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LPS regulate ERK1/2-dependent signaling in cardiac fibroblasts via PKC-mediated MKP-1 induction

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Abstract

Activation of MAPK pathways by angiotensin II (Ang II) is important for cardiac fibroblast (CFB) proliferation and migration. Activity of MAP-kinases is closely controlled by a group of dual-specific MAP kinase phosphatases (MKPs). Lipopolysaccharides (LPS) and cytokines are elevated in patients with heart failure and may contribute to disease progression. In this study, we investigate the effect of LPS on Ang II-induced CFB function. Pretreatment of CFBs with LPS (1 μg/mL; 30 min) almost completely inhibited Ang II-induced DNA-synthesis and inhibited Ang II directed chemotaxis by more than 80%. Compared to controls, LPS pretreatment significantly reduced phosphorylation levels of ERK1/2- and p38 MAPK and induced MKP-1 levels. Silencing MKP-1 with antisense oligodesoxynucleotides reversed the antimitogenic effect of LPS on Ang II-induced CFB DNA-synthesis and migration. Induction of MKP-1 by LPS was inhibited by the protein kinase C (PKC)-inhibitor calphostin C, but not by the ERK1/2-pathway inhibitor PD98059, suggesting that PKC but not ERK1/2 is required for LPS-mediated MKP-1 induction in CFBs. Our data demonstrate that LPS have direct cellular effects in CFBs through an inhibition of Ang II-induced MAPK activity via PKC-mediated induction of MKP-1. This might be relevant with regard to the decreased MAPK activity and increased levels in MKPs reported during chronic heart failure in humans.

Keywords: Angiotensin II; Cardiac fibroblasts; Protein kinases; MAP kinase phosphatases; Heart failure

Lipopolysaccharides (LPS; endotoxin) are the major constituents of the outer membrane of gram-negative bacteria. Stimulation with LPS leads to the induction and secretion of several proinflammatory cytokines and lipid mediators, which in excess, lead to organ failure and death. Recently, it has been recognized that levels of LPS are elevated in chronic heart failure with acute oedematous exacerbation, possibly due to increased venous congestion and subsequent increase in gut permeability, leading to bacterial/LPS translocation [1]. These levels are well below those found in sepsis and are accompanied by increases in different cytokines (e.g., TNF-α) and soluble CD14 in vivo [1]. Even though CD14 binds LPS and initiates signal transduction, it lacks a transmembrane domain and is therefore not capable of initiating a transmembrane signal by itself [2]. Receptors for LPS, the so called Toll-like receptors TLR2 and TLR4, have been shown on cardiac myocytes and fibroblasts [3,4]. Both, TLR2 and TLR4, mediate LPS-induced intracellular signaling [5,6], leading to the activation of NF-κB and c-Jun N-terminal kinase (JNK), which initiate the transcription of several proinflammatory cytokines.

Angiotensin II (Ang II) is critical for cardiac remodeling and cardiac fibrosis [7–9]. Induction of MAP-kinases is required for Ang II-induced cardiac fibroblast (CFB) proliferation and migration [10,11].While activation of ERK1/2 MAP-kinase has been identified to enhance cell growth and migration, the stress-activated protein kinases (JNK/SAPK) and p38 MAP-kinase are involved in the inhibition of cell growth as well as the induction of apoptosis [12]. Full activity of all three MAPKs requires reversible dual phosphorylation at threonine and tyrosine residues by individual upstream MAPK-kinases (MAPKK) [13]. A family of dual specificity mitogen-activated protein kinase phosphatases (MKPs) has been identified to control ERK1/2, JNK/

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SAPK, and p38 MAPK activity [14]. So far, at least nine different mammalian MKPs have been described and named MKP-1, -2, -3, -4, PAC-1, hVH3, hVH5, B59, and MKP-X [14]. Generally, it is thought that upon MAPK activation, MKPs are induced within 30–60 min, leading to dephosphorylation and inactivation of ERK1/2, JNK, and p38 MAPK signaling [14]. Transcriptional activation of MKP-1 has been suggested to require ERK activation [15]. MKP-1 is expressed in the heart where it limits the hypertrophic response of cardiomyocytes to aortic banding and catecholamines [16].

However, the regulation and significance of MKP-1 in cardiac fibroblasts (CFBs) are poorly understood. While some cytokines (e.g., IL6) have been shown to synergistically interact with Ang II to increase CFB proliferation/migration and Ang II-mediated hypertrophy [17], little is known about the potential crosstalk of LPS and Ang II. In the present study we therefore investigated the effects of LPS on Ang II-mediated cellular function. We find that LPS-pretreatment inhibits CFB DNA-synthesis and migration via induction of MKP-1, thereby inhibiting Ang II-induced MAPK activity.

Materials and methods

Materials

Angiotensin II was purchased from Bachem and bacterial lipopolysaccharides (from *Escherichia coli*) were from Sigma. Calphostin C and PD98059 were from Biomol. Cell culture media and materials were from Gibco. All other chemicals were purchased from Sigma. Phosphorothioate-conjugated antisense oligodesoxynucleotiodes (asODNs) targeting MKP-1 were costume made and purified (>98%) by HPLC (TRI-MOLBIOL). The liposomal transfection reagent OligofecTAMINE was from Gibco. The following antibodies were used: actin (Sigma), protein phosphatase 1 (PP1; BD Bioscience), protein phosphatase 2A (PP2A; BD Bioscience), and rabbit-polyclonal MAP kinase phosphatase-1 (MKP-1; Santa Cruz). Levels of signaling pathway induction were assessed by the use of phospho-specific antibodies, recognizing ERK1/2 MAP-kinase when phosphorylated at threonine 202 and tyrosine 204 (Promega), p38 MAP-kinase when phosphorytated at threonine 180 and tyrosine 182 (Cell Signaling), and $p70^{s6}$-kinase when phosphorylated at threonine 421 and serine 424 (Cell Signaling).

Methods

Isolation and culture of rat cardiac fibroblasts. Adult cardiac fibroblasts were prepared from Sprague–Dawley rats as described [18,19]. Fibroblasts were grown in DMEM/F12 with 10% FBS until they reached confluency, then detached by trypsin treatment (0.5%), and split 1:4. All experiments were performed in the 2nd–5th passage after starvation in serum free DMEM/F12 containing insulin (5 μg/mL), transferrin (5 μg/mL), and selenium (5 ng/mL) for 24 h. Thereafter cells were incubated with LPS, Ang II or inhibitors for various time intervals, as indicated in Results. Culture of rat vascular smooth muscle cells (VSMCs) has been described recently [20]. All experiments were done in triplicates with different preparations of cells.

Liposomal transfection of rat cardiac fibroblasts. The specificity of the MKP-1 phosphorothioate-conjugated oligodesoxynucleotiode (ODN) sequences used in this study has been demonstrated by Duff et al. [21]. For antisense, sense, and scrambled 20-mer ODNs with the following sequences were used: 5′-GGAACTCAGTGGAACTCAGG-3′ for antisense, 5′-CCTGAGTTCCACTGAGTTCC-3′ for sense, and 5′-AGG TCCTGAAAGCGAAGTCG-3′ for scrambled. Transfection was done with the help of OligofecTAMINE reagent used according to the manufacturer's instructions. Briefly, subconfluent (passage 2–5) CFBs were growth arrested overnight in serum-free DMEN/F12. Cells were then incubated in fresh serum-free DMEN/F12 supplemented with ODNs (0.4 or 1 μmol/L) for 6 h. After this period, 0.5% FCS was added and cells were maintained in 0.5% FCS–DMEN/F12 with ODNs for an additional 18 h. Following this 24 h transfection period in serum-deficient medium, cells were used in experiments (see Results). Successful transfection was determined with the use of an FITC-conjugated asODNs control by immunofluorescence and was >75%. Cell viability was assessed by trypan-blue staining.

Western blot. Immunoblotting was done as described previously [20]. Briefly, proteins were extracted in RIPA-buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing freshly dissolved protease inhibitors (Complete EDTA-free, Boehringer). Up to 50 μg of proteins was mixed with sample buffer and applied on 8% SDS–polyacrylamide gel electrophoresis. Following migration, proteins were transferred onto nitrocellulose membranes (BioRad) which were then incubated overnight at 4 °C with the primary antibody. Afterwards membranes were incubated with appropriate secondary antibodies coupled with IgG-peroxidase (Jackson Lab.). Peroxidase activity product was revealed with the ECL-method (Amersham). Semiquantitative densitometry was done using the NIH program 1.62 and is expressed in arbitrary units (A.U.).

BrdU assay. CFBs were starved in serum-free DMEM/F12 medium for 48 h. LPS treatment was started 30 min before stimulation with Ang II (0.1 and 1 μmol/L) or diluent. BrdU was added after 24 h for another 8 h period. Then supernatants were discarded and the cells were fixed and prepared for measurement with a commercially available ELISA (Boehringer).

Migration assay. Chemotaxis experiments were performed as described previously [22]. CFB chemotaxis was examined in transwell cell culture chambers (Gibco) using a gelatin-coated (0.2%) polycarbonate membrane with 8 μm pores. The number of CFBs per high power field (HPF, magnification 320×) that migrated to the lower surface of the filters was determined microscopically. Four–six randomly chosen HPFs were counted per filter. Experiments were performed in triplicate and repeated at least three times.

Statistical analysis. Analysis of variance, paired or unpaired *t* tests were performed for statistical analysis as appropriate. Statistical significance was designated at a probability value of less than 0.05.

Results

LPS inhibits the mitogenic effects of angiotensin II via inhibition of MAP-kinase

To investigate the effects of LPS on Ang II-induced DNA-synthesis and migration of CFBs, cells were preincubated with 1 μg/mL LPS for 30 min, followed by 24 h stimulation with Ang II (0.1 and 1 μmol/L). We found that LPS almost completely inhibits Ang II-induced DNA-synthesis of CFBs (Table 1A; $p < 0.05$ vs. controls). Furthermore, LPS preincubation (1 μg/mL; 30 min) significantly inhibited the Ang II-directed (1 μmol/L) migration through gelatine-coated membranes by more than 80% (Table 1B; $p < 0.05$ vs. controls). To identify the signaling pathways involved,

Table 1A
DNA-synthesis was determined by BrdU uptake using an commercially available ELISA

	Diluent	LPS 1 μg/mL
Control	100.2 ± 5.4	91.4 ± 6.3
Ang II 1 μmol/L	314.3 ± 21.8*	98.8 ± 8.7#
Ang II 0.1 μmol/L	243.8 ± 11.0*	106.4 ± 10.2#

LPS (1 μg/mL) was given 30 min before the addition of Ang II (0.1 and 1 μmol/L). Cells were then maintained for 24 h with Ang II or diluent and BrdU was then added for another 8 h. Values are expressed in percent of control (%±SEM; *$p < 0.05$ vs. control, #$p < 0.05$ vs. Ang II).

Table 1B
Chemotaxis experiments were performed as described previously [11]

	Diluent	LPS 1 μg/mL
Control (5% FCS)	1.2 ± 0.4	1.4 ± 0.5
Ang II 1 μmol/L	4.7 ± 0.8*	1.6 ± 0.3#

Cells were pretreated for 30 min with LPS (1 μg/mL) before starting chemotaxis experiments. Values are expressed in cells per highpower field ($n \pm$ SEM; *$p < 0.05$ vs. control, #$p < 0.05$ vs. Ang II).

CFBs were pretreated (30 min) with 1 μg/mL LPS followed by stimulation with Ang II (1 μmol/L; 30 min). Compared to Ang II alone, LPS significantly reduced phosphorylation levels of ERK1/2- and p38 MAP-kinases in response to Ang II (both $p < 0.05$), whereas phosphorylation of the ribosomal S6 kinase ($p70^{s6K}$) by Ang II (1 μmol/L) was not inhibited (Fig. 1).

Silencing MKP-1 reverses the inhibitory effects of LPS on Ang II-induced DNA-synthesis and migration of rat cardiac fibroblasts

Activity of MAP-kinases has been demonstrated to be closely controlled by the dual specificity mitogen-activated protein kinase phosphatase MKP-1 [14]. Thus, immunoblotting revealed that incubation of CFBs with LPS results in a transient induction of MKP-1 gene expression at 30–60 min (1 μg/mL, Figs. 2A and C). This increase of MKP-1 by LPS was concentration-dependent, with a significant increase at 1 μg/mL (30 min incubation; Figs. 2B and D). Levels of the ubiquitously expressed serine/threonine protein phosphatases PP1 and PP2A were not altered by LPS treatment (Fig. 2B). These data indicated that the observed inhibitory effects of LPS on Ang II-induced phosphorylation of ERK1/2 and p38 MAPK (Fig. 1) and the concurrent inhibition of CFB DNA-synthesis and migration (Tables 1A and 1B) result from an LPS-mediated increase of MKP-1. To test our hypothesis, we used specifically designed antisense ODNs to silence MPK-1 levels in CFBs. Fig. 3A demonstrates that antisense ODNs targeting MKP-1 inhibited MKP-1 protein expression at a concentration of 1 μmol/L, whereas transfection with the corresponding sense and scrambled ODN sequences had no effect ($p < 0.05$ vs. sense and scrambled sequences). Transfection of CFBs with antisense ODNs (1 μmol/L) specifically reversed LPS (1 μg/mL; 30 min)-mediated inhibition of Ang II-induced DNA-synthesis (Fig. 3C; $p < 0.05$ vs. sense and scrambled) to almost Ang II levels ($p =$ n.s.). Similar results were found for Ang II-

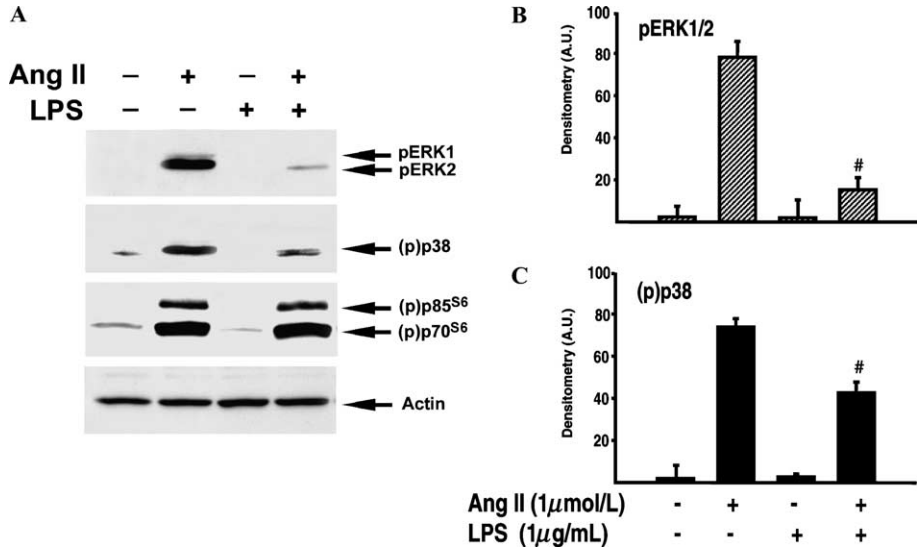

Fig. 1. To investigate the signaling pathways involved, CFBs were stimulated with Ang II (1 μmol/L; 30 min), LPS (1 μg/mL; 30 min), or Ang II after preincubation with LPS (1 μg/mL; 30 min). Western blot analysis showed that Ang II rapidly induced phosphorylation of ERK1/2, p38 MAPK, and $p70^{s6K}$, whereas LPS alone had no effect (A). Preincubation of CFBs with LPS resulted in a significant decrease in the levels of phospho-(p)ERK1/2 and (p)p38 MAPK, whereas levels of (p)$p70^{s6K}$ were not influenced. Semiquantitative densitometry for pERK1/2 (B) and (p)p38 MAPK (C) is shown (#$p < 0.05$).

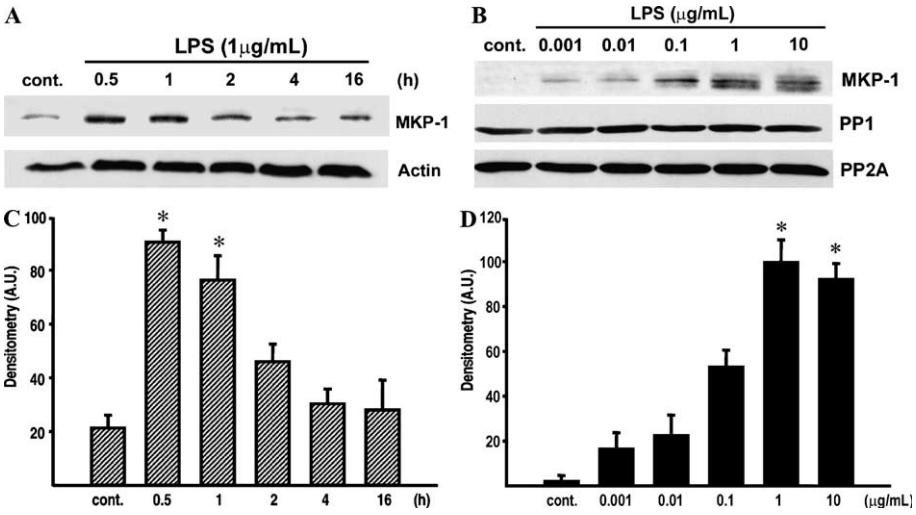

Fig. 2. LPS induces MKP-1 expression in a time- and concentration-dependent manner, whereas protein levels of the protein phosphatases PP1 and PP2A are not affected by LPS. CFBs were incubated with LPS (1 μg/mL) for various times (30 min to 16 h). Immunoblotting revealed a transient increase of MKP-1 levels at 30–60 min (A). Densitometric measurements are depicted in (C) ($*p < 0.05$). The increase in MKP-1 levels was concentration dependent, with a significant increase at 1 μg/mL (30 min stimulation, (B), densitometry is demonstrated in (D); $*p < 0.05$).

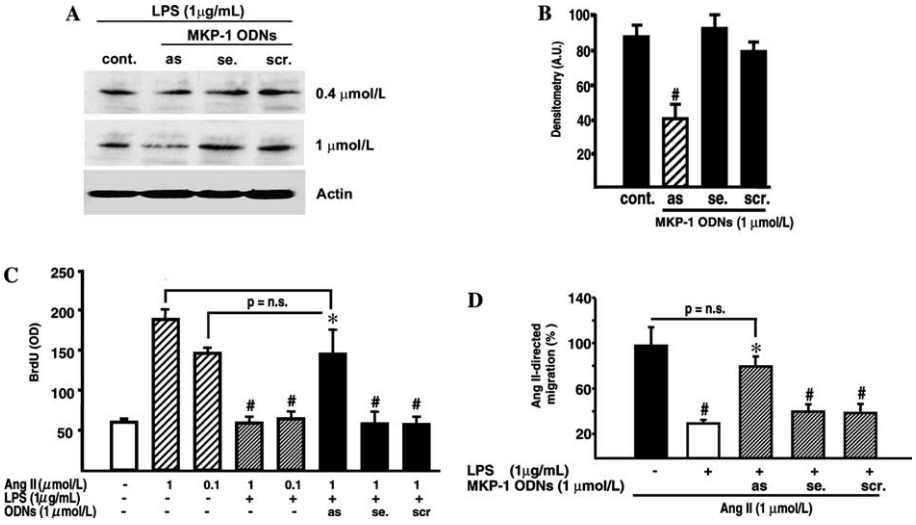

Fig. 3. Silencing of MKP-1 protein levels was done by transfection of CFBs with antisense (as) ODNs. Specificity of sequences used is demonstrated by immunoblotting with antibody against MKP-1 (A; densitometry depicted in B, $\#p < 0.05$ vs. control (cont.), sense (se.), and scrambled (scr.) ODNs). Equal loading of membranes is demonstrated by actin reblotting. Transfection of CFBs with asODNs reversed the LPS-mediated inhibition of Ang II-induced BrdU-incorporation to almost Ang II levels (C; $p =$ n.s. vs. Ang II alone), whereas se. and scr. ODN sequences had no effect (C; $\#p < 0.05$ vs. Ang II alone). Similar results were obtained for Ang II-directed chemotaxis (D; $*p =$ n.s. vs. Ang II alone; $\#p < 0.05$ vs. LPS alone and vs. control).

directed chemotaxis of CFBs, where MKP-1 antisense ODNs reversed the antimigratory effects of LPS (1 μg/mL; 30 min; Fig. 3D, $p < 0.05$ vs. sense and scrambled; $p =$ n.s. vs. control).

MKP-1 induction by LPS requires PKC signaling in rat cardiac fibroblasts

Both, ERK1/2 and PKC, might be involved in MKP-1 regulation [21,23]. Thus, to explore the signaling pathway of LPS-mediated MKP-1 induction in CFBs, we performed pharmacological inhibition experiments. CFBs were incubated with the ERK1/2-pathway inhibitor PD98059 (30 μmol/L) or the protein kinase C-inhibitor calphostin C (100 nmol/L) for 30 min and then stimulated with 1 or 10 μg/mL LPS for 30 min in the presence of the inhibitor. While the ERK1/2-pathway inhibitor PD98059 did not affect LPS-mediated induction of MKP-1, the PKC-inhibitor calphostin C significantly decreased LPS-induced MKP-1 levels ($p < 0.05$

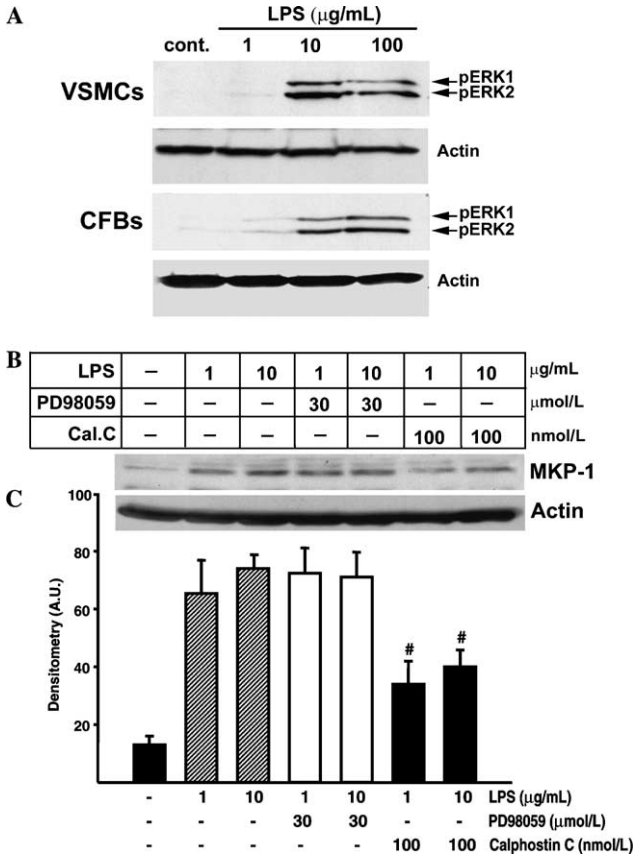

Fig. 4. ERK/12 is induced by LPS treatment at concentrations above 1 μg/mL in VSMCs and CFBs (A). Equal loading of membranes is demonstrated by reblotting with actin. To investigate the signaling pathways involved, CFBs were preincubated for 30 min with the ERK1/2-pathway inhibitor PD98059 or the PKC-inhibitor calphostin C (Cal.C) and then stimulated with LPS (1 μg/mL; 30 min). Immunoblotting revealed the inhibition of LPS-induced MKP-1 levels by calphostin C, whereas PD98059 had no effect (B). Densitometry is shown in C (#$p < 0.05$ vs. PD98059 and vs. control).

vs. PD98059 and controls), indicating that PKC activation is required for LPS signaling (Figs. 4B and C). Yamakawa et al. [24] reported that in VSMCs LPS induced phosphorylation of ERK1/2 occurred only at concentrations higher than 1 μg/mL (Fig. 4A). We observed comparable results in CFBs, since the LPS concentration used throughout this study (1 μg/mL) had no effect on ERK1/2-phosphorylation levels, whereas a significant increase was found at higher LPS concentrations of 10 and 100 μg/mL (Fig. 4A).

Discussion

The major finding of the present study is that LPS treatment of CFBs inhibits Ang II-induced MAPK activity via upregulation of MKP-1, consecutively inhibiting Ang II-induced ERK1/2 and p38 MAPK phosphorylation. The activity of the ribosomal $p70^{s6K}$, which lies on a distinct pathway [25], was not affected by LPS. Activity of ERK1/2 [10,11] and p38 MAPK [26,27] is necessary for Ang II-induced CFB cell proliferation and chemotaxis. In accordance, we show that LPS treatment inhibits Ang II-induced DNA-synthesis and migration of CFBs. These inhibitory effects were reversed by silencing LPS-induced MKP-1 protein levels with specific antisense oligodesoxynucleotides, restoring MAPK activity, thus further supporting the notion that activity of MAPK is crucial for CFB proliferation and migration.

MKP-1 is a widely expressed prototype of MKPs and has equal substrate specificity towards ERK1/2 and p38 MAPK [14]. Generally, it is believed to be a downstream target of ERK1/2, which translocates to the nucleus and there activates MKP-1, providing a negative feedback mechanism [14]. In VSMCs, MKP-1 induction by Ang II coincides with the inactivation of ERK1/2 [21] and the activation of ERK1/2 by Ang II was prolonged by silencing MKP-1 activity with antisense oligodesoxynucleotides [28]. An ERK1/2-dependent MKP-1 regulation is further supported by the use of the specific ERK1/2-pathway inhibitor PD98059, which inhibited Ang II-stimulated MKP-1 expression [29]. Still, enzymes other than MKPs have been implicated in the downregulation of MAPK activity. For example, the serine/threonine protein phosphatases 1 (PP1) and PP2A have been shown to regulate MAPK signaling [30]. Results obtained in the present study did not show any significant regulation of these protein phosphatases by LPS, excluding a significant contribution.

In contrast to an ERK1/2-dependent MKP-1 induction, it was recently shown in macrophages that activation of MKP-1 by LPS requires protein kinase C (PKC) activity. Activation of MKP-1 was blocked with the use of PKC-inhibitors and PKCε-specific antisense oligodesoxynucleotides [23]. Furthermore, in VSMCs, induction of phosphorylation of ERK1/2 by LPS has been shown to occur only at a concentration of 10 μg/mL [24], which is 10-fold higher than what was used in this study and not found in chronic heart failure [1]. Comparable results were obtained in the present study: even though LPS induced MKP-1 and inhibited Ang II-induced ERK1/2 and p38 MAPK phosphorylation at a concentration of 1 μg/mL, it did not induce ERK1/2 activity in VSMCs and CFBs, whereas higher concentrations (10 and 100 μg/mL) led to a vigorous phosphorylation. Induction of MKP-1 gene expression by 1 μg/mL LPS was inhibited by the PKC-inhibitor calphostin C, whereas the ERK1/2-pathway inhibitor PD98059 had no effect. These data indicate that PKC-activity is required for MKP-1 induction by LPS, which can occur in CFBs in an ERK-independent manner.

Several studies demonstrate the downregulation of MAPK activity in chronic heart failure in humans, a situation when low LPS concentrations might have

potential pathophysiological effects [1] and the renin–angiotensin-system is activated. In explanted hearts from patients with end-stage heart failure due to dilative cardiomyopathy decreased activity of p38 MAPK [31–33] and ERK1/2 has been reported [32], whereas JNK activity was unchanged [31]. Furthermore, chronic heart failure is paralleled by increases in the levels of MKPs in humans, which might potentially contribute to decreased MAPK activity [32]. Thus, multiple MAP-kinase pathways might be differentially regulated and stage specific activated in chronic heart failure in humans. Still, in contrast to the negative-crosstalk between LPS and the ERK1/2 and p38 MAPK branches of the Ang II signaling pathway demonstrated in the present study, a study by Yokoyama et al. [34] recently demonstrated that Ang II and LPS may also engage in a similar signaling on CFBs. Thus, in CFBs, both, Ang II and LPS, induce TNF-α biosynthesis [34], which potentially contributes to heart failure progression.

In conclusion, our study indicates that LPS-stimulated MKP-1-induction of CFBs interferes with Ang II signaling pathways, leading to an imbalance of Ang II-induced signaling via MAPK, which significantly impairs CBF function such as proliferation and motility. The in vivo significance of increased LPS levels reported in heart failure needs to be further elucidated.

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