 3 h. Immunodetection of Cox-2 was carried out in whole cell lysates. (**D**) Protein levels are shown as the percentage of Cox-2 protein expression after LPA stimulation. The results are expressed as the means $\pm$ S.D. for at least three experiments. Control (Co), 10 $\mu$M LPA for 3 h (LPA), 200 ng/ml C2IN–C3 for a pre-incubation period of 3 h (C3-3h), 200 ng/ml C2IN–C3 for a pre-incubation period of 6 h (C3-6h).

Treatment with 5 ng/ml toxin B led to a time-dependent disappearance of Rac1 protein, as determined by Western-blot analysis of whole cell lysates or cellular membranes (Figures 3A and 3B). Rac1 disappeared within less than 1 h, indicating that the monoglucosylated protein was degraded rapidly. Protein levels of other proteins, such as $\beta$-tubulin or p38 MAP kinase, were not affected by toxin B. However, treatment with toxin B increased p38 phosphorylation, indicating an activation of this stress-related kinase. Whereas Rac1 was located primarily within cellular membrane fractions, RhoA and Cdc42 were mainly detected in the cytosol (Figure 3B). Upon treatment with toxin B, a time-dependent increase in RhoA and Cdc42 in the particulate fraction of mesangial cells was observed, indicating that the monoglucosylated and thus inactive proteins were trapped at cellular membranes. Inactivation of the Rho proteins was followed by profound changes in the actin cytoskeleton, also reflected by the inactivation of proteins linked to cytoskeletal organization, such as FAK and paxillin. Tyrosine phosphorylation of both proteins was reduced when the cells were treated with toxin B (5 ng/ml) for 3 h (Figure 3C). Concomitantly, the actin cytoskeleton was dissolved. After 1 h, the fibres retracted and holes became apparent in the dense stress fibre network. After 2–3 h, the original cell shape was lost, and short actin fibres and the remains of the cytoplasm were concentrated around the nucleus (Figure 3D). Similar morphological effects were also observed when mesangial cells were treated with cytochalasin D, which leads to the depolymerization of the actin cytoskeleton by preventing the binding of the G-actin molecules to the growing end of actin fibres (Figure 3D).

Toxin B-mediated inhibition of Cox-2 expression might have been attributed to impaired signalling due to the destruction of actin filaments. Therefore, the effect of toxin B on gene expression was compared with that of cytochalasin D. However, pre-incubation with cytochalasin D (1 $\mu$g/ml) for 1 h did not affect LPA-mediated Cox-2 protein expression in mesangial cells (Figures 4A and 4B). This indicated that the effect of toxin B on Cox-2 expression was not transmitted by cytoskeletal alterations.

### Expression of LPA receptors and coupling to $G_i$-proteins are not affected by toxin B

The reduction of Cox-2 protein concentration to baseline levels by toxin B might have been due to the down-regulation of LPA receptors at the cell surface. Therefore, we tested whether LPA receptors were present and whether they could still be activated after toxin B treatment. As a read-out system, LPA-mediated release of $Ca^{2+}$ ions from intracellular stores was determined. Previous investigations [12,31] have shown that LPA-mediated release of $Ca^{2+}$ from intracellular stores is pertussis toxin sensitive in mesangial cells. Single-cell measurements of adherent fura-2-

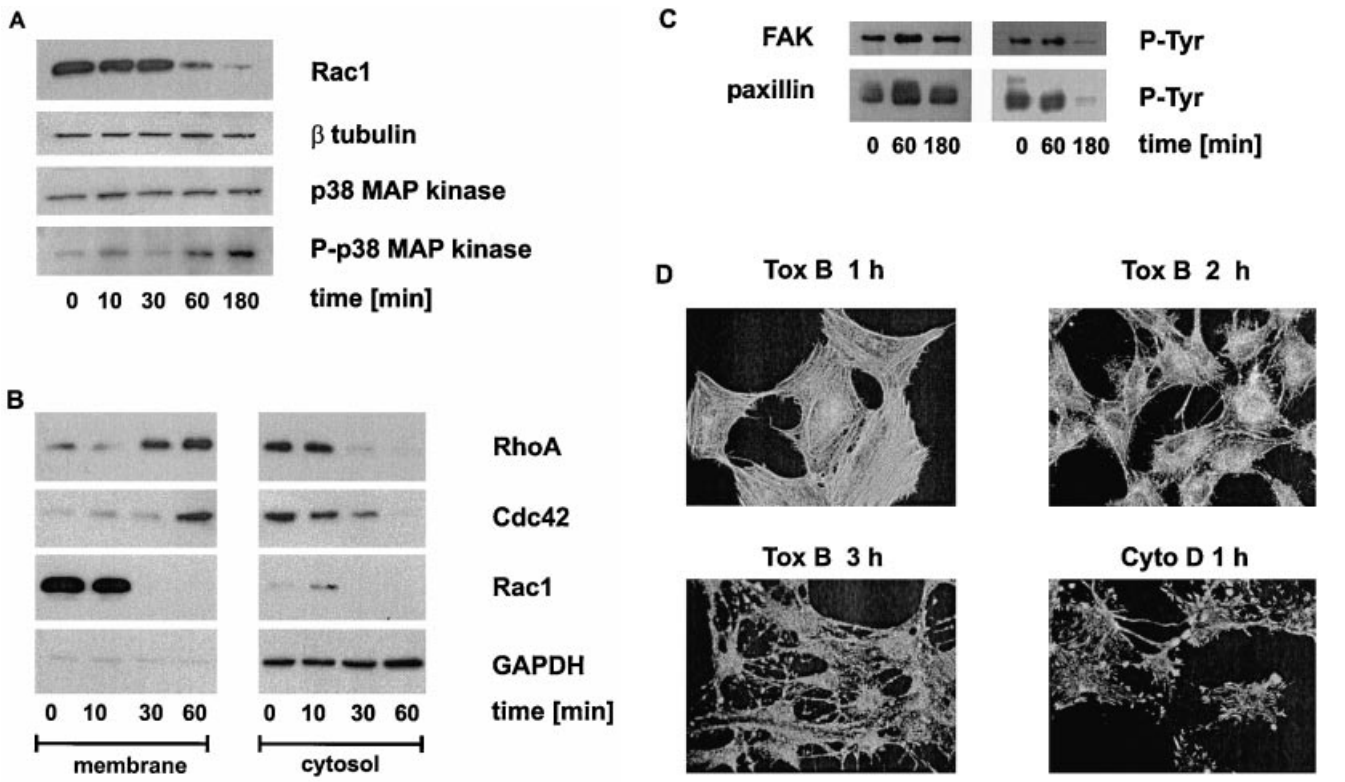


**Figure 3 Toxin B inactivates Rho GTPases and leads to rearrangements of cytoskeletal structures**

Mesangial cells were incubated with 5 ng/ml toxin B for the indicated periods. (**A**) Whole cell lysates were analysed by Western blotting using a primary antibody specifically against Rac1. The same sample was further analysed for $\beta$-tubulin, p38 MAP kinase and phosphorylated (P-) p38 MAP kinase. (**B**) Membranes and cytosol were separated by differential centrifugation. RhoA, Cdc42 and Rac1 contents of the particulate and the cytosolic fraction were determined by Western-blot analysis. Each blot is a representative for at least three independent experiments. (**C**) Mesangial cell lysates were used for immunoprecipitation with a combination of anti-FAK and anti-paxillin antibodies. Proteins were separated by SDS/PAGE and probed with anti-FAK (FAK), anti-paxillin (paxillin) or anti-(P-Tyr) antibodies. The proteins that were detected with the anti-(P-Tyr) antibody co-migrated with FAK or paxillin. (**D**) Mesangial cells were grown on 8-well multi-test glass slides. After treatment with toxin B (Tox B; 5 ng/ml) or cytochalasin D (Cyto D; 1 $\mu$g/ml) for the indicated time periods, cells were fixed and stained with a rhodamine–phalloidin complex.

loaded mesangial cells revealed a rapid increase in $Ca^{2+}$ ions in the cytosol after stimulation with 10 $\mu$M LPA. Pre-incubation of the cells with toxin B (5 ng/ml) for 1 h or 3 h did not alter LPA-mediated calcium activation (Figure 5). This indicated that toxin B did not interfere with LPA receptor expression or $G_i$-protein coupling.

Furthermore, despite the strong morphological alterations, treatment with toxin B did not affect cell viability. Toxin B-treated cells showed no signs of apoptosis or necrosis, which was assessed by DNA staining using the dye Hoechst 33258 and measurement of lactate dehydrogenase release respectively (results not shown).

### Activation of p42/44 MAP kinase is targeted by toxin B, but unaffected by C2IN–C3 or cytochalasin D

Previous studies [12] have shown that inhibition of p42/44 MAP-kinase activation by PD98059 interfered with LPA-mediated induction of Cox-2. Pre-incubation of mesangial cells with toxin B for 3 h prevented activation of p42/44 MAP kinases by LPA, whereas C2IN–C3 and cytochalasin D were without effect (Figure 6A). The activated status of p42/44 MAP kinase was determined using an antibody directed against the dually phosphorylated form of p42/44 MAP kinase.

Inactivation of the Rho GTPases by toxin B occurred after 1 h when cytoskeletal effects were barely detectable. Therefore, the effects of toxin B on LPA-mediated p42/44 MAP-kinase activation and Cox-2 transcription were also analysed after a pre-incubation time of 1 h (Figure 6B). There was a close correlation between the inhibition of p42/44 MAP-kinase activation determined after 5 min of LPA stimulation, and the induction of Cox-2 mRNA expression determined after 1 h of LPA stimulation. These results strongly suggested that inhibition of Rac and/or Cdc42 impaired p42/44 MAP-kinase activation. A role for p42/44 MAP kinase in Rac and Cdc42 signalling was supported by the analysis of CNF1-mediated Cox-2 induction (Figures 6C and 6D). PD98059 inhibited CNF1-mediated induction of Cox-2 mRNA. In contrast, the compound Y27632, which inhibits kinases downstream of RhoA, had no effect.

## DISCUSSION

In mesangial cells, as in many other cell types, LPA binds to receptors of the edg family, which are coupled to different types of trimeric G-proteins [5]. As described previously [12,18,32], LPA activates p42/44 MAP kinase and induces the expression of the early response genes Egr-1 and Cox-2 via pertussis toxin-sensitive $G_i$-proteins, whereas pertussis toxin-insensitive G-proteins are involved in the induction of CTGF. Incubation of mesangial cells with toxin B from *C. difficile* interfered with

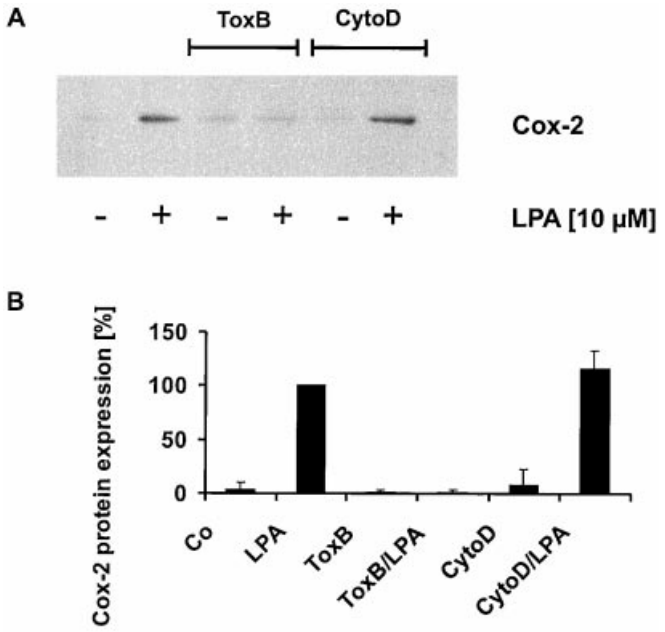


**Figure 4 Cox-2 protein expression is not dependent on an intact actin cytoskeleton**

Mesangial cells were left untreated, were pre-incubated with 5 ng/ml toxin B for 3 h (ToxB) or with 1 $\mu$g/ml cytochalasin D for 1 h (CytoD). Cells were either stimulated with 10 $\mu$M LPA for 3 h (+) or not stimulated (−). Cell lysates were then analysed by Western blotting using a anti-(Cox-2) specific antibody. (**A**) Representative result from four (CytoD) and six (ToxB) independent Western-blotting experiments. (**B**) Protein levels are shown as the percentage of Cox-2 protein expression after LPA stimulation. The results are expressed as the means ± S.D. for at least four experiments.

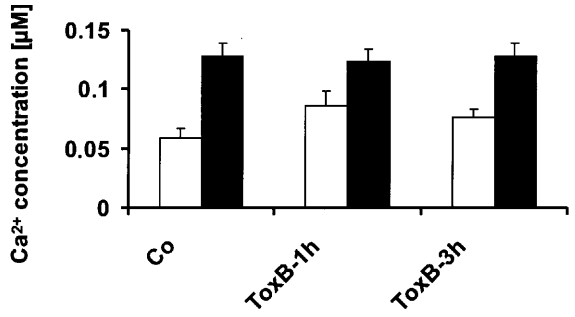


**Figure 5 LPA-receptor expression and receptor–$G_i$ coupling are not affected by toxin B**

To determine intracellular $Ca^{2+}$ levels, single cells were analysed. Mesangial cells were either left untreated (Co) or incubated with 5 ng/ml toxin B for 1 h (ToxB-1h) or 3 h (ToxB-3h). Open bars represent unstimulated cells and filled bars those which were stimulated with 10 $\mu$M LPA. Bars express the concentration of free intracellular $Ca^{2+}$ in $\mu$M and are the means ± S.D. for four to six cells.

the induction of all three early response genes. More specifically, RhoA was shown to mediate the induction of CTGF [18]. To clarify the role of RhoA in the induction of endogenous Cox-2, a cell permeable C2IN–C3 fusion protein [21], which specifically inhibits RhoA, RhoB and RhoC, was used in the present study. Treatment of mesangial cells with C2IN–C3 disassembled the cytosolic actin cytoskeleton, leaving actin fibres below the plasma membrane intact. However, specific inhibition of RhoA by C2IN–C3 had no effect on LPA-mediated p42/44 MAP-kinase activation and Cox-2 expression. Furthermore, induction of Cox-2 by CNF1, which activates Rho, Rac and Cdc42 [30], was not inhibited by the compound Y27623, which inhibits Rho kinases [33]. Based on this evidence, RhoA was excluded as a mediator of the effects of toxin B on Cox-2 expression. A link between RhoA or RhoB and Cox-2 was reported in other experimental settings. Using NIH 3T3 fibroblasts, Slice and co-workers [10,34] showed that activated $G_{13}$ was coupled to RhoA and Cox-2 promoter activity. Likewise, activation of the Cox-2 promoter by microtubule-interfering agents, such as taxol, was prevented by overexpression of dominant negative RhoA in an epithelial cell line [35]. However, induction of endogenous Cox-2 expression was shown only in RhoB overexpressing colon carcinoma cell lines, which express Cox-2 constitutively [11]. Differences may exist between activation of a transfected promoter and induction of gene expression in a physiological context, as exemplified by Beltman et al. [36]. In rat-1 fibroblasts, they observed inhibition of a serum-response-element-driven promoter construct by C3 transferase, but no interference with c-*fos* mRNA induction, indicating that RhoA is not required for physiological expression of c-*fos*. Similarly, dexamethasone activated a transiently expressed Cox-2 promoter construct in mesangial cells, in contrast with its known inhibitory function in Cox-2 regulation [37]. Thus, caution is warranted to transfer promoter studies to the physiological situation. Although, our results in the present study indicate that RhoA is not involved in the regulation of LPA-mediated Cox-2 expression, this does not exclude a role in a different cellular context, which needs to be defined in more detail.

Morphological changes due to a disruption of the actin cytoskeleton by toxin B became apparent after 2–3 h. Within the same period, proteins associated with the regulation of the cytoskeleton, such as FAK and paxillin, were inactivated, as shown by decreased tyrosine phosphorylation. Thus, after a pre-incubation period of 3 h, the cells barely resembled the originally spread and adherent mesangial cells. However, the cells were not dead or apoptotic, and there was no general impairment of LPA signalling. LPA-mediated activation of phosphatidylinositol-specific phospholipase C and the release of calcium from intracellular stores, which are also mediated by $G_i$ proteins in mesangial cells [12,31], were not affected by toxin B treatment. A similar separation of calcium release and MAP-kinase activation was observed for $G_{q/11}$-mediated P2Y-receptor signalling in endothelial cells [38], where calcium release was observed to be independent and MAP-kinase activation to be dependent on cell anchorage.

The strong morphological changes induced by toxin B suggested that the disruption of the cytoskeleton itself might interfere with LPA-mediated signalling, which involves translocation of kinases, such as p42/44 MAP kinase, into the nucleus. However, treatment of mesangial cells with C2IN–C3 disassembled the actin cytoskeleton, but did not interfere with these processes. Furthermore, cytochalasin D did not interfere with LPA-mediated p42/44 MAP-kinase activation or with the induction of Cox-2. The missing effect of cytochalasin D on p42/44 MAP-kinase activation is in agreement with findings of Della Rocca et al. [39], which showed that cytochalasin D interferred with $G_{q/11}$-coupled p42/44 MAP-kinase activation, but not $G_i$-coupled signalling.

Cytoskeletal effects of toxin B were observed after prolonged incubation periods. Inactivation of Rho proteins, however, occurred within a shorter time period. It has been shown that glucosylation of RhoA by toxin B inhibits cycling of the proteins between the cytosol and the membranes by trapping the in-

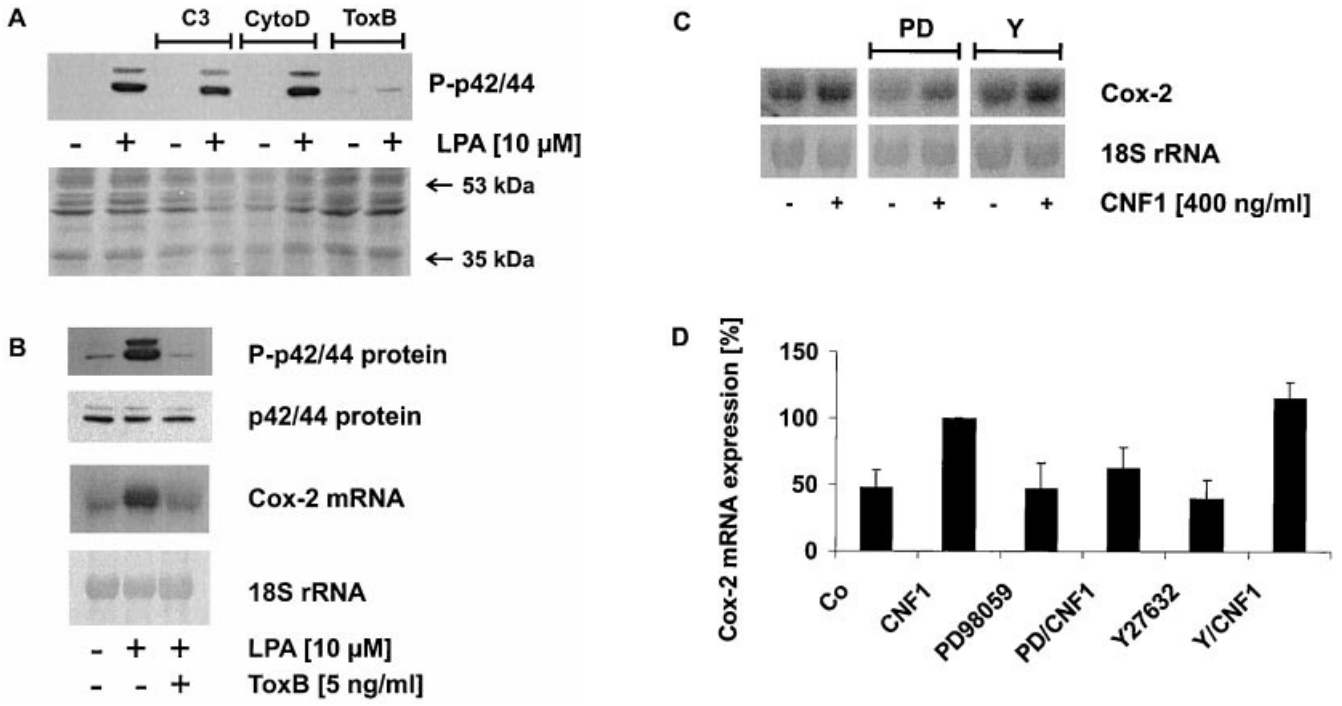


**Figure 6 Activation of p42/44 MAP kinase is targeted by toxin B, but not C3 transferase**

(**A**) Mesangial cells were pre-incubated with 200 ng/ml C2IN–C3 (C3) for 6 h, 1 $\mu$g/ml cytochalasin D (CytoD) for 1 h or 5 ng/ml toxin B (ToxB) for 3 h, and then stimulated with 10 $\mu$M LPA for 5 min (+) or not (−). Whole cell lysates were analysed by Western blotting to determine MAP-kinase activation with antibodies against the phosphorylated/activated forms of the p42/44 protein (P-p42/44). To control for equal loading the blot was stained with Amido Black. The blot is a representative result for four independent experiments. (**B**) Mesangial cells were pre-incubated with 5 ng/ml toxin B for 1 h and then stimulated with 10 $\mu$M LPA for 5 min to analyse the phosphorylation status of p42/44 MAP kinase, and for 1 h to detect Cox-2 mRNA levels. p42/44 protein and 18 S rRNA are shown as a control for equal loading. (**C**) A representative Northern-blot analysis shows Cox-2 mRNA expression induced by CNF1 (400 ng/ml, 4 h). Before stimulation the cells were incubated with 10 $\mu$M PD98059 (PD) or 10 $\mu$M Y27632 (Y) for 1 h. (**D**) To compare different experiments, stimulation with CNF1 was set to 100%. Results are expressed as the means ± half range (Y) or means ± S.D. (PD98059) of two to three experiments. Control (Co), stimulation with 400 ng/ml CNF1 for 4 h (CNF1), 1 h pre-incubation with 10 $\mu$M PD98059 (PD) or 10 $\mu$M Y27632 (Y). Expression of Cox-2 in the presence of PD98059 was not significantly different when compared with control cells.

activated protein in the membranes [40]. Thus the translocation of RhoA was taken as a measure of RhoA inactivation by toxin B in mesangial cells. It occurred between 30 and 60 min of incubation with toxin B. Within the same period, Cdc42, which was primarily located in the cytosol in non-treated mesangial cells, was also transferred to cellular membranes. Immunoreactive Rac1 disappeared when the cells were treated with toxin B, as has been described by Clerk et al. [41]. As the antibody was able to recognize the modified Rac1 (results not shown), the disappearance of Rac1 was attributed to a rapid degradation of the glucosylated protein. A pre-incubation time of 1 h was sufficient for toxin B to interfere with LPA-mediated Cox-2 induction, as well as p42/44 MAP-kinase activation, suggesting a role for Rac and/or Cdc42 upstream of p42/44 MAP-kinase activity. This was confirmed by the effect of PD98059, an inhibitor of p42/44 MAP-kinase activation, on CNF1-mediated induction of Cox-2, which was due to activation of Rac and/or Cdc42. p38 is another member of the MAP kinase family that has been related to LPA-mediated Cox-2 expression [12]. Activation of this kinase, however, was not inhibited by toxin B, but rather increased. The long-lasting activation of p38 MAP kinase was consistent with previous reports on p38 MAP kinase activation by C3 transferase in rat-1 fibroblasts [36] and by toxin A in monocytes [42]. Activation of p38 MAP kinase was interpreted as a stress response of the cells, but was also related to the development of inflammation and mucosal damage.

By the exclusion of Rho, Rac and/or Cdc42 were identified as mediators of the toxin B and CNF1 effects on Cox-2 expression. As no specific and selective inhibitors of these proteins are available, much less is known about their cellular functions when compared with RhoA. Overexpression of either Rac1 or Cdc42 led to an activation of c-Jun N-terminal kinase, but not p42/44 MAP kinase (e.g. [43,44]). However, in combination with activated Raf1 kinase, Rac1 and Cdc42 were implicated in p42/44 MAP-kinase activation in fibroblasts: overexpression of active Rac1 or Cdc42 and Raf1 synergistically activated p42/44 MAP kinase [45]. In a recent study in cardiac myocytes, Rac1 was shown to promote activation of p42/44 MAP kinase by increasing the association of MEK-1 (MAP kinase/extracellular signal-regulated kinase kinase) and activated c-Raf [41]. Furthermore, the serine/threonine PAK1 (p21-activated kinase 1) has been implicated in the cross talk between Rho proteins and the Raf1–MEK-1–p42/44 MAP-kinase signalling cascade [46]. Which of these mechanisms mediates the interaction of Rac and/or Cdc42 with the p42/44 MAP-kinase cascade in mesangial cells remains to be established.

Thus our results in the present study link the GTPases Rac and/or Cdc42 to the activation of p42/44 MAP kinase and endogenous Cox-2 expression. There was no evidence for a role of RhoA in the agonist-induced Cox-2 expression, indicating that the role of Rho proteins in Cox-2 induction may depend on the cell type and stimulus investigated.

The technical assistance of R. Rinke is highly appreciated. This work was supported by grants from the Deutsche Forschungsgemeinschaft [grant numbers Go 413 and SFB423(TP B3)].

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Received 10 July 2001/17 October 2001; accepted 7 December 2001