Heme Oxygenase-1 Inhibits the Proliferation of Pancreatic Stellate Cells by Repression of the Extracellular Signal-Regulated Kinase1/2 Pathway

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ABSTRACT

Activation of pancreatic stellate cells (PSCs) is the key process in the development of pancreatic fibrosis, a common feature of chronic pancreatitis and pancreatic cancer. In recent studies, curcumin has been shown to inhibit PSC proliferation via an extracellular signal-regulated kinase (ERK)1/2-dependent mechanism. In addition, curcumin is a potent inducer of the cytoprotective enzyme heme oxygenase-1 (HO-1) in other cell types. Therefore, the aims of this study were to 1) characterize the effect of curcumin on HO-1 gene expression in PSCs, 2) explore whether HO-1 induction contributes to the inhibitory effect of curcumin on PSC proliferation, and 3) clarify the involvement of the mitogen-activated protein kinase (MAPK) family in this context. Cultured rat PSCs were incubated with curcumin and assessed for HO-1 up-regulation by Northern blot analysis, immunoblotting, and activity assays. The effect of HO-1 on platelet-derived growth factor (PDGF)-induced PSC proliferation and MAPK activation was determined by immunoblotting, cell proliferation assays, and cell count analyses. Curcumin induced HO-1 gene expression in PSCs in a time- and dose-dependent manner and inhibited PDGF-mediated ERK1/2 phosphorylation and PSC proliferation. These effects were blocked by treatment of PSCs with tin protoporphyrin IX, an HO inhibitor, or transfection of HO-1 small interfering RNA. Our data provide evidence that HO-1 induction contributes to the inhibitory effect of curcumin on PSC proliferation. Therefore, therapeutic up-regulation of HO-1 could represent a mode for inhibition of PSC proliferation and thus may provide a novel strategy in the prevention of pancreatic fibrosis.

Pancreatic fibrosis is a common histopathological feature in chronic pancreatitis and pancreatic cancer. It is now generally accepted that pancreatic stellate cells (PSCs) play a crucial role in the fibrogenic process (Omary et al., 2007). In response to profibrogenic stimuli, PSCs undergo transdifferentiation from quiescent phenotypes into highly proliferative myofibroblast-like cells, which express the cytoskeletal protein α-smooth muscle actin and synthesize increased amounts of extracellular matrix components (Apte et al., 1998; Bachem et al., 1998). Recent studies have identified platelet-derived growth factor (PDGF)-BB as the most potent mitogen for pancreatic stellate cells in culture, and its effects have been extensively studied (Apte et al., 1998; Luttenberger et al., 2000). PDGF stimulates PSC cell proliferation by activating key effectors, including ras, raf, and the extracellular signal-regulated kinase (ERK)1/2 cascade (Jaster et al., 2002; Masamune et al., 2003a). Data from recent studies suggest that p38 mitogen-activated protein kinase (MAPK) and c-Jun NH2-terminal kinase (JNK) signaling pathways might also play a role in PSC proliferation ((Masamune et al., 2003b, 2004). Thus, modulation of MAPK activation is considered a potential strategy to inhibit PSC growth. Heme oxygenases (HOs) are the rate-limiting enzymes in...
the degradation of heme into carbon monoxide, iron, and biliverdin (Tenhunen et al., 1968). Biliverdin is subsequently metabolized to bilirubin by the enzyme biliverdin reductase. To date, three distinct members of the HO-family have been identified (Ryter et al., 2006a). HO-1, also known as heat shock protein 32, is highly inducible by a variety of compounds and different physiologic and pathophysiologic stimuli, including volatile anesthetics, heavy metals, oxidant stress, hemorrhagic shock, and others (Schmidt et al., 2004, 2006; Ryter et al., 2006a). By contrast, HO-2 is constitutively expressed. Recent data indicate that the third isoform (HO-3) probably represents a pseudogene originating from HO-2 transcripts without functional relevance. Although interest in HO-1 originally centered on its heme-degrading function, recent findings indicate that HO-1 exerts other biologically important functions. Data from current studies suggest that HO-1 plays a vital role in control of cell growth and differentiation (Durante, 2003). However, the effect of HO-1 on cell proliferation is highly variable and seems to be cell-type specific. For example, it has been shown that HO-1 could have proproliferative effects in endothelial and tumor cells and potent antiproliferative effects in vascular and airway smooth muscle cells (Duckers et al., 2001; Li Volti et al., 2002; Taille et al., 2003; Marinissen et al., 2006). However, the development of therapeutic strategies that use the beneficial effects of increased HO activity has been hampered by the fact that most “classical” pharmacological inducers such as cobalt protoporphyrin could perturb organ function themselves (Schmidt, 2007). The plant-derived polyphenolic compound curcumin is a potent inducer of HO-1 in different cell types including vascular endothelial and neuronal cells (Motterlini et al., 2000; Scapagnini et al., 2002). In a recent study, curcumin has been shown to block pancreatic stellate cell activation (Masamune et al., 2006). Inhibition of PSC proliferation by this curry pigment led us to hypothesize that this effect might be due to its ability to induce HO-1.

To test this assumption, the aims of this study were as follows: 1) to characterize the effect of curcumin on HO-1 gene expression in PSCs; 2) to explore whether HO-1 induction contributes to the inhibitory effect of curcumin on PSC proliferation; and 3) to clarify the involvement of MAPK pathways in this context.

**Materials and Methods**

**Reagents.** Collagenase P and 5-bromo-2′-deoxyuridine (Brdu) cell proliferation kits were purchased from Roche Diagnostics (Mannheim, Germany). Iohexol (Nycomed) was obtained from Nycomed (Oslo, Norway). Hank’s buffered salt solution was obtained from Invitrogen (Karlsruhe, Germany); rat PDGF was from R&D Systems (Minneapolis, MN); and tin protoporphyrin IX (SnPP) was from Frontier Scientific Europe (Carnforth, UK). Isecove’s modified Dulbecco’s medium (IMDM) and supplements for cell culture were obtained from Invitrogen (Karlsruhe, Germany); rat PDGF was from R&D Systems (Minneapolis, MN); and tin protoporphyrin IX (SnPP) was from Frontier Scientific Europe (Carnforth, UK). Isecove’s modified Dulbecco’s medium (IMDM) and supplements for cell culture were obtained from Invitrogen. The polyclonal rabbit antibodies against p38, anti-phospho-JNK, anti-total-JNK, anti-phospho-MAPKAPK-2, or c-Jun and were analyzed by laser fluorescence Kit (GE Healthcare). For normalization, blots were reprobed with antibodies to detect total amounts of GAPDH, ERK1/2, p38 MAPK, JNK, MAPKAPK-2, or c-Jun and were analyzed by laser scanning densitometry (Personal Densitometer; GE Healthcare). All results shown are representative of experiments with at least n = 3 different cell preparations.

**Determination of HO Enzyme Activity.** HO enzyme activity was measured as described previously for tissue extracts with some minor modifications (Schmidt et al., 2007). In brief, culture-activated PSCs were starved from serum for 24 h. On the next day, cells were pretreated with curcumin and/or SnPP for 4 h before stimulation with PDGF for 4 h. The resulting cell pellets were dissolved in 30 μl of 5:1 K/Na 100 mM phosphate buffer with 2 mM MgCl2 (HO activity buffer). These extracts were used to quantitate HO enzyme activity in a reaction volume of 500 μl, containing 100 μl of liver cytosol (source of biliverdin reductase), 0.8 mM nicotinamide dinucleotide phosphate, 20 μM hemin, 2 mM glucose-6-phosphate, and 0.002 U/μl glucose-6-phosphate dehydrogenase. The reaction was performed at

Horse radish peroxidase-conjugated antibodies and the ECL kit were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other reagents were purchased from Sigma Chemie (Deisenhofen, Germany) unless indicated otherwise.

**Isolation and Culture of PSCs.** All animal procedures were performed in accordance with the Institute of Laboratory Animals Resources (1996). Rat PSCs were prepared from the pancreas tissues of male Wistar rats (Charles River, Sulzfeld, Germany) weighing 250 to 300 g according to the procedure described by Shiini et al. (2002). In brief, the pancreas was digested with 0.03% collagenase P in Hank’s buffered salt solution. The resultant suspension of cells was centrifuged in a 13.2% iohexol gradient at 1400 g for 20 min. Stellate cells separated into a fuzzy band just above the interface of the iohexol solution and the aqueous buffer. This band was harvested, and the cells were washed and resuspended in IMDM containing 10% fetal calf serum (FCS), 4 mM glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). Cell purity was almost always more than 90% as assessed by vitamin A autofluorescence. After reaching confluency, cells were harvested and replated at equal seeding densities. All experiments were performed using culture-activated cells (passages 2–4). PSCs were incubated in serum-free medium for 24 h before the addition of experimental reagents.

**RNA Isolation and Northern Blot Analysis.** Total RNA was extracted from PSCs using the RNAeasy kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s recommendations. Aliquots of total RNA (10 μg/lane) were size-fractionated on an agarose gel (1% agarose, 90 ml of diethylpyrocarbonate-treated water, 1.8 ml of formaldehide, 10 ml of MOPS/ethylenediaminetraaceteate), transferred to a nylon membrane (Hybond-N; GE Healthcare) by capillary blotting in 20% sodium saline citrate (3 mol/l NaCl, 0.3 mol/l sodium citrate), and cross-linked to the membrane by UV irradiation. The membrane was preincubated for 30 min in hybridization solution (ExpressHyb TM; Clontech, Mountain View, CA) and incubated overnight at 68°C with a 32P-labeled (Prime-It labeling kit; Stratagene, La Jolla, CA). HO-1 cDNA probe. All blots were stripped and reprobed with an 18S ribosomal RNA cDNA to verify equal loading and transfer of the RNA.

**Western Blot Analysis.** PSCs were plated on 6-well culture plates in 10% FCS/IMDM and grown to 80% confluence. After 24 h quiescence in serum-free medium, cells were treated as indicated. Total cell lysates were prepared by addition of 100 μl of SDS buffer (250 mM Tris (pH 6.8), 10% SDS, 500 mM dithiothreitol, 50% glycerol, and 0.5% bromphenol blue). Twenty micro liters of total cellular extracts were separated on a 7.5% SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membranes were blocked with 5% skim milk in TWEEN 20%phosphate-buffered saline and incubated with the indicated protein-specific antibodies overnight at 4°C. After incubation with a horse radish peroxidase-conjugated anti-rabbit or antimouse Ig antibody, proteins were visualized using the Enhanced Chemiluminescence Kit (GE Healthcare). For normalization, blots were reprobed with antibodies to detect total amounts of GAPDH, ERK1/2, p38 MAPK, JNK, MAPKAPK-2, or c-Jun and were analyzed by laser scanning densitometry (Personal Densitometer; GE Healthcare). All results shown are representative of experiments with at least n = 3 different cell preparations.
37°C for 1 h in the dark and stopped by addition of 500 μl of chloroform. For extraction of bilirubin, the tubes were mixed thoroughly followed by centrifugation at 13,000 rpm for 5 min. The chloroform layers were scanned on a spectrophotometer at 464 nm minus the background at 530 nm. Based on the protein content within the reaction volume, the results of the measurements were expressed as formation of bilirubin (picomole) per milligram of protein within 1 h. Spleen tissue of rats served as a positive control but had no influence in data presentation. The HO activity assay was performed in duplicate with n = 3 separate cell preparations, respectively.

**Determination of Cell Proliferation.** Cell counts. Cultured PSCs were passaged twice and replated at equal seeding densities in 24-well culture plates. After a 24-h serum deprivation, triplicate wells of cells were then exposed to curcumin and/or SnPP for 4 h followed by a 24-h stimulation with PDGF. Cells incubated in medium without PDGF served as controls. Cells were washed twice in phosphate-buffered saline, harvested by trypsinization using 0.5% trypsin-0.2% EDTA, resuspended in 200 μl of culture medium, and counted using a Casy TT cell counter according to the manufacturer’s instructions (Schafer System, Reutlingen, Germany). Cell count analyses were performed in quadruplicate with n = 3 separate cell preparations, respectively.

**DNA synthesis (incorporation of BrdU).** Serum-starved PSCs (~80% density) were left untreated or treated with PDGF in the presence of curcumin and/or SnPP at the indicated concentrations. Cell proliferation was evaluated with a BrdU-based enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer’s instructions. This colorimetric immunoassay is based on the measurement of BrdU incorporation during DNA synthesis. After 8-h incubation with the indicated substances, cells were labeled with BrdU for 20 h at 37°C. Cells were fixed and incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. After adding the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine, BrdU incorporation was determined by measuring the optical densities at 370 nm minus the background at 492 nm. BrdU assays were performed in quadruplicate with at least n = 3 separate cell preparations, respectively.

**Design of Small Interfering RNA and Transfection of PSCs.** PSCs of the third passage (1 x 10⁵/ml) were transfected with 100 nM hmoxl-4 small interfering RNA (siRNA) directed against hmoxl-mRNA: sense (5′ > 3′) r(UUA UGA UAU CUA AUA A)3dTTP, antisense r(UUA UUA UAU CUA AUA A)3dTTP, or AllStars nonselecting siRNA (both obtained from Qiagen GmbH) using Hiperfect transfection reagent (Qiagen GmbH) according to the manufacturer’s recommendations. Twelve hours after transfection, the medium was changed and PSCs were incubated in serum-free IMDM for 24 h before treatment.

**Statistical Analysis.** Results are expressed as means ± S.E.M. for the indicated number of separate cell preparations per experimental protocol. Data were analyzed using one-way analysis of variance followed by the Student Newman-Keuls post hoc test. Differences between groups were considered to be significant at p < 0.05. Analyses were performed using the SigmaStat statistical software package (Systat Software, Inc., San Jose, CA).

**Results**

**Curcumin Induces HO-1 Gene Expression in PSCs.** As shown in Fig. 1A, exposure of culture-activated PSCs to curcumin (0–10 μM) resulted in a concentration-dependent increase in HO-1 protein expression with maximal levels at 5 μM. However, administration of curcumin at 10 μM did not induce HO-1 protein expression compared with baseline levels. The concentration of curcumin that produced the strongest increase in HO-1 expression (5 μM) was chosen to determine the effect of curcumin on HO-1 gene transcription (Fig. 1B) and translation (Fig. 1C) over time. Treatment of PSCs with curcumin for 2, 4, 8, 16, 24, and 48 h resulted in a time-dependent increase in HO-1 mRNA and protein levels, as shown by Northern and Western blot analyses, respectively. HO-1 mRNA was already apparent after 2 h of treatment and further increased after 4 and 8 h. It then gradually decreased over time until the bands disappeared after 24 and 48 h. HO-1 protein expression was detectable after 4 h of curcumin exposure and peaked at 16 h, reaching a steady state until at least 48 h. The administration of vehicle alone had no effect on HO-1 mRNA and protein expression (Fig. 1, B and C).

**Curcumin Increases HO-1 Protein Expression and HO Activity in PDGF-Stimulated PSCs.** The effects of curcumin and the HO inhibitor SnPP on HO-1 protein expression and HO enzyme activity in PDGF-stimulated PSCs are shown in Fig. 2. Compared with untreated controls, in-
versus 0.1% FCS; 
attenuated its inhibitory effect on PSC proliferation as shown 
regulation in PDGF-stimulated PSCs. By contrast, trans-
lencing siRNA had no effect on curcumin-induced HO-1 up-
gene expression. As shown in Fig. 4A, transfection of nonsi-
HO-1, we transfected PSCs with HO-1 siRNA to block
The inhibitory effect of curcumin on PSC proliferation was
ation, whereas SnPP (20
In contrast, curcumin inhibited PDGF-induced PSC prolifer-
ment resulted in a significant increase in PSC proliferation.
Compared with untreated controls, PDGF (20 ng/ml) treat-
cubation with the mitogen PDGF (20 ng/ml) for 24 h slightly
enhanced HO-1 protein expression (Fig. 2A) and HO activity (Fig. 2B). The administration of curcumin (5 μM) signifi-
cantly increased HO-1 expression and HO activity. SnPP (20 μM) caused a slight elevation in HO-1 protein expression but
significantly inhibited HO enzyme activity in curcumin-
treated cells.

**HO-1 Mediates the Inhibitory Effect of Curcumin on
PSC Proliferation.** To investigate the effect of HO-1 on PSC
growth, cell proliferation in response to PDGF stimulation was assessed using BrdU incorporation assays (Fig. 3A), cell
count analyses (Fig. 3B), and native microscopy (Fig. 3C). Compared with untreated controls, PDGF (20 ng/ml) treat-
ment resulted in a significant increase in PSC proliferation.
In contrast, curcumin inhibited PDGF-induced PSC prolifer-
ation, whereas SnPP (20 μM) treatment alone had no effect. The inhibitory effect of curcumin on PSC proliferation was
significantly attenuated by blockade of HO enzyme activity with SnPP. To further investigate whether curcumin medi-
ates the inhibitory effect on PSC proliferation specifically via
HO-1, we transfected PSCs with HO-1 siRNA to block **HO-1** gene expression. As shown in Fig. 4A, transfection of nonsi-lencing siRNA had no effect on curcumin-induced HO-1 up-
regulation in PDGF-stimulated PSCs. By contrast, trans-
fec tion with anti-HO-1 siRNA abolished HO-1 protein expres-
sion in response to curcumin treatment and significantly
attenuated its inhibitory effect on PSC proliferation as shown
by BrdU assays and cell count analyses (Fig. 4, B and C).

**PDGF Induced MAPK Activation in PSCs.** In agreement
with previous studies (Jaster et al., 2002; Masamune et
al., 2003b, 2004), PDGF (20 ng/ml) stimulation of cultured
PSCs induced a rapid activation of ERK1/2 (Fig. 5A), p38
MAPK (Fig. 5B), and JNK (Fig. 5C). As reported by
Masamune et al. (2004), PDGF induced phosphorylation of the 46-
but not the 54-kDa isoform of JNK. In subsequent experi-
ments, PSCs were stimulated for 10 min with PDGF (20
ng/ml).

**Inhibition of ERK1/2 and JNK Decreases PDGF-In-
duced PSC Proliferation.** To elucidate the involvement of
the ERK1/2, p38 MAPK, or JNK pathway on PSC prolifera-
ion rate, culture-activated PSCs were incubated with differ-
ent MAPK inhibitors. The MEK inhibitor PD98059 (20 μM)
blocked PDGF-induced ERK1/2 activation at 10 min (Fig. 6A) and significantly inhibited PDGF-induced PSC proliferation (Fig. 6D), confirming data from Jaster et al. (2002). To inves-
tigate the effect of SB203580 on p38 MAPK activity, phos-
phorylation of MAPKAPK-2, a downstream target of p38
(Young et al., 1997), was assessed (Fig. 6B). PDGF-induced phosphorylation of MAPKAPK-2 was abolished in the pres-
ence of SB203580 (20 μM), whereas an effect of SB203580 on
PDGF-induced PSC proliferation could not be observed (Fig.
6D). To demonstrate the inhibitory effect of SP600125 on
JNK activity, c-Jun serine 73 phosphorylation was assessed by
Western blot analysis using a phosphospecific antibody
(Fig. 6C). SP600125 at 20 μM suppressed both PDGFinduced c-Jun phosphorylation and PSC proliferation.

**HO-1 Up-Regulation Prevents ERK1/2 Activation in
PSCs.** To clarify the role of HO-1 in curcumin-induced
MAPK signaling, we examined the effects of HO inhibition on
PDGF-induced MAPK activation. As indicated by immuno-
blotting, curcumin significantly reduced the phosphorylation of ERK1/2 induced by PDGF (Fig. 7A). In sharp contrast, the
level of phosphorylated p38 was significantly increased by
curcumin treatment (Fig. 7B), whereas the phosphorylation of JNK was not affected (Fig. C). The application of SnPP
completely abolished the inhibitory effect of curcumin on
ERK1/2 activation and tends to decrease the level of phos-
phorylated p38 MAPK after curcumin treatment. However, this tendency did not reach statistical significance.

**Discussion**

The main results of this study indicate that up-regulation
of **HO-1** gene expression by the plant-derived polyphenolic
compound curcumin inhibits PDGF-induced pancreatic stel-
late cell proliferation. In addition, our data provide first
evidence that HO-1 takes part in the control of pancreatic stellate
cell growth by inhibition of the ERK1/2 signaling
pathway.

Accumulating evidence suggests that pancreatic fibrosis
plays an integral role in the development of chronic pancre-
atitis and pancreatic cancer (Bachem et al., 2005). Prolifer-
ation of PSCs and the expansion of their pool are essential
parts of the fibrogenic process (Omary et al., 2007). Therefore,
understanding the molecular mechanisms underlying
PSC proliferation could offer potential therapeutic targets for
the treatment and prevention of pancreatic diseases. In a
recent study, Masamune et al. (2006) have demonstrated
that curcumin, the yellow pigment in curry, inhibits PSC
proliferation. Furthermore, other groups provided evidence
that compounds like statins, taurine, or the green tea extract epigallocatechin gallate could also block the proliferation of PSCs in experimental in vitro or in vivo models, but the exact cellular mechanisms are still not fully understood (Jaster et al., 2003; Asaumi et al., 2006; Shirahige et al., 2007). Note that all of these mentioned substances are potent inducers of the cytoprotective enzyme HO-1 in several cell types, but no information is available concerning their effects on HO-1 in the pancreas (Olszanecki and Marcinkiewicz, 2004; Hsu et al., 2006; Wu et al., 2006). This could be of major importance because up-regulation of HO-1 or administration of its metabolites bilirubin or carbon monoxide have been reported to exert antiproliferative effects in different organ systems (Ryter et al., 2006b). Given the above, it seems reasonable to speculate that the inhibitory effect of curcumin on PSC proliferation is considerably linked to its ability to induce HO-1 expression. The results of our study support this concept. We have demonstrated for the first time that administration of curcumin leads to an up-regulation of HO-1 gene expression in culture-activated PSCs. This induction becomes evident on the protein level after 4 h of incubation and persists over at least 48 h, confirming data obtained from other cell lines.

**Fig. 3.** Effect of curcumin and/or SnPP on PDGF-induced PSC proliferation. Serum-starved PSCs were treated with curcumin (5 μM) and/or SnPP (20 μM) for 4 h followed by a 24 h stimulation with PDGF (20 ng/ml). A, DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Results are expressed as percentage of control values observed in PSCs not incubated with PDGF. **, p < 0.01 versus 0.1% FCS; **, p < 0.01 versus PDGF; *, p < 0.05 versus PDGF + curcumin. B, PSC proliferation assessed by cell count analysis. Results are expressed as a percentage of control values (cells not incubated with PDGF). **, p < 0.05 versus 0.1% FCS; *, p < 0.05 versus PDGF; *, p < 0.05 versus PDGF + curcumin. C, native microscopy. Original magnification, 10× objective. All histograms shown are representative of the results for experiments with n = 3 separate cell preparations.
Effect of HO-1 on PDGF-induced PSC proliferation. PSCs were transfected with siRNA directed against hmxol-mRNA (HO-1 siRNA) or nonsilencing siRNA not targeting any gene product (Control siRNA). After transfection, cells were incubated in serum-free medium for 24 h. Curcumin (5 μM) was added to the cells for 4 h followed by a 24-h stimulation with PDGF (20 ng/ml). A, HO-1 protein expression was assessed by Western blot analysis. The membrane was stripped and reprobed with an anti-GAPDH protein-specific antibody. B, DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Results are expressed as percentage of control values observed in PSCs not incubated with PDGF (n = 3). **, p < 0.01 versus 0.1% FCS; ***, p < 0.01 versus PDGF + control siRNA; *, p < 0.05 versus PDGF + curcumin + control siRNA (n = 3). C, PSC proliferation assessed by cell count analysis. Results are expressed as percentage of control values (cells not incubated with PDGF). ***, p < 0.01 versus 0.1% FCS; ***, p < 0.05 versus PDGF + control siRNA; *, p < 0.05 versus PDGF + curcumin + control siRNA (n = 3).

Fig. 4. Effect of HO-1 on PDGF-induced PSC proliferation. PSCs were transfected with siRNA directed against hmxol-mRNA (HO-1 siRNA) or nonsilencing siRNA not targeting any gene product (Control siRNA). After transfection, cells were incubated in serum-free medium for 24 h. Curcumin (5 μM) was added to the cells for 4 h followed by a 24-h stimulation with PDGF (20 ng/ml). A, HO-1 protein expression was assessed by Western blot analysis. The membrane was stripped and reprobed with an anti-GAPDH protein-specific antibody. B, DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Results are expressed as percentage of control values observed in PSCs not incubated with PDGF (n = 3). **, p < 0.01 versus 0.1% FCS; ***, p < 0.01 versus PDGF + control siRNA; *, p < 0.05 versus PDGF + curcumin + control siRNA (n = 3). C, PSC proliferation assessed by cell count analysis. Results are expressed as percentage of control values (cells not incubated with PDGF). ***, p < 0.01 versus 0.1% FCS; ***, p < 0.05 versus PDGF + control siRNA; *, p < 0.05 versus PDGF + curcumin + control siRNA (n = 3).

Fig. 5. Effect of PDGF on MAPK activation in PSCs. PSCs of the third passage were starved from serum for 24 h before stimulation with PDGF (20 ng/ml) for the indicated periods of time. Total cell lysates were prepared, and the levels of phosphorylated ERK1/2 (P-ERK1/2; A), p38 MAPK (P-p38; B), and JNK (P-JNK; C) were assessed by Western blot analysis. Accuracy of loading and transfer was confirmed by reprobing using antibodies recognizing total ERK1/2, total p38 MAPK, and total JNK.

PDGF is a potent proproliferative mediator of PSCs, and curcumin has recently been shown to inhibit PDGF-induced PSC proliferation (Masamune et al., 2006). In the present study, we show that the inhibitory effect of curcumin on PDGF-stimulated DNA synthesis and cell growth was associated with a profound up-regulation of HO-1 gene expression in these cells. Moreover, this effect was significantly reduced in the presence of the HO inhibitor SnPP or by transfecting PSCs with HO-1 siRNA, indicating a direct involvement of HO-1. It is interesting to note that PDGF itself slightly enhanced HO-1 expression and activity in PSCs. These data are in agreement with observations made in airway smooth muscle cells (Taille et al., 2003). It is possible that induction of HO-1 protein expression with a subsequent increase in HO activity represents a feedback mechanism by which PSCs regulate cell growth in response to mitogenic stimuli from PDGF, as described in the vascular smooth muscle (Durante et al., 1999).
versus 0.1% FCS; FCS only). The data represent means of the untreated control (0.1% FCS). The results are expressed as a percentage of the untreated control (0.1% FCS) assessed by BrdU assays after a 24-h incubation with PDGF (20 ng/ml).

**Figure 6.** Effect of PD98059, SB203580, and SP600125 on PDGF-induced MAPK activation and PSC proliferation. Serum-starved PSCs were pre-treated with PD98059 (20 μM), SB203580 (20 μM), or SP600125 (20 μM) for 1 h before exposure to PDGF. Representative Western blot analysis of ERK1/2 (A), MAPKAPK-2 (B), and c-Jun (C) phosphorylation after a 10-min stimulation with PDGF (20 ng/ml). To demonstrate equal loading, the membranes were stripped and reprobed with antibodies against total ERK1/2, total MAPKAPK-2, and total c-Jun. D, cell proliferation was assayed by BrdU assays after a 24-h incubation with PDGF (20 ng/ml). The results are expressed as a percentage of the untreated control (0.1% FCS only). The data represent means ± S.E.M. (n = 3). ***p < 0.001 versus 0.1% FCS; ***, p < 0.01 versus 0.1% FCS; &&, p < 0.01 versus PDGF.

The crucial involvement of the Ras-Raf-ERK1/2 signal transduction pathway in PDGF-induced PSC proliferation is well documented (Jaster et al., 2002; Masamune et al., 2003a). Furthermore, it has been reported that p38 MAPK and JNK signaling pathways might play a role in PSC proliferation (Masamune et al., 2003, 2004). In the present study, we found that pharmacological inhibition of ERK1/2 and JNK, but not p38 MAPK, led to a decrease in PSC proliferation, confirming data obtained in hepatic stellate cells (Schnabl et al., 2001). In tumor cells, curcumin has been demonstrated to inhibit phosphorylation of JNK (Aggarwal et al., 2003). However, we did not observe inhibition of JNK activation after curcumin treatment in our experimental setting, which might be explained by the different cell type. By contrast, we could detect a marked activation of p38 MAPK after curcumin treatment. This phenomenon could be due to an accumulation of carbon monoxide in response to HO activation. It has been shown in vascular smooth muscle cells that carbon monoxide activates the p38 MAPK signaling pathway (Otterbein et al., 2003). Taking this into account, it is conceivable that the observed raise in HO activity after curcumin treatment increases the level of cellular carbon monoxide, thereby activating the p38 MAPK. This might also explain why blockade of the HO enzyme by administration of SnPP decreased the level of phosphorylated p38 MAPK. Masamune et al. (2006) recently showed that the inhibitory effect of curcumin on PSC proliferation correlates with decreased ERK1/2 phosphorylation. In light of this result and our observation that curcumin induces HO-1 in PSCs, we assumed that the curcumin-induced inhibition of ERK1/2 phosphorylation could be due to HO-1 induction. In this study, we show that the curcumin-mediated decrease in ERK1/2 activation is paralleled by HO-1 up-regulation and could be prevented by administration of the HO inhibitor SnPP, suggesting that HO-1 up-regulation suppresses the ERK1/2 signaling pathway.

In contrast to many established experimental HO-1 inducers such as cobalt protoporphyrin, which could have a multitude of undesirable or toxic side effects, up-regulation of HO-1 by natural compounds seems more suitable for therapeutic purposes. In this regard, curcumin has already been used recently in a number of clinical studies. Cheng et al. (2001) reported no treatment-related toxicity after oral administration of up to 8 g per day that yields curcumin serum levels of 1.77 ± 1.87 μM. In the present study, we observed profound antiproliferative effects on PSC growth after incubation with 5 μM curcumin, a concentration which is very similar to that observed in human serum by Cheng et al. (2001). In addition, a pilot phase I clinical trial has shown curcumin to be safe even when consumed at a daily dose of 12 g for 3 months (Goel et al., 2008). Other human studies have been performed evaluating the protective actions of curcumin in various diseases including one in pancreatitis (Hsu and Cheng, 2007). In this preliminary study including only 20 patients, Durgaprasad et al. (2005) demonstrated a significant reduction in lipid peroxidation markers in the blood of patients with tropical pancreatitis after curcumin therapy compared with placebo (Durgaprasad et al., 2005). Nevertheless, well-designed clinical trials with larger groups of patients are required to validate the effects of curcumin as a reasonable treatment. In this context, it would be of major interest if curcumin administration could lead to an up-regulation of HO-1 in patients, because no pharmacological compound has been identified so far, offering the potential for HO-1 induction in patients.

In conclusion, this study provides first evidence that the inhibitory effect of curcumin on ERK1/2 activation and PSC...
proliferation is mediated by induction of HO-1 gene expression. These findings suggest that up-regulation of the HO-1 enzyme could represent a potential target for pharmacological inhibition of PSC proliferation and thus may provide a novel therapeutic strategy in preventing pancreatic fibrosis.

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