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CCK1- und CCK2- Rezeptoren werden auf pankreatischen Sternzellen exprimiert und induzieren die Kollagensynthese

Dissertation

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CCK1 and CCK2 Receptors Are Expressed on Pancreatic Stellate Cells and Induce Collagen Production

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The gastrointestinal hormone cholecystokinin (CCK) can induce acute pancreatitis in rodents through its action on acinar cells. Treatment with CCK, in combination with other agents, represents the most commonly used model to induce experimental chronic pancreatitis. Pancreatic stellate cells (PSC) are responsible for pancreatic fibrosis and therefore play a predominant role in the genesis of chronic pancreatitis. However, it is not known whether PSC express CCK receptors. Using real time PCR techniques, we demonstrate that CCK1 and CCK2 receptors are expressed on rat PSC. Interestingly both CCK and gastrin significantly induced type I collagen synthesis. Moreover, both inhibit proliferation. These effects are comparable with TGF-β-stimulated PSC. Furthermore, the natural agonists CCK and gastrin induce activation of pro-fibrogenic pathways Akt, ERK, and Src. Using specific CCK1 and CCK2 receptor (CCK2R) inhibitors, we found that Akt activation is mainly mediated by CCK2R. Akt activation by CCK and gastrin could be inhibited by the PI3K inhibitor wortmannin. Activation of ERK and the downstream target Elk-1 could be inhibited by the MEK inhibitor U0126. These data suggest that CCK and gastrin have direct activating effects on PSC, are able to induce collagen synthesis in these cells, and therefore appear to be important regulators of pancreatic fibrogenesis. Furthermore, similar to TGF-β, both CCK and gastrin inhibit proliferation in PSC.

Acute and chronic pancreatitis are responsible for a significant morbidity and mortality. Although studies have shown that pancreatic acinar cells activate the mechanisms leading to inflammation and organ destruction in acute pancreatitis (1), another cell type, called pancreatic stellate cells (PSC),[3] play a pivotal role in the process leading to pancreatic fibrosis and chronic pancreatitis (2). These fibroblast-like cells represent only a minority of cells in the normal pancreas. Growth factors, G-protein-coupled receptors, and toxins activate PSC, leading to proliferation, migration, and production of extracellular matrix constituents including collagen, thereby inducing the organ changes characteristic of chronic pancreatitis (3–8). These cellular effects are mediated by central signal transduction cascades including Akt, ERK, and Src. The gastrointestinal hormone cholecystokinin (CCK) was among the first gastrointestinal hormones discovered. CCK binds to two receptors (CCK1R and CCK2R) that are expressed in many tissues, including the exocrine pancreas (9). The stimulation of CCK receptors by the natural agonists CCK (comparable affinity for CCK1R and CCK2R) and gastrin (1000-fold higher affinity for CCK2R) regulate many physiological and pathophysiological processes (9). Most notably, CCK induces experimental acute pancreatitis in rodents, a process mediated by the CCK1R expressed on pancreatic acinar cells. The pathophysiology of CCK-induced acute pancreatitis and the underlying signal transduction pathways have been extensively studied in whole animals and isolated acinar cells in the last two decades (10). Repeated administration of CCK, or its agonist cerulein, was used in many studies to induce different aspects of chronic pancreatitis, including activation of PSC and fibrosis. In these studies, it had been assumed that chronic pancreatitis is the result of repeated organ inflammation caused by multiple inductions of acute pancreatitis by CCK. Moreover, some studies found that patients suffering from chronic pancreatitis have higher plasma CCK levels than healthy controls (11–13) and this observation was the rationale of a clinical study of CCK-antagonist loxiglumide in chronic pancreatitis (14). Despite these experimental and clinical findings pointing to a role of CCK in chronic pancreatitis, surprisingly, no study has considered the possibility of a direct effect of CCK or CCK agonists on PSC. Even the expression of CCK receptors has not been studied on PSC. To systemically analyze the possible direct role of CCK and CCK receptors in the pathophysiology of chronic pancreatitis, we studied the expression of CCK1R and CCK2R on PSC, as well as the effect of stimulation of these receptors on collagen production, proliferation, and on pro-fibrogenic signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (150–250 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Akt Ser(P)^{473}, anti-caspase 3, Src family (Tyr^{416}), ERK (Thr^{202}/Tyr^{204}), U0126, and ELK-pS383 were from Cell Signaling Technology (Beverly, MA). Anti-goat horseradish peroxidase (HRP) conjugate, anti-actin, anti-p21 Ser^{146}, anti-p21, anti-p27, and anti-rabbit HRP-conjugate antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p27 Ser^{10} was from Abcam (Cambridge, UK). Collagen antibody was from Southern Biotech (Birmingham, AL). α-SMA antibody was from Dako (Denmark). Chamber slides and anti-PY20 antibody were purchased from BD Biosciences (San Jose, CA). L-364,718 and LY288513 were from Tocris (Ellisville, MO). Wortmannin,

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[3] The abbreviations used are: PSC, pancreatic stellate cell; CCK, cholecystokinin; α-SMA, smooth muscle actin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

TGF-β RI kinase inhibitor VIII, and PI3Kγ inhibitor II were from Calbiochem (Nottingham, UK). Iscove was from Biochrome AG (Berlin, Germany). SDS and ammonium sulfate were from Bio-Rad. Tris, acrylamide (40%), and glycin were from Carl Roth (Karlsruhe, Germany). Protein ladder was from Fermentas (Burlington, Canada). Agarose G, BCA protein assay, and SuperSignal West Dura were from Fisher Scientific. Films were from Amersham Biosciences. Donkey anti-goat Alexa Fluor 488, donkey anti-mouse Alexa Fluor 596, rabbit anti-goat Alexa Fluor 596, donkey anti-mouse Alexa Fluor 488, Hanks' balanced salt solution, FCS, PenStrep, NEAS, Dulbecco's PBS, and ETDA were from Invitrogen. Cell culture dishes were from Sarstedt (Nümbrecht, Germany). Protease, HEPES, benzamidine, orthovanadate, leupeptin, PMSF, and aprotinin were from Sigma. Protran nitrocellulose membranes were from Whatman. DNase and collagenase were from Roche Applied Science. CCK1R and CCK2R antibodies were from Everest Biotech (Oxfordshire, UK).

Animal Care—Animals were cared according to the guidelines and under supervision of the local animal welfare board.

Tissue Preparation—Pancreatic stellate cells were isolated using intraductal injection of enzyme solution followed by cell isolation and concentration of stellate cells by gradient centrifugation as reported previously (15, 16). Briefly, the common bile duct was intubated via the papilla using a 24-French syringe and 8 ml of an enzyme mixture (800 mg/liter of collagenase P, 400 mg/liter of Protease, and 200 mg/liter of DNase). Pancreases were excised injected with enzymes and cut into pieces. The tissue was disrupted by re-suspension in new enzymes and incubation at 37 °C for 10 min. Finally, cells were isolated using a 150-μm mesh. Isolated cells were subjected to Histodenz gradient centrifugation as described previously to isolate pancreatic stellate cells. Stellate cells were cultured for 7 days, then subjected to stimulants as described.

Stellate Cell Lysis—After incubation with stimulants and/or inhibitors, stellate cells were lysed in lysis buffer (0.5 mM NaF, 0.2 mM EDTA, 10% glycerol, 10 mM benzamidine, 0.2 mM sodium orthovanadate). Lysates were centrifuged at 10,000 $\times$ *g* for 15 min at 4 °C, and protein concentration was measured using the BCA reagent. Equal amounts of samples were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation—After cell lysis, equal amounts of samples were incubated overnight with agarose G and 4 μg of the specific antibody diluted in lysis buffer. Then samples were centrifuged, washed three times, and analyzed by SDS-PAGE and Western blotting.

Western Blotting—Western blotting was performed as described previously.

Whole cell lysates were subjected to SDS-PAGE using Tris glycine gels. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (50 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) at room temperature for 1 h. Membranes were incubated overnight with primary antibody, washed three times in washing buffer (50 mM Tris/HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% Tween 20) for 5 min, and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were washed again three times in washing buffer for 5 min, incubated with chemiluminescense detection reagents and finally exposed to film. The intensity of the protein bands was measured using ImageJ analysis. When reprobing was necessary, membranes were incubated in Stripping buffer (Pierce) for 30 min at room temperature, washed twice for 10 min in washing buffer, blocked for 1 h in blocking buffer at room temperature, and re-probed as described above.

Immunocytochemistry—To detect CCK1R and CCK2R in isolated PSC, cells were grown on glass chamber slides, fixed with acetone, blocked in 2% rabbit-PBS, and immunostained. The sequence was 1:500 CCK1R and CCK2R, and rabbit anti-goat Alexa Fluor 488 at 1:200. α-SMA was counterstained using donkey anti-mouse Alexa Fluor 596. To detect the effects of CCK and gastrin on PSC, cells were grown on glass chamber slides. After serum starvation, cells were stimulated for 24 h with no additions, 1 nM CCK, or 1 μM gastrin (stimulants were replaced after 12 h). Then cells were fixed in acetone and immunostained. Staining sequence for collagen type I was goat anti-collagen I (1:50) and donkey anti-goat Alexa Fluor 488 (1:200). Staining for the α-SMA sequence was mouse anti-α-SMA (1:100) and donkey anti-goat Alexa Fluor 594 (1:200). Nuclei were counterstained with bisbenzimide. Pictures were taken using the IPLab3 software. For the collagen experiments we applied the same exposure time and magnification.

Immunohistology—To detect CCK1R and CCK2R in rat pancreatic tissue, we snap froze pancreatic tissue in liquid nitrogen. 5-μm slides were then fixed in acetone, blocked in 2% rabbit PBS, and immunostained. Staining sequence was 1:500 CCK1R and CCK2R, and 1:200 for α-SMA. Secondary antibodies were rabbit anti-goat Alexa Fluor 596 for the CCK receptors and donkey anti-mouse Alexa Fluor 488 for α-SMA. Nuclei were counterstained using bisbenzimide.

All micrographs were taken using a Leica DM LB fluorescence microscope equipped with a Retiga I300 camera. We acquired the pictures using the IPLab3 software.

ELISA—Indirect ELISA was performed essentially as described by Kordes *et al.* (35). Stellate cells were grown in 12-well plates until confluence. After serum starvation, cells were stimulated for 24 h in serum-free medium containing 50 μg/ml of ascorbic acid (Sigma) with no additions, 2 ng/ml of TGFβ1 (Peprotech, Hamburg, Germany), 1 nM CCK, and 1 μM gastrin. Stimulants were replaced after 12 h. The supernatants were collected and diluted (1:4) in PBS. 50 μl/wells were used to coat Microtiter plates (Nunc Maxisorp, Langenselbold, Germany) overnight at 4 °C. Plates were blocked with 5% milk powder in PBS/T for 1 h. Plates were washed and incubated with anti-type I collagen for 2 h. Subsequently, the secondary anti-goat HRP antibody was used. Unbound secondary antibody was washed away and 100 μl/well of tetramethylbenzidine substrate (TMB Plus, Kementec, Taestrup, Denmark) were added. Enzymatic reaction was stopped by adding 50 μl/well of 0.2 N H_2SO_4. Plates were read on a standard plate reader at 540 nm. Purified type I collagen (Serva, Heidelberg, Germany) served as the control.

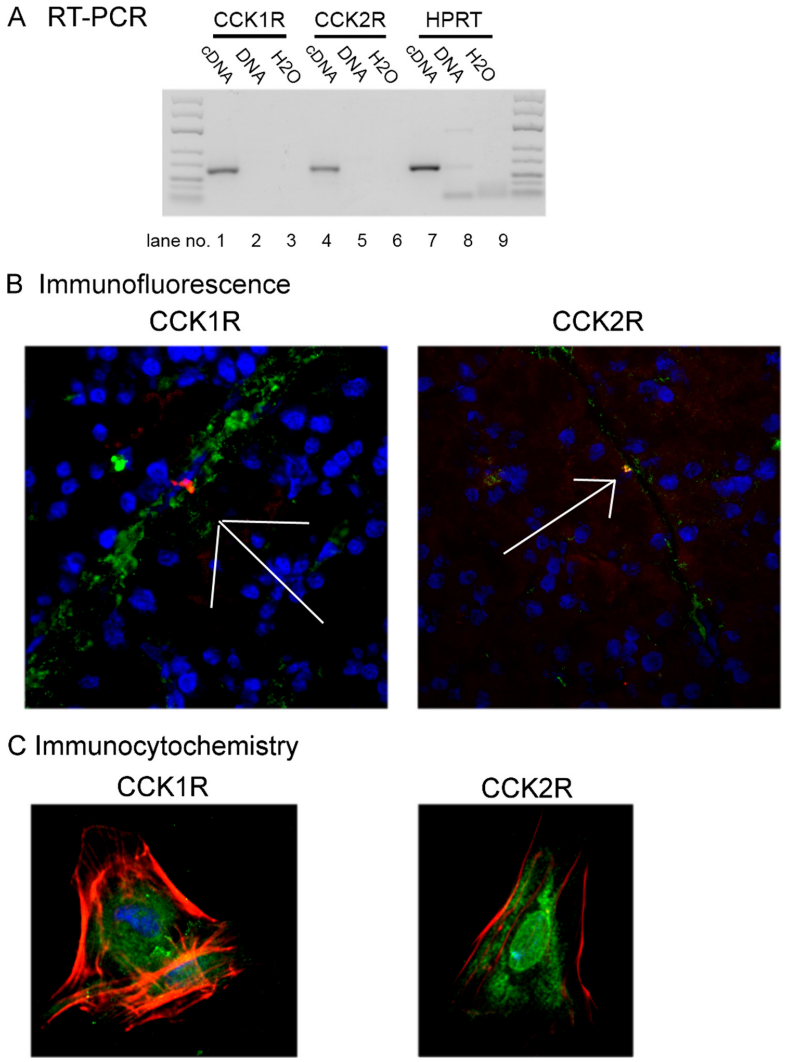

FIGURE 1. **CCK2 and CCK1 receptors are expressed on rat PSC.** *A*, rat PSC were cultured for 7 days, RNA was isolated, transcribed, and samples submitted to RT-PCR. Negative controls (genomic DNA and water) were included. Expected product sizes are 93 (*CCK2R*) and 118 bp (*CCK1R*). A representative experiment of a total of 3 independent experiments is shown. *B*, rat pancreatic tissue was snap frozen, fixed in acetone, and 5-μm sections were immunostained. Shown is a merged image of the CCK1R/CCK2R-stained tissue (*red*), counterstained with α-SMA (*green*). Magnification, $\times$200. *C*, rat PSC were cultured, trypsinized, and plated on glass chamber slides. Cells were acetone fixed and immunostained for CCK1R/CCK2R and α-SMA. Magnification, $\times$200.

Thymidine Uptake—Rat PSC were isolated and 50,000 cells/well were seeded on 12-well plates. When reaching 60% confluence, cells were serum starved and then stimulated for 24 h in serum-free medium containing 2 μCi/ml of [^{3}H]thymidine (PerkinElmer Life Sciences) with no additions, 2 ng/ml of TGF, 1 nM CCK, and 1 μM gastrin. After 24 h cells were harvested (Brandel Harvester, Unterföhring, Germany) and [^{3}H]thymidine incorporation was measured (Hidex Plate Chameleon, Straubenhardt, Germany).

MTT Assay—Rat PSC were isolated and cultured. Cells were trypsinized and 100,000 cells/well were seeded in 6-well plates. When reaching subconfluence, cells were serum-starved and stimulated with no additions, 2 ng/ml of TGF, 1 nM CCK, or 1 μM gastrin for 24 h. Stimulating factors were replaced after 12 h. Thereafter, 100 μg/ml of MTT (Sigma) were added and cells were incubated for 2 h. Supernatants were removed and 250 μl/well of DMSO (Sigma) was added. Reaction products were transferred to microtiter plates and read in a standard microplate reader at 570 nm.

Statistical Analysis—Data are presented as mean $\pm$ S.E. and were analyzed using the Student's *t* test for unpaired data using the Prism software (GraphPad). *p* values <0.05 were considered significant.

RESULTS

Rat PSC Express CCK1 and CCK2 Receptors—The presence of mRNA for CCK1 and CCK2 receptors was assessed by RT-PCR in PSC cultured for 7 days. According to the manufacturer of the primers used, specific bands of 118 (CCK1R) and 93 bp (CCK2R) were expected. As shown in Fig. 1*A*, expression of CCK1R mRNA was found in PSC (*lane 1*), but absent in the genomic DNA (*lane 2*) and negative control using water (*lane 3*). Similarly, CCK2R mRNA was found in PSC (*lane 4*), whereas the corresponding controls using genomic DNA (*lane 5*) or water (*lane 6*) did not show a corresponding band. We conclude that mRNA for both CCK1R and CCK2R is expressed in rat PSC.

Furthermore, the presence of both receptors was confirmed by immunohistology (Fig. 1*B*) and immunocytochemistry (Fig. 1*C*). α-SMA as a tissue-specific marker was used to confirm localization on PSC. Snap frozen rat brain sections served as positive control for the antibodies.

CCK Causes Collagen Production in PSC—Upon stimulation by growth factors and several G protein-coupled receptors, PSC have been reported to produce extracellular matrix proteins including type I collagen, leading to pancreatic fibrosis, a constant feature of chronic pancreatitis. To date TGF-β has been the most potent fibrogenic stimulus described in PSC. To assess

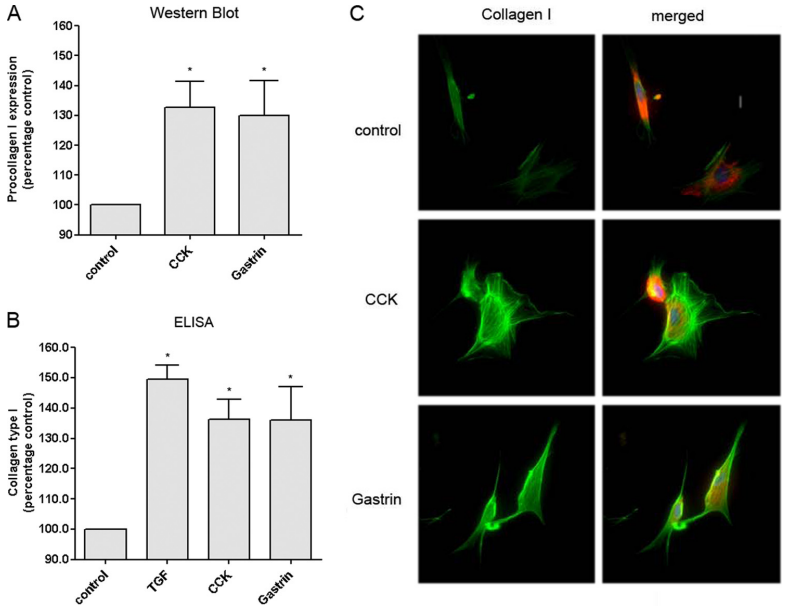

FIGURE 2. **Effect of CCK and gastrin on collagen type I synthesis in rat PSC.** *A*, rat PSC were cultured, serum-starved, and stimulated without additions, or with 1 nM CCK or 1 μM gastrin for 24 h (stimulants were replaced every 12 h) and lysed. Lysates were analyzed by Western blot for procollagen type I. Western blots were scanned and densitometry was performed. The values represent the mean ± S.E. of 6 experiments and are expressed as percent of the pre-treatment values. (*, $p < 0.05$ *versus* control). *B*, rat PSC were cultured, serum-starved, and stimulated without additions, or with 2 ng/ml of TGF, 1 nM CCK, or 1 μM gastrin for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean ± S.E. of 5 experiments and are expressed as percent of the pre-treatment value. (*, $p < 0.05$ *versus* control). *C*, rat PSC were cultured, trypsinized, and plated on glass chamber slides. Serum-starved cells were stimulated without additions, or with 1 nM CCK or 1 μM gastrin (stimulants were replaced every 12 h) for 24 h. Then cells were acetone fixed and immunostained for collagen type I and α-SMA. Micrographs were taken by using same exposure time and magnification (×200).

whether CCK or gastrin can stimulate collagen type I production in PSC (Fig. 2) and to compare its action to TGF-β we performed Western blot analysis and indirect ELISA upon stimulation with CCK and gastrin (Western blot, ELISA) and TGF-β (ELISA). After 24 h of stimulation, CCK (1 nM) caused a 136.3% increase ($p < 0.05$) in procollagen type I production. Similarly, gastrin (1 μM) caused a 136% ($p < 0.05$) increase. TGF-β caused an increase in collagen production of 149%. Furthermore, to demonstrate that collagen type I production was specifically induced in PSC, we performed immunocytochemistry for collagen type I and α-SMA (Fig. 2, *right panel*). These results demonstrate that CCK and gastrin induced collagen production by rat PSC, and that collagen synthesis was comparable, and not statistically significantly different from TGF-β-stimulated collagen production.

CCK and Gastrin Inhibit Proliferation in PSC—Some profibrogenic factors like TGF-β are also known to inhibit proliferation of PSC. To assess if CCK and gastrin have similar effects in inhibiting proliferation we performed thymidine uptake and MTT assays. After 24 h of stimulation TGF-β inhibited proliferation by 15.91%, CCK by 18.1%, and gastrin by 14.45% over controls (Fig. 3*A*). These results demonstrate that CCK and gastrin have similar effects to TGF-β in inhibiting proliferation of PSC. To elucidate if apoptosis or cell cycle arrest were involved in inhibition of proliferation upon stimulation, we performed caspase-3 assays, as well as p21 and p27 analysis. The missing cleavage of caspase-3 and the up-regulation of p21 and p27 suggest that G_1 cell cycle arrest, not apoptosis, is the likely mechanism involved. To address the question whether the up-regulation of p21 and p27 was due to an increase in transcriptional level or phosphorylation, we performed qPCR and Western blot analysis. As shown in Fig. 3*C*, p21 up-regulation is in part due to an increase in the transcriptional level and in part due to phosphorylation. Unlike p21, p27 is mostly regulated by phosphorylation (Fig. 3*D*).

Effect of CCK and Gastrin on Central Pro-fibrogenic Signal Transduction Pathways in Rat PSC—CCK has been reported to activate multiple signal transduction pathways including Akt, ERK, and Src in rat pancreatic acini 1 (7, 24). These central signal transduction pathways mediate activation of PSC and pancreatic fibrogenesis (5, 16, 25, 26). We analyzed whether CCK can activate Akt, ERK, and Src signal transduction pathways in rat PSC. Subsequently, we characterized the time course of this effect using a CCK concentration that caused maximal Akt activation (1 nM). CCKs effect on Akt was maximal at 2.5 min (3.8 ± 1.6-fold increase), significant at 5 min (2.3 ± 1.2-fold increase) with values returning to baseline after 10 min (Fig. 4*A*). CCK also (1.7 ± 0.8-fold) increased ERK phosphorylation after 2.5 min, with significant stimulation (1.8 ± 0.06-fold, $p = 0.0018$) after 5 min, stimulation slightly above baseline (1.4 ± 0.8-fold) after 10 min, and values comparable with baseline thereafter (Fig. 4*A*). Moreover, CCK caused significant stimulation (1.4 ± 0.3-fold, $p < 0.05$) of Src family kinases after 2.5 min with maximal stimulation (1.7 ± 0.3-fold, $p = 0.014$) at 5 min and stimulation slightly above baseline at 10 and 30 min (Fig. 4*A*). These data demonstrate that 1 nM CCK caused significant stimulation of the fibrogenic signal transduction pathways of Akt, ERK, and Src in rat PSC. Similar to CCK, gastrin activated Akt, ERK, and Src family kinases in rat PSC (Fig. 4*A*). Gastrin (1 μM) caused maximal Akt stimulation at 2.5 min, significant stimulation at 5 and 10 min with values returning to baseline after 30 min (Fig. 4*A*). Similarly, gastrin (1 μM) caused maximal ERK phosphorylation after 2.5 min with values returning to baseline after 3 min (Fig. 4*A*). Gastrin had a more complex effect on activation of Src family kinases with moderate stimulation after 2.5 and 5 min, no significant stimulation at 10 min, and strong stimulation at 30 min (Fig. 4*A*). These data

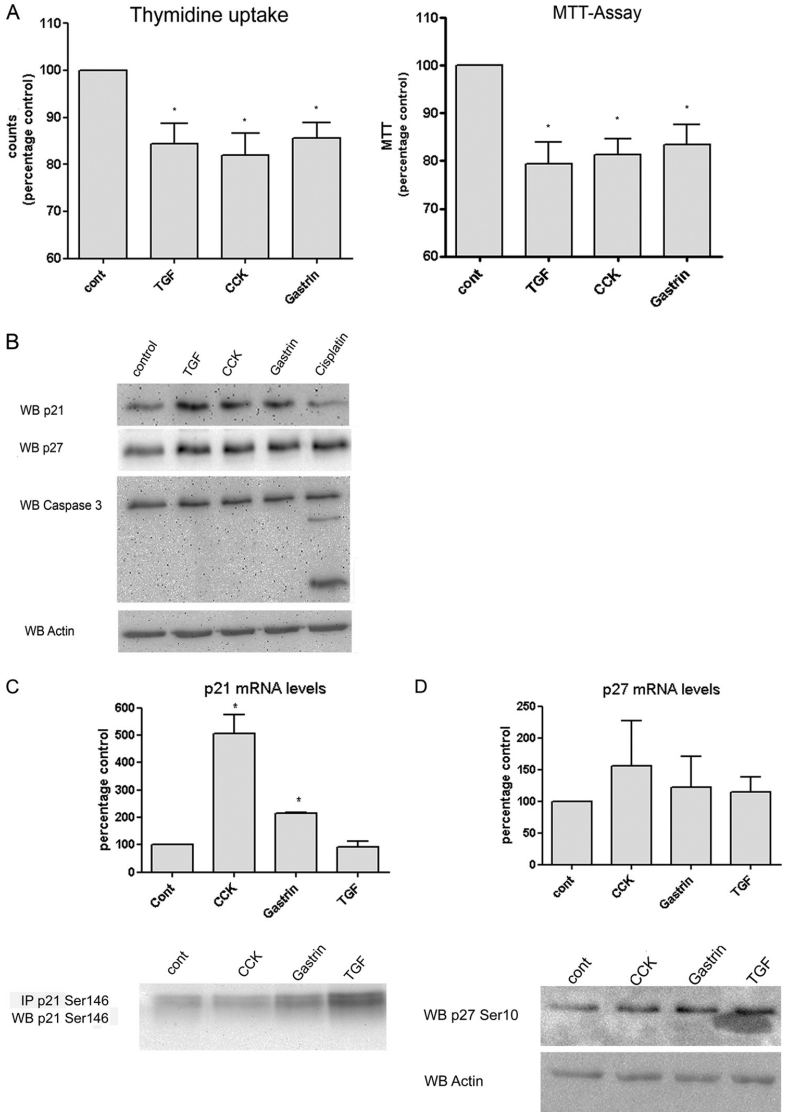

FIGURE 3. **Effect of CCK, Gastrin and TGFbeta1 on proliferation of PSC.** *A, thymidine uptake*: 50,000 cells/well were seeded, stimulated for 24 h, and [^{3}H]thymidine incorporation measured. The values represent the mean ± S.E. of 5 experiments and are expressed as percentage of the control. *, $p < 0.01$ *versus* control. *MTT assay*, rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated, and the MTT assay performed. The values represent the mean ± S.E. of 5 experiments and are expressed as percentage of the control. *, $p < 0.01$ *versus* control. *B*, rat PSC were cultured, serum starved, and stimulated for 24 h with no additions, ow with 2 ng/ml of TGF, 1 nM CCK, 1 μM gastrin, 100 μM cisplatin, and then lysed. Lysates were analyzed by Western blot (*WB*) for p21, p27, and caspase 3. β-Actin served as loading control. Shown is a representative experiment. *C*, rat PSC were cultured, serum starved, and stimulated for 24 h with no additions, or with 2 ng/ml of TGF, 1 nM CCK, and 1 μM gastrin. Cells were lysed/harvested and immunoprecipitations (*IP*) were performed using anti-p21 Ser^{146}. Western blots were then analyzed for anti-p21 Ser^{146}. RNA from harvested cells was isolated, reverse transcribed, and real time PCR for p21 mRNA was performed. The values indicated represent the mean ±S.E. of 4 experiments and are expressed as percentage of the control. (*, $p < 0.05$ *versus* control). *D*, rat PSC were cultured, serum starved, and stimulated for 24 h with no additionsm or with 2 ng/ml of TGF, 1 nM CCK, and 1 μM gastrin. Cells were lysed/harvested and Western blots were performed using anti-p27 Ser^{10}. β-Actin served as loading control. RNA from harvested cells was isolated, reverse transcribed, and real time PCR for p27 mRNA was performed. The values indicated represent the mean ±S.E. of 4 experiments and are expressed as percentage of the control.

demonstrate that, similar to CCK, gastrin stimulated Akt, ERK, and Src in rat PSC.

CCK and Gastrin Modulate Akt Activation in a Dose-dependent Manner in Rat PSC—We have recently shown that CCK1R induced a biphasic modulation of Akt activity in rat pancreatic acini depending on the CCK concentration (18). Furthermore, recent publications have shown an important role of Akt in PSC activation (6, 19). Phosphorylation of Akt at serine 473 has been shown to closely correlate with Akt activity in numerous studies (20, 21). Therefore, we examined the effect of different doses of CCK on Akt Ser^{473} phosphorylation in rat PSC (Fig. 4*B*). Doses as low as 1 pM CCK caused a significant increase in Akt Ser^{473} phosphorylation. This response was maximal at 1 nM (2.36 ± 0.45-fold increase) and then decreased to be just slightly above control at 1 μM. These results suggest that CCK has a complex effect on Akt activation in rat PSC: low doses of CCK induce significant Akt activation, whereas higher doses of CCK partially reverse this activation. To assess the effect of CCK2R on Akt activation in rat PSC, we studied the effect of the CCK2R-preferring agonist gastrin on Akt serine 473 phosphorylation (Fig. 4*B*). Gastrin (0.1 nM) caused a significant ($p < 0.01$) increase in Akt Ser^{473} phosphorylation and this effect was maximal at 1 μM (2.25 ± 0.40-fold). These data suggest that CCK2R stimulated Akt activation in rat PSC.

CCKs Action on Collagen Production, Akt Activation Was Mediated by Both Receptors but Primarily by the CCK2R in Rat PSC—Although CCK1R binds CCK with high affinity (K_d in the nanomolar range) and gastrin with low affinity (K_d in the micromolar range), the CCK2R had almost equal affinity for gastrin and CCK (for review, see Ref. 22). To determine whether CCK and gastrin effects are mediated by CCK1R or CCK2R, we used L364 and LY288513 as specific CCK1R and CCK2R inhibitors, respectively (for

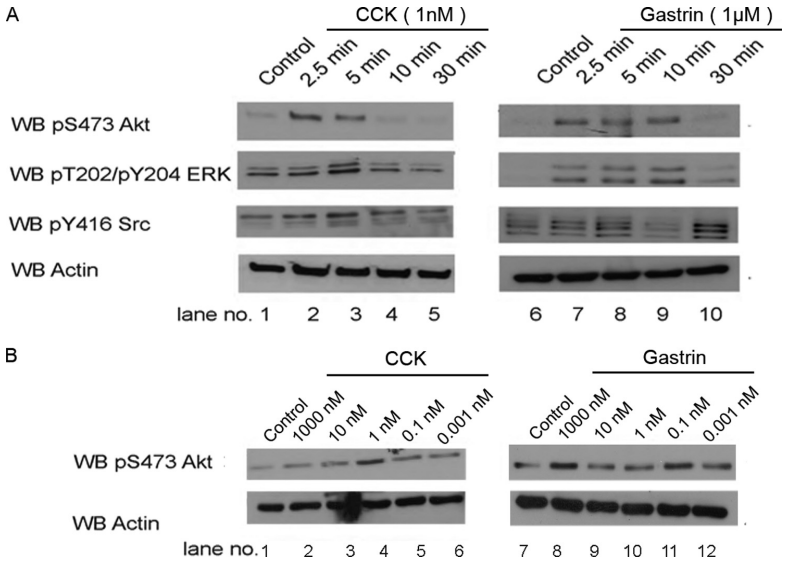

FIGURE 4. **Effect of CCK and gastrin on central fibrogenic signal transduction pathways in rat PSC.** *A*, rat PSC were cultured for 7 days and stimulated without additions (*control*) with 1 nM CCK (*left panels*) or with 1 μM gastrin (*right panels*) and then lysed. Lysates were analyzed by Western blot (*WB*) for pAkt, pERK, and pSrc. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 3 experiments. *B*, rat PSC were cultured for 7 days and treated with CCK (*left panel*) and gastrin (*right panel*) for 5 min. Proteins were analyzed for phosphorylated Akt by Western blot. Blots were stripped and analyzed for actin. Shown are representative results of a total of 6 experiments.

review of CCKR inhibitors, see Ref. 9). The CCK1R inhibitor L364 caused a moderate, but significant ($p < 0.001$) inhibition of Akt phosphorylation by 1 nM CCK (Fig. 5*A*, *second versus fourth lane*) and had a similar effect on Akt phosphorylation by 1 μM gastrin (Fig. 5*A*, *seventh versus ninth lane*). CCK2R inhibitor LY288513 strongly inhibited ($p < 0.0001$) Akt phosphorylation caused by 1 pM CCK (Fig. 5*A*, *second versus third lane*) and almost completely suppressed the effect of 1 μM gastrin on Akt phosphorylation (Fig. 5*A*, *seventh versus eighth lane*). A combination of both inhibitors inhibited Akt phosphorylation to a greater and statistically significant extent than the inhibition of one receptor alone, showing clearly that both receptors are involved (Fig. 5*A*, *fifth* and *tenth lane*). The same inhibitors were used to evaluate CCK1 and -2 receptor inhibition on collagen synthesis and proliferation (Fig. 5*B*). Similar to the results on Akt activation inhibition of CCK2R leads to a greater decrease of collagen synthesis than inhibition of CCK1R; simultaneous inhibition of both receptors reduces collagen production back to unstimulated levels, whereas proliferation raises production to unstimulated levels. These results clearly show that the inhibitory effect of CCK and gastrin is mediated through CCK1 and CCK2 receptors.

Akt Activation by CCK and Gastrin Are Mediated by PI3K in Rat PSC—Akt activation by growth factors is generally mediated by PI3K in most experimental systems. In rat pancreatic acini, Akt activation by the high affinity state of the CCK1R is mediated by PI3K (18). Therefore, we wanted to study if PI3K mediates Akt activation by CCK and gastrin in rat PSC. The highly specific PI3K inhibitor wortmannin (10 μM) caused a significant decrease of basal Akt Ser^{473} phosphorylation in rat PSC (Fig. 6*A*, *second versus first lane*) and almost completely suppressed Akt Ser^{473} phosphorylation caused by CCK and gastrin. These data demonstrate that CCK- and gastrin-stimulated Akt phosphorylation are mediated by PI3K in rat PSC. PI3K consists of two major components: p85, which is the regulatory site and p110, the catalytic domain (27). To study the events involved in CCK- and gastrin-mediated PI3K activation, we inhibited the β and γ subunits of G protein-coupled receptor targeting p110 using the PI3Kγ inhibitor II. As shown in Fig. 6*C* no inhibition was seen. On the other hand, inhibition of the Src pathway using the inhibitor PP2 leads to a marked decrease in CCK and gastrin-stimulated p85 activation (Fig. 6*B*). This result shows that CCK and gastrin activation of the PI3K is regulated by Src-dependent activation of the p85 subunit.

ERK Mediates Activation of Transcription Factor Elk-1 in Response to Gastrin and CCK in Rat PSC—The transcription factor Elk-1 has been reported to mediate ERK-induced c-*fos* transcription in response to growth factors, leading to cellular growth and proliferation in some cell systems (28, 29). This pathway has been reported to mediate PSC activation by alcohol and acetaldehyde (30). Therefore, we wanted to investigate whether CCK and gastrin cause Elk-1 activation and whether this activation is mediated by ERK. Our experiments show that both CCK and gastrin caused a significant increase in Elk-1 Ser^{383} phosphorylation, reflecting activation of Elk-1 (Fig. 7). As reported for pancreatic acini (17), the specific MEK inhibitor U0126 inhibited ERK activation induced by CCK and gastrin (Fig. 7). Moreover, U0126 completely inhibited stimulation of Elk-1 phosphorylation induced by CCK and gastrin (Fig. 7). These data demonstrate that, similar to findings reported in other cells, in rat PSC, transcription factor Elk-1 is a downstream target of the MEK-ERK pathway activated by CCK and gastrin.

Recent studies (31, 32) have shown that beyond its localization in the nucleus, transcription factor Elk-1 can be found in the cytosol. We did not find a cytosolic fraction of Elk-1 in PSC and subsequently no nuclear transition upon stimulation with CCK or gastrin (data not shown).

Pathways Involved in CCK and Gastrin-stimulated Collagen Production and Inhibition of Proliferation—As shown in Fig. 2, CCK and gastrin stimulate collagen production in PSC. Fig. 3 shows the inhibitory effect on proliferation of CCK and gastrin.

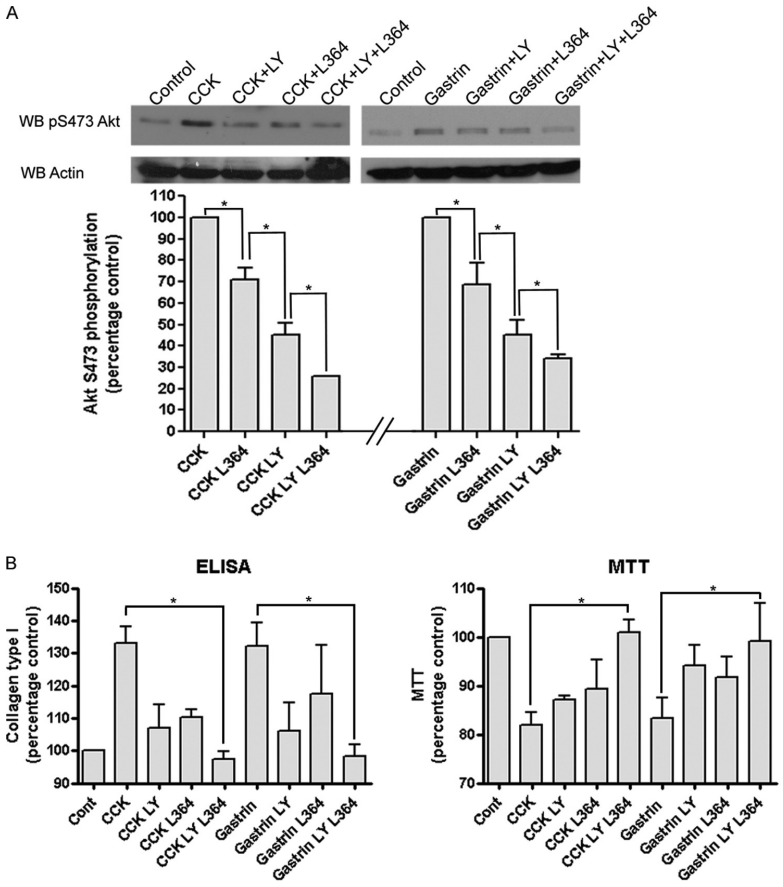

FIGURE 5. **Effect of CCK1R and CCK2R inhibitor on Akt activation, collagen synthesis, and proliferation mediated by CCK or gastrin in rat PSC.** *A*, rat PSC were cultured for 7 days, serum starved, and preincubated without additions, 1 μM CCK1R inhibitor L364 for 2.5 min, 10 nM CCK2R inhibitor LY288513, or a combination of both for 5 min. Cells were stimulated without additions (*control*), 1 nM CCK for 5 min or 1 μM gastrin for 5 min and then lysed. *Upper panel,* lysates were analyzed by Western blot for Akt Ser(P)^{473}. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 6 experiments. *Lower panel*, Western blots (*WB*) were scanned and densitometry was performed. The values represent the mean ± S.E. of 6 experiments and are expressed as fold of the maximal stimulation achieved with CCK or gastrin alone (*, $p < 0.05$). *B*, *ELISA*: rat PSC were cultured for 7 days, serum starved, and preincubated with 1 μM L364 for 2.5 min, or with 10 nM CCK2R LY288513 for 5 min, or with the combination. Cells were stimulated without additions, or with 1 nM CCK or 1 μM gasrin for 24 h. Stimulants and inhibitors were replaced after 12 h. Indirect ELISA using cell culture supernatants was performed. *MTT assay*: rat PSC were cultured for 7 days, serum starved, and preincubated with 1 μM L364 for 2.5 min, with 10 nM CCK2R LY288513 for 5 min, or with the combination. Cells were stimulated without additions, or with 1 nM CCK or 1 μM gasrin for 24 h. Stimulants and inhibitors were replaced after 12 h. MTT assays were performed. The values represent the mean ± S.E. of 3 experiments and are expressed as percentage of the control. *, $p < 0.05$ *versus* control.

Furthermore, we were able to show that CCK and gastrin stimulate different signaling pathways. Therefore we next wanted to elucidate which pathways are involved in CCK- and gastrin-stimulated collagen production and inhibition of proliferation. As shown in Fig. 8, *A* (CCK) and *B* (gastrin), inhibition of Src with the specific inhibitor PP2 leads to a statistically significant decrease in collagen synthesis in PSC, whereas inhibition of MEK and PI3K shows only a trend to decrease collagen production without reaching statistical significance. Fig. 8, *C* (CCK) and *D* (gastrin), shows that the same intracellular mechanisms are involved in inhibiting proliferation.

DISCUSSION

Our results clearly demonstrate that both CCK1 and CCK2 receptors are expressed in rat PSC. First, using the RT-PCR technique, we found that mRNA for both CCK1R and CCK2R is present in rat PSC. For both PCR, the specificity of the primer-target interaction was confirmed by the presence of a specific band in agarose-gel electrophoresis (Fig. 1). Furthermore, an interaction with possible contaminations of genomic DNA can be ruled out by the absence of a specific band in controls using genomic DNA (Fig. 1*A*). Furthermore, we were able to show the presence of both receptors using immunohistochemistry and immunocytology, also showing co-localization with α-SMA (Fig. 1, *B* and *C*). Most importantly, the data show that both receptors are involved in crucial cellular functions such as collagen synthesis and proliferation. Our data demonstrate for the first time that CCK and gastrin cause a significant increase in collagen I synthesis (Fig. 2). Synthesis of type I collagen contributes to organ fibrosis in the liver and pancreas. Therefore, our finding that CCK receptors have a direct effect on PSC by increasing collagen synthesis is of particular interest, because it demonstrates for the first time that CCK receptors can induce pancreatic fibrosis. CCK serum levels are often elevated in patients with chronic pancreatitis. A direct fibrogenic effect of CCK could be an important mediator of pancreatic fibrosis, suggesting that CCK receptors could be an interesting therapeutic target in patients with chronic pancreatitis. To date, the cytokine TGF-β had been the most potent stimulator of collagen synthesis in PSC (35). As shown in Fig. 2, the effect of CCK and gastrin on collagen synthesis was comparable with TGF-β-stimulated collagen production supporting their potential role in development of pancreatic fibrosis. Although expression of other matrix proteins, such as fibronectin, TIMP-1, and MMP has been described in PSC (36), CCK and gastrin were unable to stimulate the production of fibronectin and TIMP1.

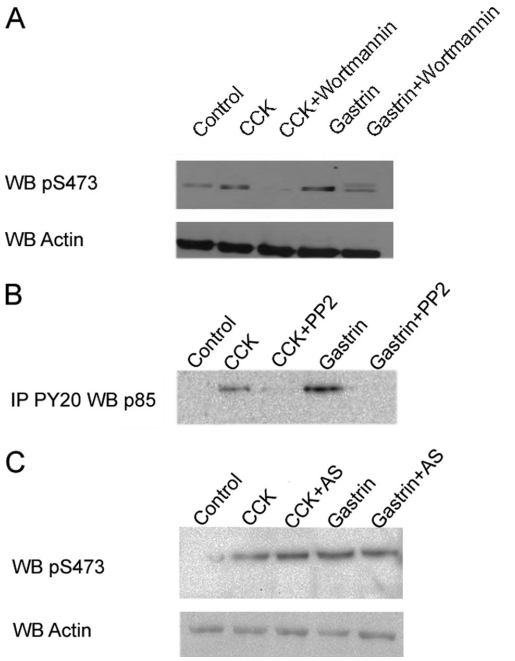

FIGURE 6. **Effect of CCK and gastrin on PI3K and Akt.** *A*, rat PSC were cultured for 7 days and preincubated without additions or with 10 μM wortmannin for 30 min. After this preincubation, cells were stimulated without additions (*control*) or with 1 nM CCK for 5 min or with 1 μM gastrin for 5 min, and then lysed. Lysates were analyzed by Western blot (*WB*) for Akt Ser(P)^{473}. Blots were stripped and analyzed for actin. *B*, rat PSC were cultured for 7 days and preincubated without additions or with 10 μM PP2 for 60 min. After this preincubation, cells were stimulated without additions (*control*), with 1 nM CCK for 5 min or with 1 μM gastrin for 5 min, and then lysed. Immunoprecipitation (*IP*) was performed using anti-PY20 antibody. Western blots were then analyzed for p85. *C*, rat PSC were cultured for 7 days and preincubated without additions or with 2 μM PI3Kγ inhibitor II for 60 min. After this preincubation, cells were stimulated without additions (*control*), or with 1 nM CCK for 5 min or 1 μM gastrin for 5 min, and then lysed. Western blots were analyzed for Akt Ser(P)^{473}. Blots were stripped and analyzed for actin.

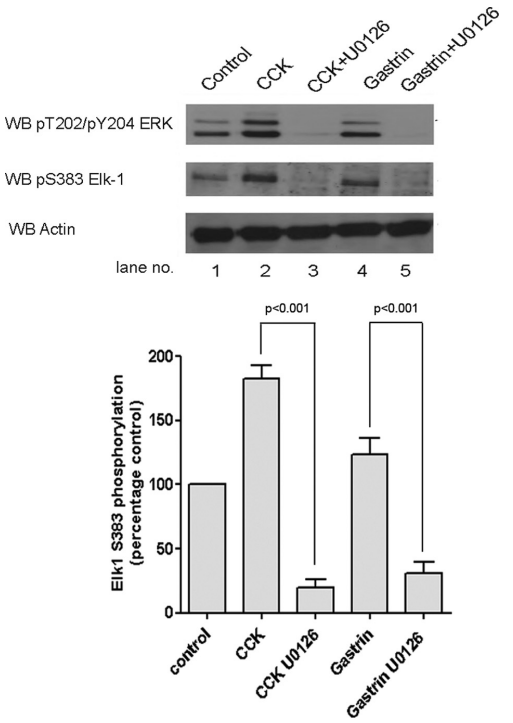

FIGURE 7. **Effect of MEK inhibitor U0126 on ERK and Elk-1 activation mediated by CCK or gastrin in rat PSC.** Rat PSC were cultured for 7 days, serum starved, and preincubated without additions or with 20 μM U0126 for 60 min. After this preincubation, cells were stimulated without additions (*control*), or with 1 nM CCK for 5 min or 1 μM gastrin for 5 min, and then lysed. *Upper panel*, lysates were analyzed by Western blot (*WB*) for pERK and pElk-1. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 3 experiments. *Lower panel*, Western blots were scanned and densitometry was performed. The values represent the mean ± S.E. of 3 experiments and are expressed as fold of the pre-treatment values.

To further evaluate the functional role of CCK and gastrin in PSC, we examined proliferation. Similar to TGF-β, and in contrast to other cytokines like PDGF (35), both CCK and gastrin inhibited proliferation. As shown in Fig. 3, the inhibitory effect is unlikely to be caused by apoptosis. However, accumulation of p21 and p27 upon stimulation with CCK, gastrin, and TGF-β supports the hypothesis that the mechanism involves G_1 cell cycle arrest. p21 is considered one of the most important effector molecules of p53, but is also known to be regulated via many p53 independent pathways. It is known to be regulated on the transcriptional level and to undergo posttranslational modifications, such as phosphorylation (37). p27 is regulated by several different independent mechanisms. Phosphorylation plays a major role: for example, phosphorylation of Ser^{10} leads to export from the nucleus and phosphorylation of Thr^{187} marks the protein for degradation (37, 38). As shown in Fig. 3, *C* and *D*, p21 is regulated on the transcriptional and protein levels, whereas p27 is regulated by phosphorylation upon stimulation with CCK and gastrin in PSC. To address the question which profibrogenic pathways are involved, we investigated the Akt, Src, and ERK pathways.

Our results demonstrate that rat PSC express functional CCK1 and CCK2 receptors (Figs. 4*B* and 5, *A* and *B*). Because numerous studies have found that rat acinar cells express only CCK1R, expression of both CCK1R and CCK2R in rat PSC was unexpected. Previous studies using CCK1R antibodies in rat, pig, and human pancreas (33) or CCK1R and CCK2R autoradiography in human pancreas (35) did not describe expression of CCK1R on fibroblast-like cells. Because PSC represent only a minority of cells in the normal pancreas, single positive PSCs on thin sections might be missed. Therefore, our data demonstrating expression of both CCK1R and CCK2R on rat PSC are compatible with these previous studies.

Our finding that CCK2R can activate PI3K and Akt is consistent with previous studies showing PI3K activation by gastrin in AR42J cells (40) and CCK2R-transfected COS-7 cells (41). Our data suggest that CCK1R could also have an effect on Akt phosphorylation, but studies with specific CCK1R agonists are needed to clearly define that effect.

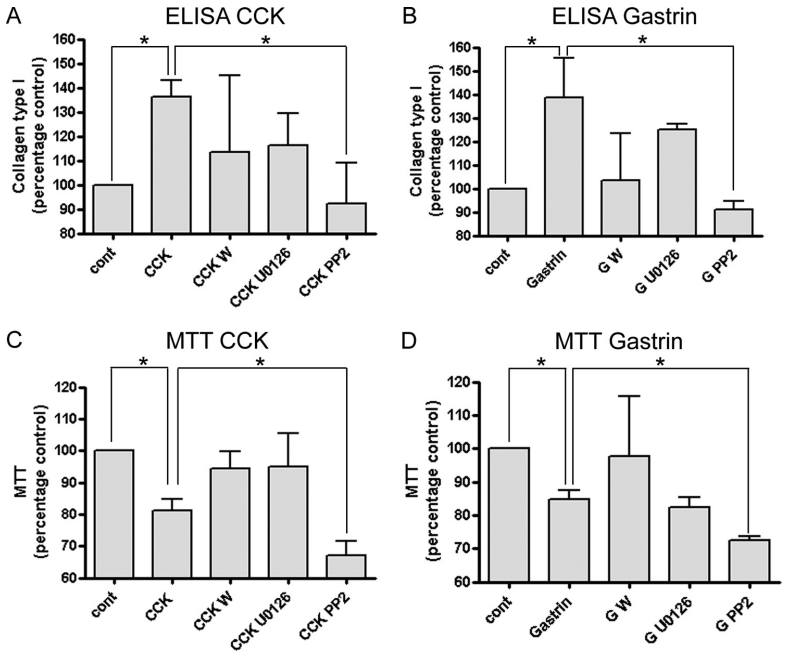

FIGURE 8. **Pathways involved in CCK and gastrin-stimulated collagen production and inhibition of proliferation.** *A*, rat PSC were cultured, serum starved, and stimulated without additions, or preincubated with wortmannin (30 min, 10 μmol), PP2 (10 μmol 60 min), U0126 (20 μmol 60), and stimulated with 1 nM CCK for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean ± S.E. of 4 experiments and are expressed as percent of the pre-treatment value. *B*, rat PSC were cultured, serum starved, and stimulated without additions, or preincubated with wortmannin (30 min, 10 μmol), PP2 (10 μmol 60 min), U0126 (20 μmol 60), and stimulated with 1 μM gastrin for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean ± S.E. of 4 experiments and are expressed as percent of the pre-treatment value. *C*, *MTT assay*: rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated with 1 nM CCK, and the MTT assay was performed. The values represent the mean ± S.E. of 5 experiments and are expressed as percentage of the control. *, $p < 0.01$ *versus* control. *D*, *MTT assay*: rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated with 1 μM gastrin, and MTT assay was performed. The values represent the mean ± S.E. of 5 experiments and are expressed as percentage of the control. *, $p < 0.01$ *versus* control. *GW*, gastrin + wortmannin.

Our data demonstrate that both CCK and gastrin cause activation of the MEK-ERK pathway in rat PSC, leading to activation of the transcription factor Elk-1. This is compatible with studies showing activation of ERK by CCK in rat pancreatic acini (10) and with studies showing activation of ERK by CCK and gastrin in AR42J pancreas cancer cells (42). Our finding that gastrin and CCK induce significant ERK activation is particularly relevant because PSC activation and proliferation caused by PDGF, one of the strongest known activators of PSC, is mediated by ERK (16, 43). We have shown that both CCK and gastrin cause reproducible activation of Src family kinases (Fig. 4*A*). This finding is compatible with studies showing activation of Src kinases in rat pancreatic acini by CCK (mediated by CCK1R)(24) and with studies showing activation of Src kinases by gastrin in AR42J and Panc-1 pancreas cancer cells (44–46) as well as in other multiple cells. Activation of Src family kinases by CCK and gastrin in rat PSC is interesting because Src family kinases activate the JAK2-STAT pathway after PDGF stimulation in PSC and thereby could be important regulators of PSC proliferation (26). Furthermore, we were able to show (Fig. 8) that inhibition of Src reduces collagen synthesis and proliferation in a statistically significant manner, pointing to the Src pathway as a pivotal pathway in CCK- and gastrin-stimulated collagen production and inhibition of proliferation.

Chronic pancreatitis is a complex disease and our knowledge of the exact pathophysiologic mechanisms are still incomplete. Recent studies suggest that the development of chronic pancreatitis requires a first episode of pancreatitis (sentinel acute pancreatitis event), which, by the release of different cytokines, triggers activation of immune cells and stellate cells, leading to chronic inflammation, fibrosis, and destruction of normal organ architecture, resulting in loss of organ function (47, 48). In this process, activation of stellate cells is crucial, because these cells have been shown to be responsible for the development of pancreatic fibrosis, which is a constant feature of chronic pancreatitis. In rodent animal models, CCK is routinely used in combination with other agents to induce chronic pancreatitis (23, 49–51). These studies have supposed that the role of CCK in these models was to trigger the sentinel pancreatitis event by interacting with CCK receptors on acinar cells and inducing necrosis of acinar cells (49). Our finding that CCK can directly activate stellate cells and induce collagen production suggests that the role of CCK is more complex: 1) by its action on acinar cells, it could contribute to the induction of the sentinel pancreatitis event; and 2) by its action on stellate cells, it could serve as an important regulator of pancreatic fibrosis. In conclusion, we report for the first time that rat PSC express CCK1 and CCK2 receptors, that the natural agonists CCK and gastrin induce activation of pro-fibrotic signaling pathways PI3K/Akt, MEK/ERK, and Src, induce activation of the transcription factor Elk-1, and most notably significantly increase synthesis of type I collagen and inhibit proliferation.

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2. Darstellung der Publikation

2.1. Einführung

2.1.1. Übersicht zur chronischen Pankreatitis

Die chronische Pankreatitis ist definiert als chronische Entzündung der Bauchspeicheldrüse, die zu einer irreversiblen Zerstörung des Pankreasparenchyms mit nachfolgendem Funktionsverlust, d.h. einer exokrinen und endokrinen Pankreasinsuffizienz führt (Riemann et al. 2008). Histopathologisch läßt sich zunächst eine lymphozytär-entzündliche Fibrose, später dann eine diffuse Fibrose und Atrophie des Organs beobachten (Riede et al. 2004).
Die Inzidenz beträgt in Deutschland ca. 2-10/100000 Einwohner, wobei das Verhältnis männlich:weiblich ca. 3:1 beträgt. Als auslösende Faktoren werden verschiedene Ursachen angenommen, allerdings spielt hierbei Alkohol mit ca. 80% die wichtigste Rolle.

Ätiologie	Ursachen
Toxisch-metabolisch **80%**	**Alkohol**, Hyperkalziämie, Hyperlipidämie, Urämie, Medikamentös
Idiopathisch 15%	Ursache unbekannt
Genetisch	Mutationen im PRSS-1-, CFTR- und SPINK-1-Gen
Autoimmun	Infiltration Pankreas mit IgG4-positiven Plasmazellen
Obstruktiv	Rezidivierende Choledocholithiasis, SOD (Sphincter-Oddi-Dysfunktion), Pancreas divisum, obstruktiver Tumor

Tab. 1: Ätiologie und Ursachen der chronischen Pankreatitis (Greten et al. 2010)

2.1.2. Rolle der pankreatischen Sternzellen an der Entstehung der chronischen Pankreatitis / dem Ablauf der Entzündung

In vielen Arbeiten zur Pathogenese der akuten Pankreatitis konnte gezeigt werden, dass Mechanismen innerhalb der Azinuszellen zu Inflammation und Organdestruktion führen (Saluja et al.1989). Wie in Tab.1 zu sehen, kann diese über verschiedene Wege geschädigt werden. Die entscheidende Rolle bzw. gemeinsame Endstrecke jedoch

besteht in der intrazellulären Spaltung von Trypsinogen zu Trypsin. Exemplarisch soll dies an der autosomal-dominanten hereditären Pankreatitis beschrieben werden. Bei dieser Erkrankung wurden Mutationen im kationischen Trypsinogen-Gen beschrieben. Diese führen zu einer gesteigerten Trypsinaktivität sowie zu einer erhöhten Stabilität des aktivierten Trypsins und in Folge dessen zu intrazellulärem Stress, sowie einer lokalen Entzündung. Im Verlauf treten intra- und extrapankreatische Fettgewebsnekrosen auf, welche die Entzündung verstärken. Als Konsequenz daraus werden pankreatische Sternzellen (PSC) aktiviert.

Erstmalig 1998 näher charakterisiert, konnte diesen Zellen eine zentrale Rolle an der Entstehung der chronischen Pankreatitis zugeschrieben werden (Apte et al. 1998, Bachem et al. 1998). Die physiologische Rolle am gesunden Organ wird bislang nur unzureichend verstanden. Vermutet wird unter anderem, dass sich diese am Grundumsatz der extrazellulären Matrix beteiligen. Als gesichert gilt jedoch, dass sie im Rahmen einer Entzündung durch inflammatorische Cytokine wie TGF-ß, andere Noxen wie Acetaldehyd aber auch Trypsin aktiviert werden. Sie beginnen zu proliferieren und Cytokine, Interleukine sowie Matrixproteine, z.B. Kollagen Typ I, zu produzieren und zu sezernieren. (Apte et al. 2000, Hama et al. 2006, Masamune et al. 2005, 2009, 2010) Die Entzündung verstärkt sich selbst und führt durch Ablagerung der Kollagene zu Fibrose. Dieser Prozess wurde von Klöppel als sog. Nekrose-Fibrose-Sequenz beschrieben (Klöppel et al. 1992).

2.1.3. Rolle des Cholecystokinin

Das gastrointestinale Hormon Cholecystokinin (CCK) gehörte zu den ersten entdeckten gastrointesinalen Hormonen. CCK bindet an zwei Rezeptoren (CCK1R und CCK2R), welche in vielen Geweben einschließlich des exokrinen Pankreas exprimiert werden (Berna et al. 2007). Eine Stimulation der CCK-Rezeptoren durch die natürlichen Agonisten CCK (vergleichbare Affinität für CCK1R und CCK2R) und Gastrin (1000fach höhere Affinität für CCK2R) reguliert eine Vielzahl physiologischer und pathophysiologischer Prozesse. Am bemerkenswertesten ist, dass CCK in Nagetieren eine akute Pankreatitis induzieren kann, vermittelt durch seine Wirkung auf den CCK1R, welcher auf pankreatischen Azinuszellen exprimiert wird (Williams et al. 2002). Die Pathophysiologie der CCK-induzierten akuten Pankreatitis und die zu Grunde liegenden Signaltransduktionswege wurden in den letzten 20 Jahren ausführlich in Studien mit Tieren sowie mit isolierten Azinuszellen untersucht. Die wiederholte Gabe von CCK oder

dem Agonisten Cerulein wurde und wird in vielen Studien benutzt, um verschiedene Aspekte der chronischen Pankreatitis zu induzieren, einschließlich der Aktivierung von PSC und der Fibrogenese. Auch in diesen Studien wurde angenommen, dass die Entstehung der Fibrose ein Produkt der wiederholten Organinflammation darstellt, verursacht durch die Induktion multipler akuter Pankreatitiden durch CCK (sog. Sentinelpankreatitiden). Darüber hinaus konnte in mehreren Studien gezeigt werden, dass im Serum von Patienten welche an einer chronischen Pankreatitis litten verglichen mit gesunden Kontrollen, erhöhte CCK-Plasmaspiegel zu messen waren (Gomez et al. 1991, Schafmayer et al. 1985, Slaff et al. 1984). Trotz dieser experimentellen und klinischen Ergebnisse, die auf eine Rolle von CCK in der chronischen Pankreatitis hinwiesen, hatte bislang keine Studie untersucht, ob CCK oder eines seiner Agonisten einen direkten Effekt auf pankreatische Sternzellen haben könnte. Selbst die Expression von CCK-Rezeptoren auf PSC wurde bislang noch nicht untersucht.

2.1.4. Ziel der Arbeit

Um also eine mögliche direkte Rolle von CCK und CCK-Rezeptoren in der Pathophysiologie der chronischen Pankreatitis zu untersuchen, wurden sowohl die Expression von CCK1R und CCK2R untersucht, als auch die Effekte der Stimulation dieser Rezeptoren auf die Kollagenexpression, die Proliferation und auf profibrogene Signaltransduktionswege.

2.2. Zusammenfassung der Versuche und Ergebnisse

2.2.1. Expression von CCK1- und CCK2- Rezeptoren auf Sternzellen

Zunächst wurden pankreatische Sternzellen als Primärkultur aus Rattenpankreas gewonnen. Hierzu wurde nach duktaler Injektion einer Enzymlösung und anschließendem Verdau eine Gradientenzentrifugation durchgeführt. Die daraus gewonnenen Zellen wurden in Kultur genommen.
Der Nachweis der CCK1- und CCK2- Rezeptoren erfolgte mittels RT-PCR (Abb.1A), durch Immunofluoreszenz (Abb.1B) in kryokonservierten Pankreasschnitten sowie durch immunzytologische Färbungen an kultivierten Sternzellen (nicht gezeigt). Durch einen für Sternzellen typischen Oberflächenmarker (alpha-SMA) konnte die Spezifität der Färbung gezeigt werden.

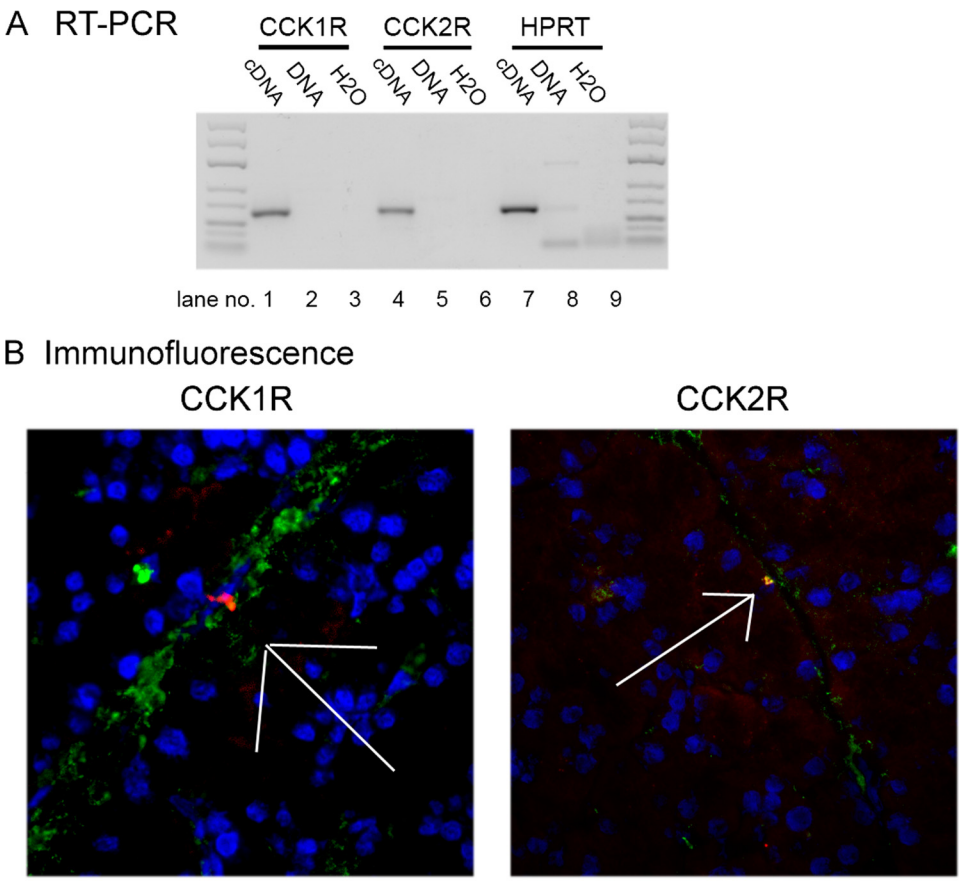

Abb.1: Nachweis von CCK1- und CCK2- Rezeptoren mittels RT-PCR und Immunhistologie.

Es konnte so erstmals gezeigt werden, dass CCK-Rezeptoren in Pankreassternzellen exprimiert werden.

2.2.2. Proliferation und Kollagenproduktion in pankreatischen Sternzellen nach CCK-Stimulation

Im Anschluss daran wurde untersucht, ob die Stimulation der Zellen mit CCK funktionelle Auswirkungen hat. Insbesondere im Hinblick auf die Funktion der Sternzellen im erkrankten Organ, wurden die Kollagenproduktion, die Proliferation sowie die Aktivierung profibrogener Signaltransduktionswege untersucht. Als positive Kontrolle diente TGF-ß, welches bislang als potentester Stimulus der Kollagensynthese in PSC galt (Kordes et al. 2005).

Als Nachweis der Kollagenproduktion wurden verschiedene Methoden angewandt. Zum einen auf Proteinebene mittels Western Blot und indirektem ELISA. Zum anderen mittles immunzytologischer Färbungen nach Stimulation, um durch eine Kolokalisation der

Kollagensynthese mit alphaSMA den Nachweis zu erbringen, dass die Synthese in Sternzellen stattfand. Wie in Abb. 2 gezeigt, stimulierten sowohl CCK als auch Gastrin signifikant die Kollagensynthese. So konnte gezeigt werden, dass die exprimierten Rezeptoren funktionell aktiv sind, und nach Aktivierung die Pankreasfibrose fördern können.

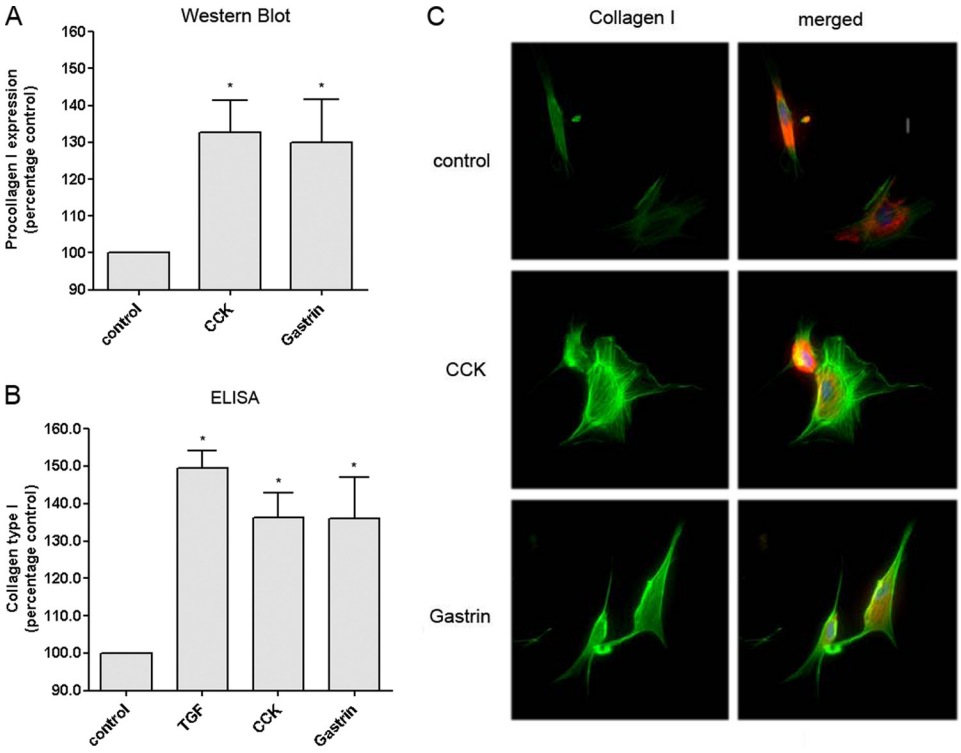

Abb. 2: Messung der Kollagensynthese in pankreatischen Sternzellen nach CCK-, Gastrin und TGF-ß - Stimulation im Western Blot (A), ELISA (B) und mittels Immunzytochemie (C)

Von einigen Stimuli wie TGF-ß ist bekannt, dass sie neben ihren profibrogenen Wirkungen das Wachstum inhibieren (Kordes et al. 2005). Um dies für CCK und Gastrin zu untersuchen, wurden Thymidin- und MTT-Assays durchgeführt. Nach 24-stündiger Stimulation konnte in beiden Verfahren gezeigt werden, dass sowohl CCK als auch Gastrin das Wachstum von PSC inhibiert, vergleichbar der Wirkung von TGF-ß (Abb.3).

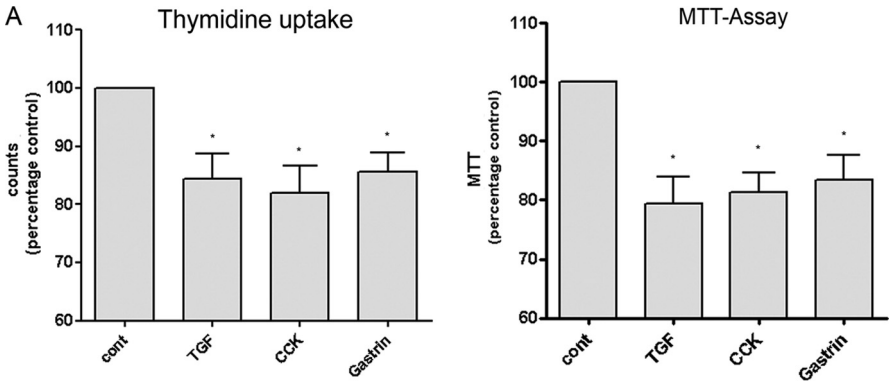

Abb. 3: Messung der Proliferation mittels Thymidinuptake (links) und MTT-Assay (rechts)

Hieran schloss sich die Frage nach dem Mechanismus der Wachstumsinhibition. Untersucht wurden zum einen die Apoptose sowie zum anderen ein Zellzyklusarrest als mögliche Ursachen. Als Marker für die Apoptose diente die Caspase-3 Spaltung, für einen Zellzyklusarrest als alternativen Mechanismus p21 und p27, beide als Marker der G1-Phase. Wie in Abb.4B zu sehen, kam es im Gegensatz zu Cisplatin-stimulierten Zellen zu keiner Caspase 3-Spaltung.

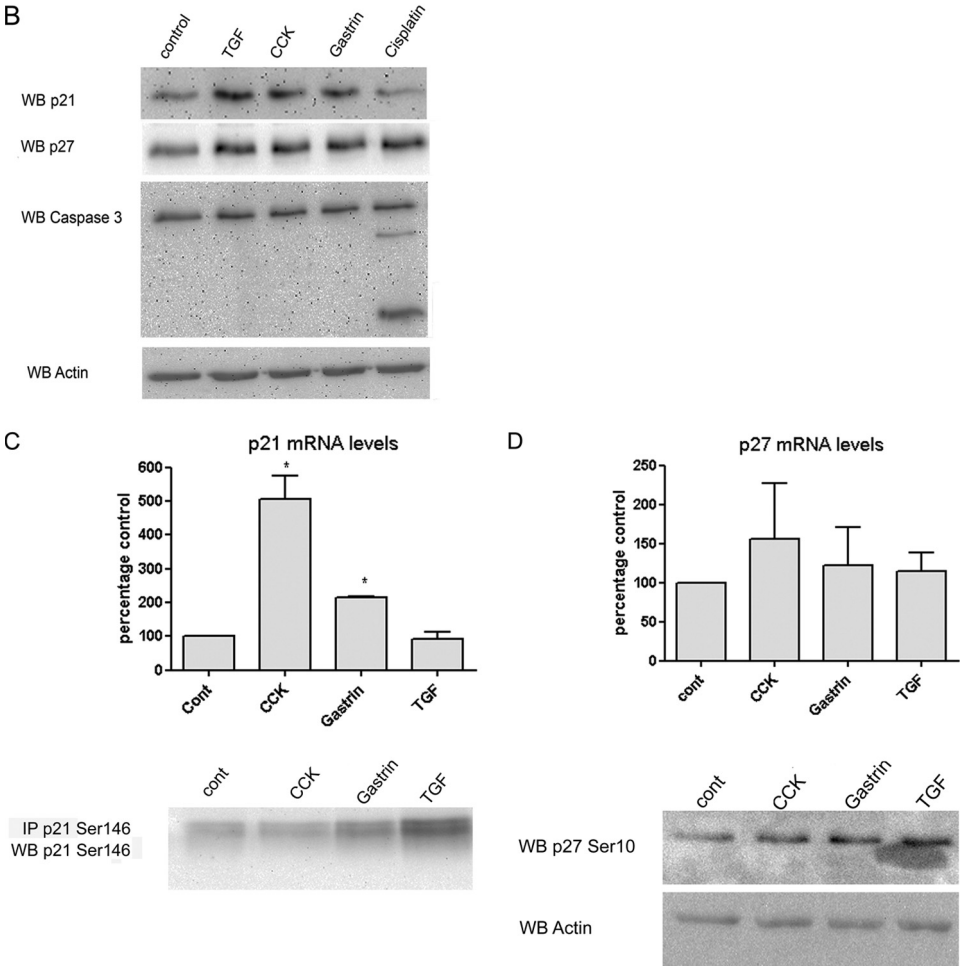

Abb.4: Messung der Apoptose mittels Caspase-3 (oben) und G1-Zellzyklusarrest mittels p21 und p27 realtimePCR (Mitte) und Western Blot (unten)

Stattdessen konnte mittels Western Blot und realtimePCR gezeigt werden, dass p21 und p27 hochreguliert wurden, so dass ein G1-Arrest als Ursache für die Wachstumsinhibition in Frage kam. Wie in Abb. 4C zu sehen, erfolgte die Regulation von p21 dabei sowohl auf Transkriptionsebene als auch durch Phosphorylierung, wohingegen p27 hauptsächlich durch Phosphorylierung reguliert wurde (Abde et al. 2008, Vervoorts et al. 2008).

2.2.3. Profibrogene Signaltransduktionswege nach CCK-Stimulation

Aus Untersuchungen an Azinuszellen ist bekannt, dass CCK vielfältige Signaltransduktionswege aktivieren kann, einschließlich Akt, ERK und Src (Masamune et al. 2010, Pace et al. 2006). In anderen Studien zur Fibrogenese des Pankreas wurden eben diese Signalwege untersucht und ihr profibrogener Einfluss beschrieben. Daher wurden die Wirkungen CCKs und Gastrins auf diese Signalwege untersucht. Es zeigte sich, dass sowohl CCK als auch Gastrin Akt, ERK und Src durch Phosphorylierung aktivieren können (nicht gezeigt).
Die Aktivierung von ERK durch CCK und Gastrin führte zur Aktivierung des Transkriptionsfaktors Elk-1. Dies war insofern von Interesse, da Elk-1 die Transkription von c-fos vermittelt, einem Mechanismus, der in der Aktivierung von pankreatischen Sternzellen durch Alkohol und Acetaldehyd involviert ist (McCarroll at al. 2003). Die ERK-Abhängigkeit der Elk-1-Aktivierung wurde mittels spezifischer ERK-Inhibitoren untersucht (nicht gezeigt).

2.2.4. Einfluss von Akt, ERK und Src auf Kollagensynthese und Proliferation

Wie oben beschrieben konnte zum einen gezeigt werden, dass nach CCK- und Gastrin-Stimulation in Sternzellen die Kollagenproduktion erhöht und die Proliferation gehemmt, sowie zum anderen die profibrogenen Signalwege PI3K/Akt, ERK und Src aktiviert wurden. In einem nächsten Schritt wurde deshalb untersucht welche Signalwege direkt an den beschriebenen Effekten - Kollagenproduktion und Wachstumshemmung - beteiligt waren. Wie aus Abb.5 zu entnehmen, kam hierbei insbesondere der Src-Kinase eine Schlüsselrolle zu. Nach Inhibition mit dem spezifischen Inhibitor PP2 konnten sowohl ein

deutlicher Rückgang der Kollagenproduktion als auch eine deutliche Wachstumsinhibition beobachtet werden.

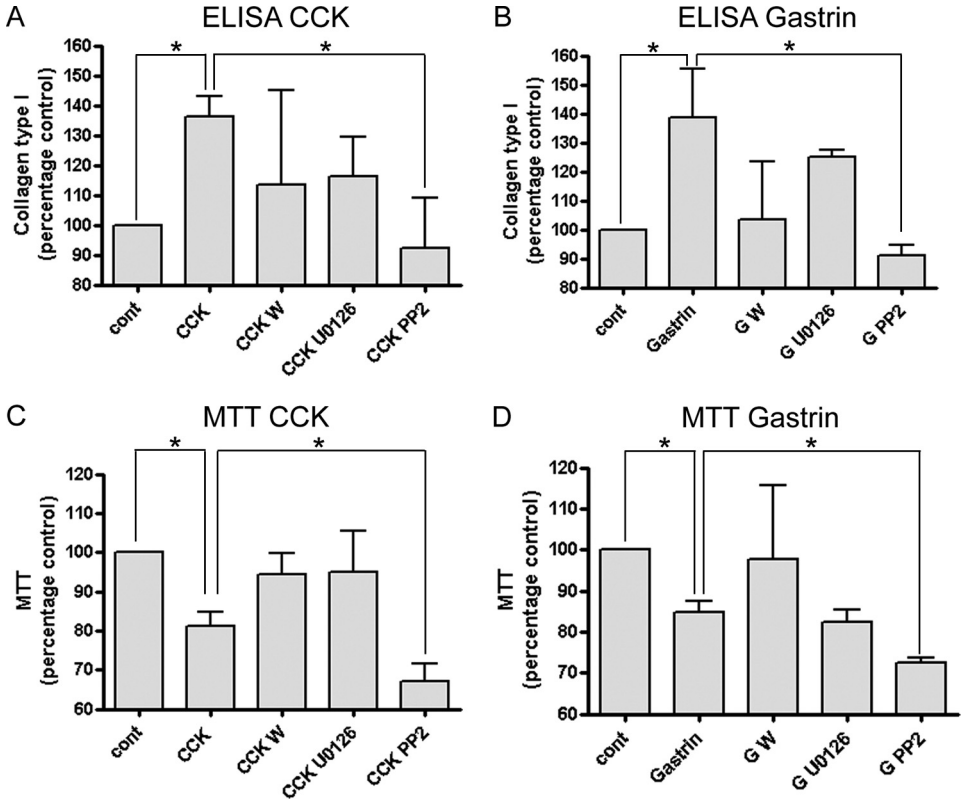

Abb. 5: Rolle der PI3-Kinase/Akt, ERK und Src in der CCK- und Gastrin-stimulierten Kollagenproduktion und Wachstumshemmung. W: Wortmannin, PI3-Kinasen-Inhibitor; U0126: ERK-Inhibitor; PP2: Src-Inhibitor

2.2.5. Einfluss der Rezeptortypen auf Akt Aktivierung und Kollagenproduktion

Es ist bekannt, dass die beiden CCK-Rezeptoren CCK und Gastrin mit unterschiedlicher Affinität binden. Während der CCK1-Rezeptor CCK mit hoher und Gastrin mit 1000fach niedrigerer Affinität bindet, hat der CCK2-Rezeptor nahezu äquimolare Bindungsaffinität. Um zu bestimmen ob die CCK-Rezeptor vermittelten Effekte auf die Akt-Aktivierung zum einen und die Kollagensynthese zum anderen nun vermehrt über CCK1R oder CCK2R vermittelt werden, wurden Versuche mit spezifischen Inhibitoren durchgeführt: L364 für CCK1R und LY288513 für CCK2R (Berna et al. 2007). In Abb.6A ist zu sehen, dass sowohl die Inhibition des CCK1R als auch des CCK2R zu einer signifikanten Inhibition der Akt-Phosphorylierung führten, wobei diese nach CCK2R-Hemmung stärker ausfiel. In ähnlicher Weise ist dies auch in Abb. 6B und 6C zu sehen: nach Inhibition des CCK2R,

konnte eine deutlichere Hemmung der Induktion der Kollagenproduktion bzw. Aufhebung der Wachstumsinhibition beobachtet werden, verglichen zur Inhibition des CCK1R. Hieraus ließ sich schließen, dass die beschriebenen Effekte sowohl durch CCK1R als auch durch CCK2R vermittelt werden, vornehmlich aber durch CCK2-Rezeptoren.

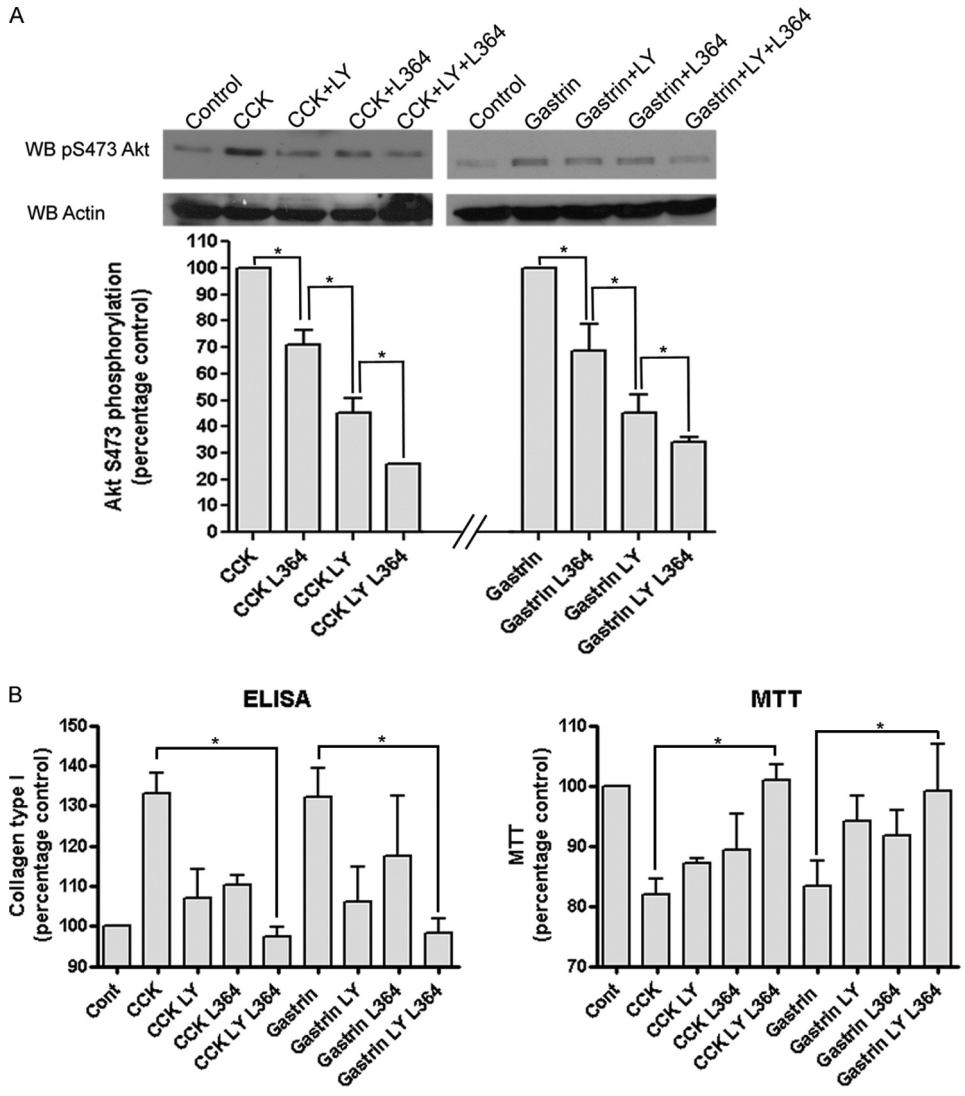

Abb. 6: Rolle der CCK1- und CCK2- Rezeptoren in der Akt-Aktivierung (A), sowie der Kollagenproduktion und Proliferation (B ELISA und MTT)

2.2.6. Zusammenfassung

Die chronische Pankreatitis ist eine komplexe Erkrankung und das Wissen über die genaue Pathophysiologie ist nach wie vor unvollständig. Aktuelle Studien beschreiben die Entwicklung hin zu einer chronischen Pankreatitis so, dass zuerst eine akute Pankreatitis erforderlich ist, als sogenanntes „Sentinel acute pancreatitis event“, welches durch die Ausschüttung verschiedener Cytokine die Aktivierung von Immunzellen und Sternzellen

triggert, um so der chronischen Entzündung Vorschub zu leisten. Dies führt zu Fibrose, zu Zerstörung der normalen Pankreasarchitektur und zum Verlust der normalen Organfunktion. Innerhalb dieses Prozesses spielt die Aktivierung pankreatischer Sternzellen eine grundlegende Rolle, da sich herausgestellt, dass diese Zellen verantwortlich für die Entstehung der Pankreasfibrose sind, dem typischem Merkmal der chronischen Pankreatitis.

In Nagetiermodellen wird CCK routinemäßig allein oder in Kombination mit anderen Stoffen eingesetzt, um eine chronische Pankreatitis zu induzieren. Diese Studien nahmen an, dass die Rolle CCKs darin bestand durch Interaktion mit CCK-Rezeptoren auf Azinuszellen die Sentinelpankreatitis auszulösen und in Folge dessen Nekrose zu induzieren.

Die Ergebnisse konnten zeigen, dass Cholecystokinin Sternzellen direkt aktivieren und die Kollagensynthese induzieren kann. Dies lässt eine komplexere Rolle von CCK in der Pathogenese der chronischen Pankreatitis vermuten. CCK könnte zum einen durch seine Wirkung auf Azinuszellen zu der Entstehung der Sentinelpankreatitis beitragen und zum anderen durch seine Effekte auf Sternzellen als ein wichtiger Regulator in der Entstehung der Fibrose fungieren.

Zusammenfassend konnte zum ersten Mal gezeigt werden, dass pankreatische Sternzellen CCK1- und CCK2- Rezeptoren exprimieren und, dass die natürlich vorkommenden Agonisten CCK und Gastrin die Aktivierung der profibrogenen Signalwege PI3K/Akt, MEK/ERK/Elk-1 und Src induzieren. Am wichtigsten jedoch, dass die Stimulation dieser Rezeptoren die Kollagensynthese signifikant erhöht sowie das Wachstum signifikant inhibiert.

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4. Lebenslauf

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Veröffentlichungen

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5. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift: .....