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STAT3-independent inhibition of lysophosphatidic acid-mediated upregulation of connective tissue growth factor (CTGF) by cucurbitacin I

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CTGF, connective tissue growth factor
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STAT, signal transducers and activators of transcription
Jak, Janus kinase

ABSTRACT

Cucurbitacins are recognised as anti-tumour agents because of their interference with STAT3 signalling, but may also affect the integrity of the actin cytoskeleton. In the present study the effect of cucurbitacin I was investigated in fibroblasts. In these cells, cucurbitacin I interfered with lysophosphatidic acid (LPA) signalling. It inhibited tyrosine phosphorylation of focal adhesion proteins and induction of connective tissue growth factor (CTGF), a potent profibrotic protein. Inhibition of Src family kinases with PP2, but not the inactive analogue PP3, also interfered with LPA-mediated tyrosine phosphorylation and induction of CTGF. Jak2–STAT3 signalling seemed to be the connecting link, because CTGF induction was sensitive to AG490, an inhibitor of Jak2, and cucurbitacin I, an inhibitor of Jak2 and STAT3. However, LPA did not activate tyrosine phosphorylation of STAT3. Furthermore, cucurbitacin I was as effective in STAT3 knock out cells as in control cells. Therefore, the inhibitory effect of cucurbitacin I was not related to inhibition of STAT3.

Immunocytochemical analysis of cucurbitacin I-treated cells revealed disassembly of F-actin fibres, reorganisation into F-actin patches and resolution of focal adhesions. The phenotypic changes resembled changes observed after treatment of the cells with cytochalasin D, which has been shown to interfere with CTGF induction. Concentrations of cucurbitacin I, which have been shown to target Jak2–STAT3 signalling, thus, profoundly affect the actin cytoskeleton and may therefore modulate cell morphology, migration, adherence and gene expression also in non-tumour cells.

1. Introduction

Cucurbitacins are compounds isolated from various plant families which have been used as folk medicines for centuries in countries such as India or China [1]. Some of the cucurbitacins are toxic, but also possess anti-inflammatory or analgesic effects. As cytotoxic substances they have been tested in several tumour models but showed a low therapeutic index. At the cellular level, cucurbitacins inhibit proliferation of several cell types such as lymphocytes, HeLa cells or endothelial cells [2–4]. The molecular mechanism of the various biological effects of cucurbitacins have not been fully investigated. Some cucurbitacins were shown to affect the structure of prostate carcinoma cells by disruption of the actin

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cytoskeleton [5]. A similar effect was also observed when proliferating endothelial cells were treated with cucurbitacins, whereas no effect was observed in quiescent, confluent endothelial cells, which organise actin into F-actin bundles forming a dense peripheral band [4]. In endothelial cells, cucurbitacin E proved to be the most potent derivative.

More recently, some cucurbitacins were shown to interfere with the Jak–STAT (Janus kinase-signal transducer and activator of transcription) signalling pathway, specifically with STAT3 signalling [6,7]. STAT3 has been implicated in malignant transformation and tumour cell survival [8], and therefore has been proposed as target structure for anti-cancer therapy (e.g. [9]). Anti-tumour effects of cucurbitacin I were thus attributed to the inhibition of the Jak–STAT3 pathway [10]. The Jak family of kinase is associated constitutively with different cytokine receptors, but was also linked to the activation of certain G-protein coupled receptors, such as angiotensin II, bradykinin or cholecystokinin CCK receptors [11–13]. Activation of Jak2–STAT3 signalling by angiotensin II is well documented in several cellular systems and seems to involve physical association between angiotensin ATI receptors and Jak2 [14] as well as intermediary signalling pathways including protein kinase C [15], Rho family GTPases [16] or Src kinase [17]. Functionally, Rho-mediated activation of Jak2–STAT3 signalling was related to cytoskeleton rearrangement, gene expression, cell migration and proliferation [18] as well as oncogenic transformation [19]. Angiotensin II-mediated activation of STAT3 by Src kinase was independent of Jak2 [17]. Direct STAT3 phosphorylation was not restricted to c-Src, but has been reported for various members of Src family kinases [20,21]. Activation of STAT3 by v-Src plays a role in cellular transformation and Src family kinase-mediated tumourigenesis (summarised in [22]).

Whether interference with Jak–STAT signalling by certain cucurbitacins and disruption of the actin cytoskeleton are independent or interdependent effects of these drugs has not yet been addressed.

Connective tissue growth factor (CTGF) is a proangiogenic and profibrotic protein, which plays a role in atherosclerosis, fibrotic disorders and also certain tumours [23,24]. In earlier studies, we have shown that RhoA, Src family kinases and alterations of the actin cytoskeleton are involved in the regulation of CTGF gene expression. By binding to G-protein coupled receptors, lysophosphatidic acid (LPA) stimulated CTGF expression in a RhoA-dependent manner, with alterations of the actin cytoskeleton being involved in the signalling pathway [25–27]. In a recent study activation of focal adhesion proteins, focal adhesion kinase (FAK), phosphatidylinositol (PI) 3-kinase and Src kinase were shown to contribute to CTGF induction upon focal adhesion clustering [28]. Given the linkage between Src kinase or Rho proteins and STAT3 signalling, we postulated STAT3 as downstream mediator involved in CTGF induction. Furthermore, cucurbitacins were expected to interfere with CTGF expression based on their ability to either interfere with Jak–STAT signalling and/or their effects on the actin cytoskeleton. Thus far, these different activities of cucurbitacins have not been investigated in parallel, and the specificity of cucurbitacin I as inhibitor of Jak/STAT signalling has not been defined in relation to the effects of this drug on the cytoskeleton.

2. Materials and methods

2.1. Materials

Cucurbitacin I, cytochalasin D, AG490, PP2 and PP3 were obtained from Merck Biosciences, Bad Soden, Germany. The inhibitors were dissolved in DMSO and appropriate solvent controls were included in all experiments. Lysophosphatidic acid (LPA) was from Sigma, Taufkirchen, Germany. Protease inhibitor cocktail was purchased from Roche Diagnostics, Mannheim, Germany. The following primary antibodies were used: polyclonal goat anti-CTGF (SC14939), polyclonal rabbit anti-STAT3 (SC482), and polyclonal rabbit anti-vinculin (SC5573) (Santa Cruz, Heidelberg, Germany); monoclonal mouse anti-paxillin (BD, Heidelberg, Germany); polyclonal rabbit anti-phospho STAT3 and monoclonal mouse anti-phosphotyrosine (p-tyr-100; New England Biolabs, Frankfurt, Germany). The peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Amersham Biosciences (Freiburg, Germany), the anti-goat secondary antibody was from Santa Cruz (Heidelberg, Germany). Anti-mouse IgG secondary antibodies coupled to Alexa 488 or Alexa 555 were from Molecular Probes, Leiden, The Netherlands.

2.2. Cell culture

Renal fibroblasts were obtained from the kidney of a 4-week-old male mouse, immortalised and established as clonal cell line by R. Faessler, Munich, Germany [29], who kindly provided these cells. The cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 4.5 g/L glucose, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were seeded in DMEM containing 1% FCS 1 day before the experiments. Fibroblasts lacking c-Src, Yes and Fyn (ATCC CRL-2459) and fibroblasts lacking Yes and Fyn (ATCC CRL-2498) were cultured and treated as the renal fibroblasts.

Wild type (STAT3-WT) and STAT3 deficient (STAT3-KO) mouse embryonic fibroblasts were generated as previously described [30]. The cells were seeded in 10% FCS for 24 h and starved with 0% FCS for 24 h before the experiments.

2.3. Western blot analysis

Cells were lysed in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (w/v) glycerol, 4 mM sodium orthovanadate and protease inhibitor cocktail. Twenty-five micrograms of total protein were separated by SDS-PAGE and electroblotted onto fluorotrans transfer membrane (Pal, Biosupport Division, Dreieich, Germany) as described previously [31]. Protein-antibody complexes were visualised by the enhanced chemiluminescence detection system (ECL, Amersham Biosciences). Blots were quantified using the luminescent image analyser LAS 100 (FujiFilm) and AIDA image analyser software (Raytest, Straubenhardt, Germany).

2.4. Immunoprecipitation

Precleared lysates were incubated with a polyclonal rabbit anti-STAT3 antibody (SC482) at 4 °C for 2 h and with protein G PLUS-agarose (SC2002, Santa Cruz, Heidelberg, Germany) for

1 h. The agarose-beads were washed three times with lysis buffer, resuspended in SDS gel loading buffer and boiled at 95 °C. Separated immunoprecipitates were analysed by Western blotting.

2.5. *Immunocytochemistry*

Actin filaments were stained with Alexa Fluor 488 phalloidin (Molecular Probes, Leiden, The Netherlands) as described previously [26]. In brief, cells were seeded on glass coverslips placed in a 35 mm Petri dish. After treatment with the stimuli, the cells were fixed with 3.5% paraformaldehyde in PBS for 10 min and then permeabilized with 0.2% TritonX-100 in PBS for 10 min at room temperature before staining with phalloidin. Focal adhesions were visualised with a monoclonal mouse anti-phosphotyrosine antibody (p-tyr-100). Actin fibres and focal adhesions were analysed by fluorescence microscopy (Leica, Wetzlar, Germany).

3. Results

3.1. *Cucurbitacin I is a potent inhibitor of LPA-induced CTGF expression*

The effect of cucurbitacin I on the expression of CTGF was investigated in a mouse renal fibroblast cell line. In a previous study we have shown that LPA-induced CTGF expression in a RhoA-dependent manner in these cells [31]. Fibroblasts were preincubated with cucurbitacin I at concentrations, 1–10 μM, which have previously been reported to inhibit Jak–STAT3 signalling pathways in different cell lines [6]. By both concentrations, LPA-induced CTGF expression was reduced significantly (Fig. 1A and C). Furthermore, AG490, an inhibitor specific for Jak2, also led to a partial inhibition of CTGF induction (Fig. 1B and C). Compared to cucurbitacin I, AG490 proved to be less potent.

3.2. *Src family kinases are involved in LPA-mediated regulation of CTGF*

Src family kinases were involved in CTGF induction upon alterations of the actin cytoskeleton in colchicine-treated fibroblasts [28] and Src family kinases are implicated in STAT3 activation [19]. Therefore, the role of these non-receptor tyrosine kinases in LPA-induced CTGF expression was investigated. Pretreatment of renal fibroblasts with the inhibitor of Src family kinases PP2 (1 μM) prevented upregulation of CTGF by LPA (Fig. 2A). The structurally related but inactive compound PP3 did not interfere with LPA-induced CTGF expression.

To further characterise the Src family kinase involved in LPA-mediated upregulation of CTGF, fibroblasts lacking the kinases c-Src, Yes and Fyn and fibroblasts reconstituted with c-Src but lacking Yes and Fyn were investigated. The sensitivity towards PP2 proved to be different in those cell lines: while only a small effect of the inhibitor was detectable in c-Src/Yes/Fyn-negative cells, there was a significant inhibition of LPA-induced CTGF expression in Yes/Fyn-negative cells, indicative of a role for c-Src in LPA-mediated upregulation of CTGF.

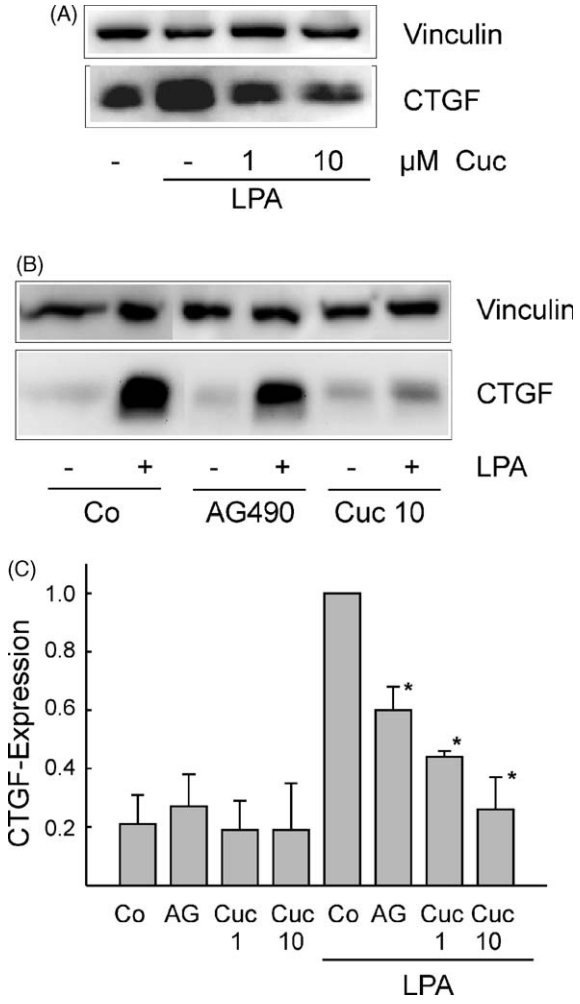

Fig. 1 – Upregulation of CTGF is inhibited by cucurbitacin I and AG490. (A and B) Renal fibroblasts were preincubated with cucurbitacin I (Cuc, 1 and 10 μM, 1 h, as indicated) and AG490 (100 μM, 1 h), and then further incubated with LPA (10 μM, 2 h). CTGF (MW 38 kDa) was detected using a polyclonal goat antibody. The blot was reprobed for vinculin to confirm equal loading. (C) CTGF expression was quantified by luminescent imager analysis. LPA-stimulated CTGF expression was set to 1. The graph shows mean ± S.D. of three experiments. *$p < 0.001$ (ANOVA, Bonferroni).

3.3. *LPA does not induce tyrosine phosphorylation of STAT3 in renal fibroblasts*

Activation of focal adhesion proteins was involved in LPA signalling as shown by a strong increase in tyrosine phosphorylation of proteins of apparent molecular weight of 125–130 kDa and several proteins of apparent molecular weights of about 72 kDa (Fig. 3A). These bands represent focal adhesion proteins as shown previously [28,32]. The upper band comigrated with focal adhesion kinase (FAK) and most likely contained additional proteins such a p130 Cas, and the lower bands comigrated with phosphorylated paxillin. The phosphorylation state of these proteins was critically

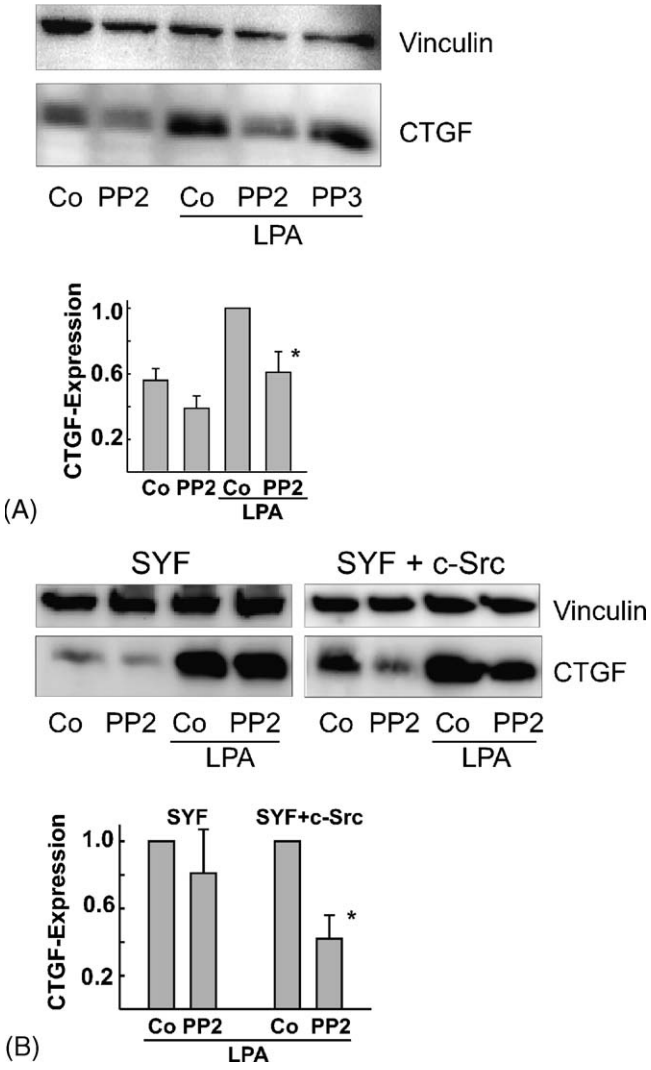

Fig. 2 – Inhibition of Src family kinases reduces LPA-induced CTGF expression and LPA-induced tyrosine phosphorylation. (A) Renal fibroblasts were preincubated with PP2 (1 μM, 30 min) or vehicle (DMSO) and stimulated with LPA (10 μM, 2 h). CTGF expression was detected using a polyclonal goat CTGF antibody. Vinculin was detected to confirm equal loading of the blot. CTGF expression was quantified by luminescent imager analysis with LPA-stimulated CTGF expression set to 1. The graph shows means ± S.D. of three experiments. $^{*}p < 0.001$ (ANOVA, Bonferroni). (B) Fibroblasts defectived in c-Src, Yes and Fyn (SYF) or defective in Yes and Fyn (SYF + c-Src) were preincubated with PP2 (1 μM, 30 min) or vehicle (DMSO) and stimulated with LPA (10 μM, 2 h). To compare different experiments, stimulation with LPA was set to 1. The graph shows means ± S.D. of three experiments. $^{*}p < 0.05$ inhibition of SYF + c-Src compared to inhibition of SYF cells (Student *t*-test).

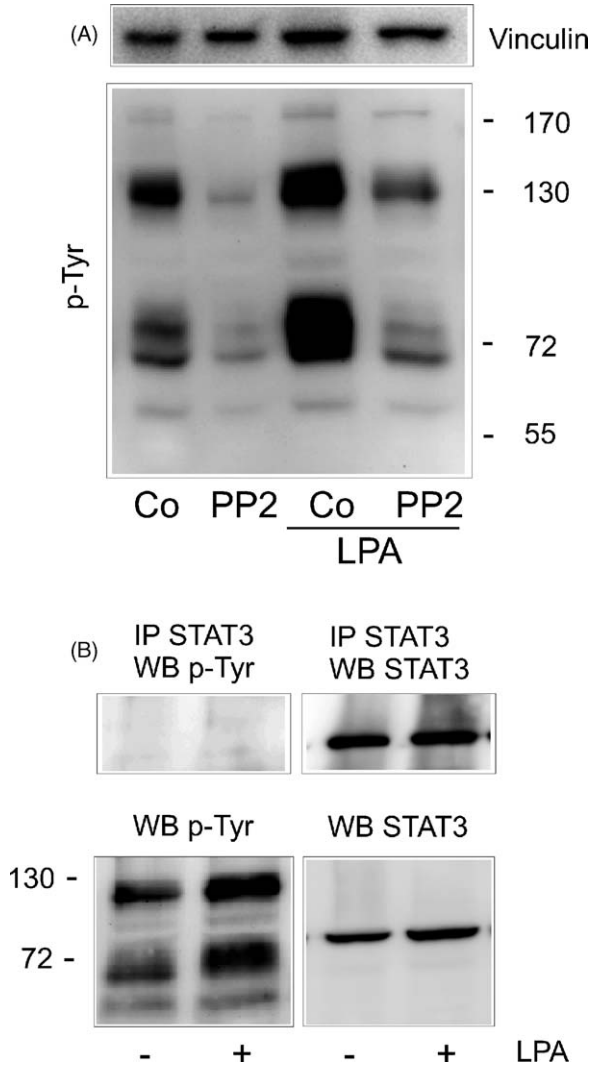

Fig. 3 – LPA activates focal adhesion proteins, but not STAT3. (A) Renal fibroblasts were preincubated with PP2 (10 μM, 30 min) or vehicle (DMSO) and stimulated with LPA (10 μM, 10 min). Tyrosine-phosphorylated proteins were detected by an antibody directed against phosphotyrosine (p-Tyr). The size of the molecular weight marker is indicated. The blot was reprobed for vinculin to confirm equal protein loading. The blot is representative of three independent experiments. (B) Renal fibroblasts were stimulated with LPA (10 μM, 10 min), lysed and immunoprecipitated with an anti-STAT3 antibody. Separated immunoprecipitates were analysed for phosphotyrosine and STAT3 (upper panel). As control aliquots of the lysates were separated by SDS-PAGE and were also analysed for tyrosine phosphorylation and STAT3 (lower panel). The position of molecular weight markers in kDa is indicated. The blots are representative of two independent experiments.

dependent on Src family kinases as shown by the inhibitor PP2. Basal phosphorylation was strongly reduced as was the increase observed after treatment with LPA (10 μM, 10 min, Fig. 3A).

To investigate the involvement of STAT3 in LPA-mediated signalling, tyrosine phosphorylation of STAT3 was assessed. Immunoprecipitation of STAT3 after treatment of the cells with LPA for 10 min and subsequent detection with

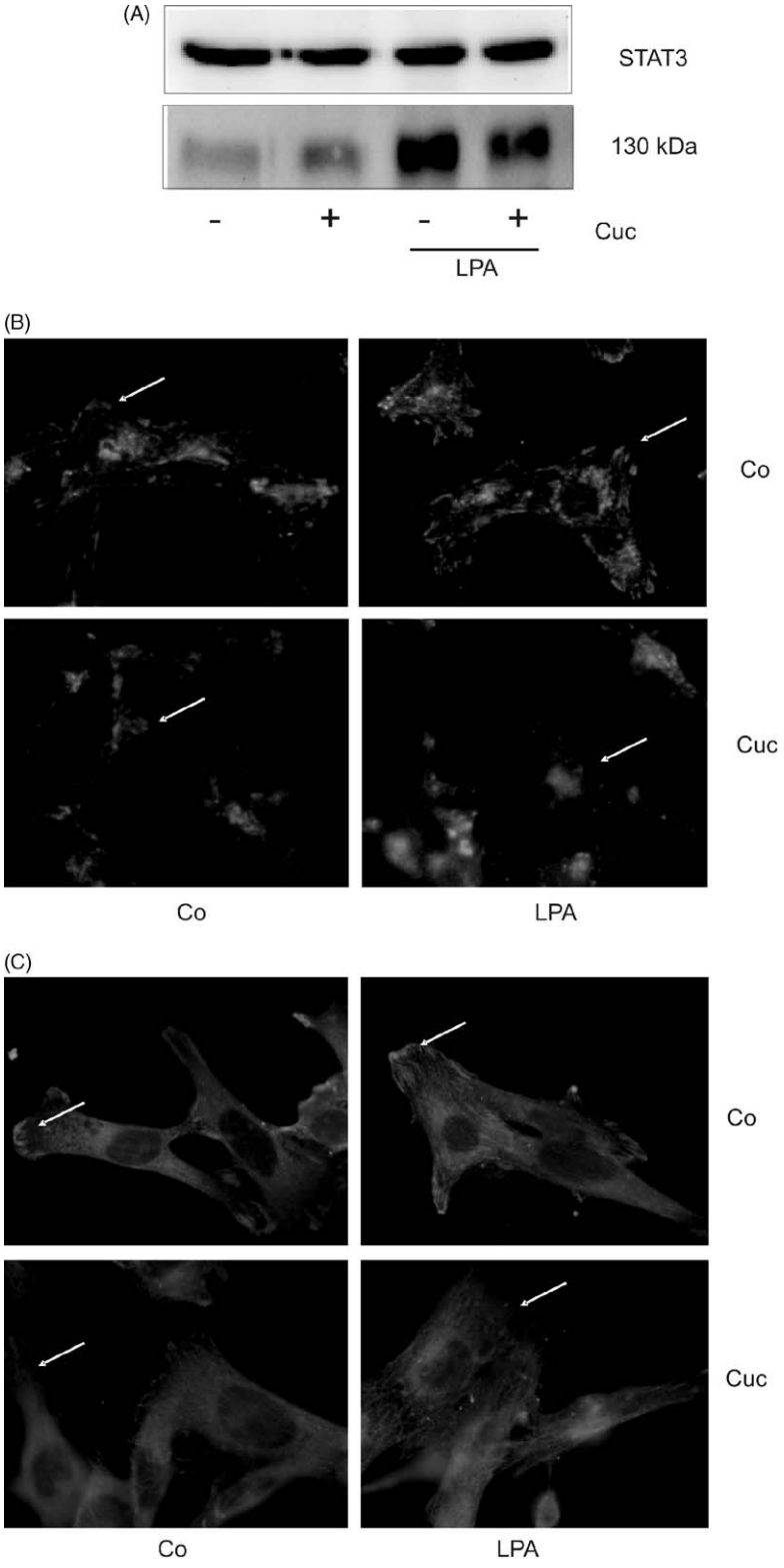

Fig. 4 – Cucurbitacin I affects structure and function of focal adhesion proteins. (A) Renal fibroblasts were preincubated with cucurbitacin I (10 μM, 1 h) and further stimulated with LPA (10 μM, 10 min). Tyrosine-phosphorylated proteins with an apparent molecular weight of 125 kDa were detected. The blot was reprobed for STAT3 to confirm equal loading. The blot is representative of two experiments. (B) Renal fibroblasts were preincubated with cucurbitacin I (1 μM, 30 min), further stimulated with LPA (10 μM, 10 min) and fixed. Tyrosine-phosphorylated proteins were detected with p-tyr-100 as primary antibody and Alexa Fluor 488-coupled secondary antibody. Original magnification 400×. (C) Renal fibroblasts were treated as in Fig. 4B. Folcal adhesions were visualised by staining with anti-vinculin and Alexa Fluor 555-coupled secondary antibody. Original magnification: 1000×.

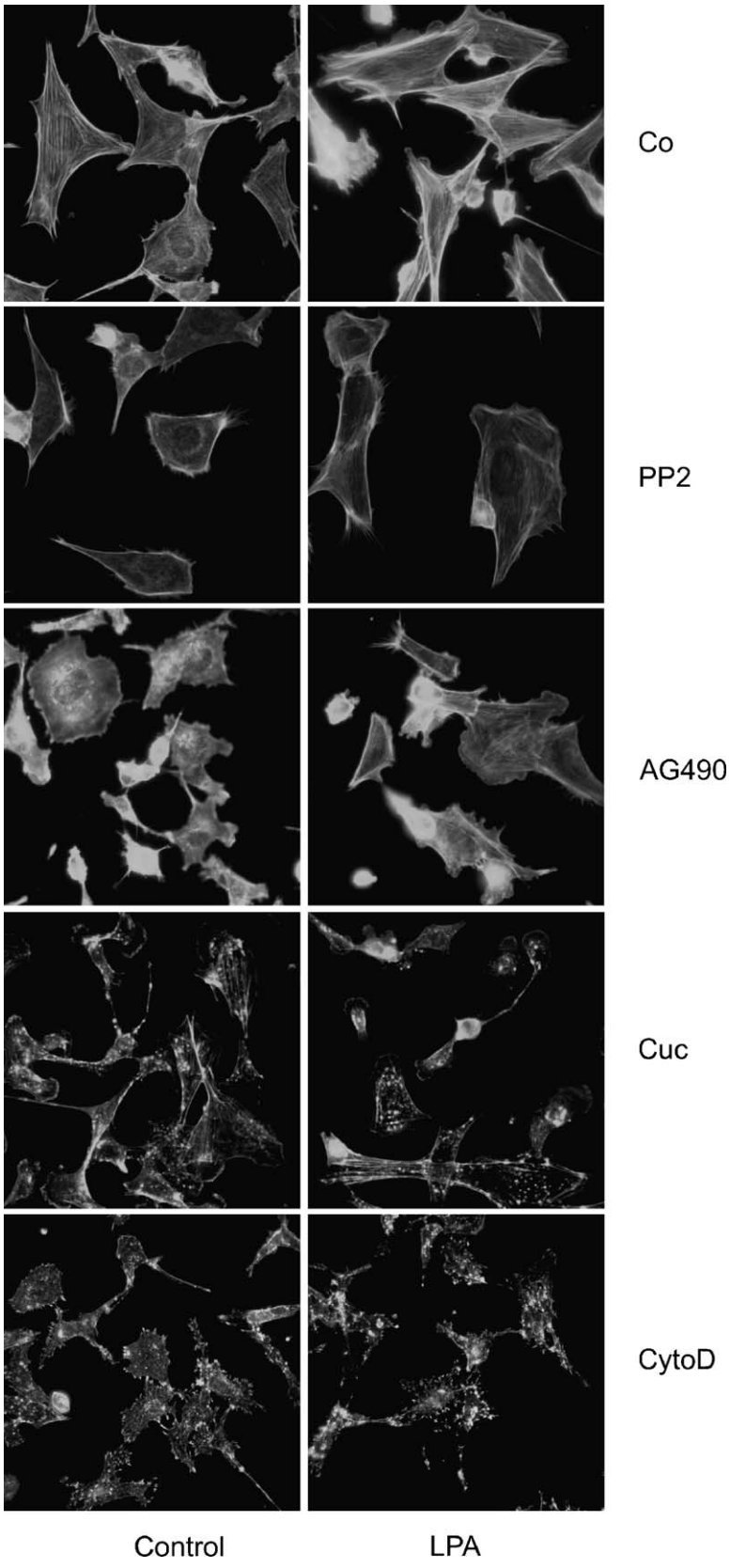

Fig. 5 – Cucurbitacin I, PP2, and AG490 differentially affect F-actin stress fibres. Renal fibroblasts were preincubated with PP2 (10 μM, 30 min), AG490 (100 μM, 30 min), cucurbitacin I (1 μM, 30 min) and cytochalasin D (1 μM, 30 min), and stimulated with LPA for 30 min. F-actin filaments were visualised with Alexa Fluor 488 phalloidin. Photographs were obtained by fluorescence microscopy and are representative of two independent experiments. Original magnification: 400×.

an antibody against phosphotyrosine did not reveal any phosphorylated STAT3 (Fig. 3B, upper panel). Western blot analysis of the same homogenates revealed tyrosine phosphorylation of focal adhesion proteins (Fig. 3B, lower panel). The presence of STAT3 was confirmed by immunodetection of STAT3 in the cellular lysates and the immunoprecipitates. Furthermore, an antibody specific for phospho-STAT3 did not detect STAT3 phosphorylation after incubation of cells with LPA for 5–30 min (data not shown).

3.4. *Cucurbitacin I alters tyrosine-phosphorylated focal adhesions*

Pretreatment of fibroblasts with cucurbitacin I reduced tyrosine phosphorylation of focal adhesion proteins. As an example, the reduction of tyrosine phosphorylation of focal adhesion proteins of apparent molecular weight of 125–130 kDa by cucurbitacin I is depicted in Fig. 4A. The changes in tyrosine phosphorylation were also visualised by immunocytochemistry. Treatment of renal fibroblasts with LPA for 30 min strongly increased tyrosine phosphorylation of focal adhesions (Fig. 4B, upper panel). In the presence of cucurbitacin I, however, no increase in tyrosine phosphorylation was detectable, and even the control cells seemed to be less structured (Fig. 4B, lower panel). The loss of focal adhesion structures became even more evident, when the cells were stained for vinculin, a protein typically located in focal adhesions. In the presence of cucurbitacin I (1 μM), focal adhesion structures disappeared and were not restored upon incubation with LPA, which enlarged focal adhesions in control cells (Fig. 4C). These data indicated that cucurbitacin had profound effects on cell structure.

3.5. *Cucurbitacin I disintegrates F-actin fibres in renal fibroblasts*

To further analyse the structural effects of cucurbitacin I, F-actin fibres were visualised by phalloidin staining. Control cells showed cortical F-actin as well as cell spanning stress fibres. Incubation with LPA for 30 min increased F-actin fibres and induced the formation of lamellipodia as expected by its activation of Rho proteins (Fig. 5). Cucurbitacin I at 1 μM rapidly disassembled F-actin fibres, forming patchy structures. Incubation with LPA could not rescue the deteriorated actin cytoskeleton. The appearance of the cytoskeleton in cucurbitacin I-treated cells closely resembled the patchy F-actin cytoskeleton observed in cells treated with cytochalasin D (1 μM). Reorganisation of the actin cytoskeleton was also observed

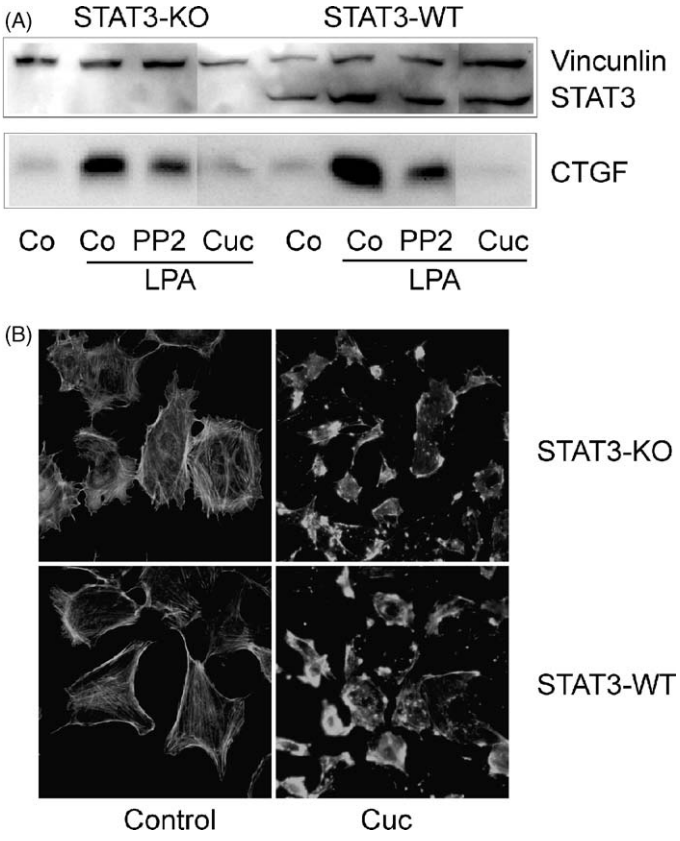

Fig. 6 – LPA-mediated CTGF induction is inhibited by cucurbitacin I in STAT3-KO cells. (A) STAT3-KO cells and STAT3-WT cells were preincubated with PP2 (10 μM, 30 min), cucurbitacin I (10 μM, 30 min) or vehicle (DMSO) and stimulated with LPA (10 μM, 2 h). CTGF expression was detected using polyclonal goat CTGF antibody. The blot was reprobed for STAT3. The blot is representative of three experiments with comparable results. (B) STAT3-KO cells and STAT3-WT cells were incubated with cucurbitacin I (1 μM, 30 min), fixed and stained with Alexa Fluor 488 phalloidin. Photographs were obtained by fluorescence microscopy (original magnification: 400×) and are representative of two independent experiments.

when the cells were treated with PP2 or AG490. Inhibition of Src family kinases with PP2 led to cell rounding and abolished the extension of F-actin fibres to the cell periphery. Phenotypic changes by AG490 were less prominent. While some cells were indistinguishable from control cells, others appeared contracted and showed reduced F-actin fibres. Incubation with LPA did not overcome these phenotypic changes.

3.6. *Cucurbitacin I interferes with CTGF induction in STAT3 knock out cells*

To further distinguish between effects of cucurbitacin I mediated by STAT3 or alterations of the actin cytoskeleton, STAT3 knock out (STAT3-KO) fibroblasts were compared to the respective wild type cells (STAT3-WT). Preincubation of STAT3 knock out and STAT3 wild type cells with cucurbitacin I prevented upregulation of CTGF by LPA (Fig. 6A). In both cell types, interference with the activity of Src family kinases inhibited CTGF induction to a comparable extent indicating mediators other than STAT3 to be involved in CTGF expression downstream of Src kinase.

STAT3 knock out cells showed multiple F-actin spikes extending into filopodia like structures (Fig. 6B). Upon treatment with cucurbitacin I (1 μM, 30 min), STAT3 wild type and knock out cells reacted identically. Within half an hour F-actin fibres were reorganised into actin clumps located all over the cells.

4. Discussion

By investigating different types of fibroblasts in vitro, cucurbitacin I was shown to interfere with LPA-mediated upregulation of CTGF. At the molecular level, involvement of STAT3 was excluded cucurbitacin I being as effective in STAT3 wild type as in STAT3 knock out cells. More likely the inhibitory effect was attributable to the complete disassembly of F-actin fibres and destabilisation of focal adhesions observed even at low concentrations of the drug.

RhoA-dependent formation of stress fibres and assembly of focal adhesions is a well known response to LPA stimulation (e.g. [33–36]). Reorganisation of focal adhesions is accompanied by activation of focal adhesion proteins including FAK or paxillin by tyrosine phosphorylation. The molecular mediators linking activated RhoA and tyrosine phosphorylation of focal adhesion proteins have not yet been identified. In Swiss 3T3 fibroblasts, Salazar et al. provided evidence that tyrosine phosphorylation of FAK or paxillin was not mediated by receptor cross talk of the LPA receptors with the epidermal growth factor (EGF) receptor [37]. In this study we could show in different types of fibroblasts that Src family kinases were involved in LPA-mediated tyrosine phosphorylation of focal adhesion proteins. These data were consistent with our previous observation that tyrosine phosphorylation of focal adhesion proteins mediated by RhoA-induced focal adhesion clustering was abrogated by PP2 in human renal fibroblasts [28]. Comparison of cells expressing c-Src and those without this kinase indicated involvement c-Src itself in LPA-mediated CTGF induction. However, a partial effect of PP2 was also observed in cells lacking the Src family kinases Yes, Fyn and c-Src suggesting an additional impact of other kinases of this family.

Activation of several G-protein-coupled receptors has been linked to Jak2–STAT3 signalling most data being available for angiotensin II [38]. Angiotensin II was shown to activate Jak2–STAT3 signalling in various cell types, including cardiac myocytes [15,39] and vascular smooth muscle cells [17,40,41]. In earlier studies, we had shown induction of CTGF by angiotensin II in human renal fibroblasts [42]. Furthermore, activation of Rho GTPases has been linked to STAT3 as downstream mediator [18]. Overexpression of RhoA or oncogenic v-Src activated tyrosine phosphorylation of Jak2 and STAT3 [19]. Most interestingly, tyrosine phosphorylation of STAT3 in v-Src overexpressing NIH 3T3 cells was sensitive to cucurbitacin I [6]. Based on these data, we had postulated STAT3 as downstream mediator of LPA-mediated activation of focal adhesion proteins, which was dependent on Src family kinases. However, we did not obtain any evidence for tyrosine phosphorylation of STAT3 upon incubation of the cells with LPA for various time intervals. Functionally, involvement of STAT3 in LPA-mediated CTGF induction was excluded by the analysis of STAT3 knock out cells. Induction of CTGF by LPA was not impaired in these cells compared to wild type cells, and was sensitive to PP2, the inhibitor of Src family kinases. Other potential down-stream mediators of Src kinase were not investigated in this study, but may include serum response factor, which can be activated by RhoA, and also by Src kinase [43,44].

Given the missing activation of STAT3, it became evident that the strong inhibition of LPA-mediated CTGF expression by cucurbitacin I was not attributable to inhibition of Jak2–STAT3 signalling, as suggested by the results of Blaskovich et al. [6]. However, cucurbitacin I led to an appearance of the F-actin cytoskeleton which resembled the appearance of cells treated with cytochalasin D or lantrunculin B, characterised by F-actin patches [25]. Similar results were reported earlier by Duncan and Duncan in prostate cancer cells and proliferating endothelial cells [4,5]. Their results are not directly comparable to the data obtained in this study as different cell types and different time intervals were used. In their experiments, cucurbitacin E was more active than cucurbitacin I and Q, which still disrupted the actin cytoskeleton of prostate tumour cells within 24 h at 50–100 nM [5]. Concomitant with the destabilisation of the F-actin fibres, focal adhesion structures disappeared in cells treated with cucurbitacin I. Whether this was a consequence of the cucurbitacin I-mediated disassembly of F-actin or whether cucurbitacin I interacted directly with focal adhesion proteins remains to be investigated. Taken together, these data indicate that cucurbitacins at concentrations which inhibit tyrosine phosphorylation of Jak2 and/or STAT3 will interfere with the integrity of tumour cells as well as tissue cells such as endothelial cells or fibroblasts, as shown in this study.

Disruption of the actin cytoskeleton was reported earlier to prevent LPA-induced upregulation of CTGF in mesangial cells and fibroblasts [45,46]. Therefore, it is concluded that cucurbitacin I inhibited CTGF induction by interference with the integrity of the actin cytoskeleton. Only a partial inhibition of CTGF induction was observed with the Jak2 inhibitor AG490. Whether the rather moderate cytoskeletal effects of AG490

contributed to the inhibition of LPA-induced CTGF expression, or whether other down stream mediators of Jak2 were responsible for the inhibition remains to be clarified.

Our data do not question the inhibition of Jak2–STAT3 signalling described in other cell types and potentially also in fibroblasts if this pathway is activated. However, they clearly indicate that alterations of the actin cytoskeleton and focal adhesions have to be taken into consideration, when the biological effects of cucurbitacins are discussed. Depending on the extent of cytoskeletal alterations these may interfere with or facilitate cellular actions such as migration or proliferation. Therefore, the consequences of cucurbitacin-induced changes of cellular structures need to be evaluated in vivo.

Acknowledgments

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