Biochemical and Biophysical Research Communications 444 (2014) 75–80

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pancreatic acinar cells-derived cyclophilin A promotes pancreatic damage by activating NF-κB pathway in experimental pancreatitis

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ARTICLE INFO

Article history:
Received 24 December 2013
Available online 14 January 2014

Keywords:
Cyclophilin A
Acute pancreatitis
Inflammatory cytokines
Necrosis
Nuclear factor-κB

ABSTRACT

Inflammation triggered by necrotic acinar cells contributes to the pathophysiology of acute pancreatitis (AP), but its precise mechanism remains unclear. Recent studies have shown that Cyclophilin A (CypA) released from necrotic cells is involved in the pathogenesis of several inflammatory diseases. We therefore investigated the role of CypA in experimental AP induced by administration of sodium taurocholate (STC). CypA was markedly upregulated and widely expressed in disrupted acinar cells, infiltrated inflammatory cells, and tubular complexes. *In vitro*, it was released from damaged acinar cells by cholecystokinin (CCK) induction. rCypA (recombinant CypA) aggravated CCK-induced acinar cell necrosis, promoted nuclear factor (NF)-κB p65 activation, and increased cytokine production. In conclusion, CypA promotes pancreatic damage by upregulating expression of inflammatory cytokines of acinar cells via the NF-κB pathway.

1. Introduction

Acute pancreatitis (AP) is a disease with high morbidity and mortality, but its complete mechanism has not been established. The initial events occur in pancreatic acinar cell, including activation of zymogens and release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, resulting in the recruitment of inflammatory cells such as neutrophils and macrophages [1,2]. One important signaling molecule, nuclear factor (NF)-κB, a nuclear transcription factor responsible for regulating the transcription of a wide variety of genes involved in immunity and inflammation [3], has been shown to play a critical role in the development of AP. Recently, Huang and colleagues demonstrated that activation of NF-κB in acinar cells by transgenic mice increased the severity of pancreatitis in mice [4].

Cyclophilin A (CypA), also known as cis-trans peptidyl-prolyl isomerase A (Ppia), is an abundant cytosolic protein (~0.25% of cellular protein) belonging to the cyclophilin family [5]. Although CypA is widely distributed in the cytoplasm, it can also be secreted into the extracellular environment as a proinflammatory product by both live and dying cells [6–8]. Indeed, extracellular CypA concentrations are elevated in the synovial fluid of patients with active rheumatoid arthritis [9] and in the serum of patients with severe sepsis [10]. *In vitro* studies have shown that extracellular CypA has a potential chemotactic effect on several human and mouse leukocyte subsets, including neutrophils, T cells and monocytes/macrophages [11–13]. Moreover, CypA can also act as a damage-associated molecular pattern (DAMP) to worsen acetaminophen-induced liver injury [8].

Acinar cell necrosis is an important pathological feature of AP. Unlike apoptosis, necrosis amplifies the tissue damage by inducing an inflammatory response. We have previously used a gene chip to examine multiple gene differential expression patterns in the pancreas and found that CypA mRNA is markedly upregulated during arginine-induced AP. Moreover, CypA is an abundant intracellular protein and released into the extracellular space when cell necrosis occurs. We hypothesized that CypA is released from necrotic acinar cells and acts as an "alarmin" to worsen the severity of AP.

This study was designed to investigate the role of CypA during experimental pancreatitis *in vivo* and *in vitro*. Our results showed that CypA promoted pancreatic damage by upregulating expression of inflammatory cytokines via activating the NF-κB pathway.

2. Materials and methods

2.1. Ethics statement

All the animal related procedures were approved by the Animal Care and Use Committee of The Tenth People's Hospital of Shanghai, Tongji University. Permit number: 2011-RES1. This study was

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http://dx.doi.org/10.1016/j.bbrc.2014.01.015

also approved by Science and Technology Commission of Shanghai Municipality (ID: SYXK 2007-0006).

2.2. Animal experiments and regents

Male Sprague–Dawley rats weighing 250 ± 30 g were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Animals were maintained in 12-h light–dark cycles at 22 °C, given water *ad libitum*, fed standard laboratory chow, and allowed to acclimatize for a minimum of 1 week. The environment was maintained at a relative humidity of 30–70%. 4% sodium taurocholate (STC; 0.1 ml/100 g) was injected in the retrograde direction of the biliopancreatic duct at a rate of 0.20 ml/min. All animals were sacrificed under anesthesia at the times described in the relevant figures. STC, caerulein, CCK, Hoechst 33342, PI, antibodies against CypA and β-actin were purchased from Sigma–Aldrich (St. Louis, MO, USA). rCypA (ab86219) and antibody against NF-κB p65 were from Abcam (Hong Kong). Unless otherwise stated, the other chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL (USA).

2.3. Isolation of pancreatic acinar cells

Pancreatic acinar cells were isolated from rats using a collagenase digestion procedure as described previously [14]. The isolated acinar cells were incubated at 37 °C under humidified conditions of 95% air and 5% CO_2 in Dulbecco's Modified Eagle's Medium/Ham F-12 Medium (DMEM/F12) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin with or without rCypA, CCK, and other agents as described in the relevant figures.

2.4. Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were cut at a thickness of 5 μm. Each tissue section was deparaffinized and rehydrated with graded ethanol. For antigen retrieval, slides were boiled in EDTA (1 mM, pH 8.0) for 15 min in a microwave oven. Endogenous peroxidase activity was blocked with a 0.3% hydrogen peroxide solution for 10 min at room temperature. After rinsing with PBS, slides were incubated overnight at 4 °C with a polyclonal antibody against CypA (1:100 dilution). The antibody binding was detected with an Envision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (Gene Tech, Shanghai, China). Sections were counterstained with hematoxylin. For negative control, isotype-matched primary antibody replaced the antibody against CypA followed by the same secondary antibody used in the other sections. Positive areas stained with CypA were examined in all specimens using a microscope (CTR 6000; Leica, Wetzlar, Germany).

2.5. Western blot analysis

For Western blot analysis, rat pancreas was rapidly ground in liquid nitrogen. The resulting powder or isolated acinar cells were reconstituted in ice-cold RIPA buffer containing 1 mmol/l phenylmethanesulfonyl fluoride (PMSF) and a cocktail of protease inhibitors (1:100 dilution). Samples were centrifuged at 4 °C for 15 min at 10,000*g*. Supernatants were recovered, and total protein was determined using the BCA method (Pierce, Rockford, LA, USA). The acinar cells supernatants were centrifuged by ultrafiltration centrifuge tube (Vivaspin 20, 3000 MWCO PES, Sartorius, Germany) at 4 °C for 100 min at 4000*g*. A 50-μg portion of protein or equal proportion of concentrated supernatant was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE), and then blotted following standard methods. Non-specific binding to the membrane was blocked by 5% (w/v) dry non-fat milk in Tris-buffered saline/0.05% Tween-20 (TBST) at room temperature for 1 h in a covered container. Blots were incubated overnight at 4 °C with rabbit polyclonal anti-CypA antibody (1:400 dilution), rabbit polyclonal anti-NF-κB p65 antibody (1:500 dilution) or a mouse monoclonal anti-β-actin (1:1000 dilution) diluted in 5% BSA. Membranes were washed with TBST and incubated with a secondary goat anti-mouse IgG horseradish peroxidase (HRP) antibody (1:2000 dilution) or goat anti-rabbit IgG-HRP antibody (1:2000 dilution) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) diluted in 5% (w/v) dry nonfat milk in TBST for 1 h at room temperature. Finally, membranes were washed with TBST, developed using the ECL detection system (Santa Cruz Biotechnology), quickly dried, and exposed to ECL film.

2.6. Quantification of cell viability

Apoptosis or necrosis in pancreatic acinar cells was determined using Hoechst 33342 or propidium iodide (PI) staining as described previously [15]. Cells were stained with 8 mg/ml Hoechst 33342 or 1 mg/ml PI and were examined directly by fluorescence microscopy. Cells with nuclei containing condensed and/or fragmented chromatin were considered to be apoptotic, while cells with swollen cytoplasm, loss of plasma membrane integrity, and stained by PI were considered to be necrotic. Cell necrosis was determined by measuring ATP levels using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) as previously described [16].

2.7. Real-time reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from each tissue or from isolated acinar cells using a modified acid guanidinium/phenol/chloroform method, as reported previously [17]. RNA was reverse-transcribed using the SuperScript II preamplification kit (Fermentas, Hanover, MD, USA) and subjected to real-time PCR using gene-specific, intron-spanning primers that were designed with software (See Table 1). Quantitative real-time RT-PCR (qRT-PCR) was performed in triplicate for each gene of interest under each triplicate experimental condition using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as a separate endogenous control to which each gene of interest was normalized. Fold changes and subsequent percent gene expression levels relative to designated control groups were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method.

2.8. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, USA). The single-stranded 3′-end biotin-labeled probe containing the NF-κB consensus site 5′-AGT TGA GGG GAC TTT CCC AGC C-3′ (binding motif underlined) was purchased from Metabion (Beyotime,

Table 1
Primer sequences used for qRT-PCR analysis.

Gene		Primer sequence (5′ → 3′)
Rat CypA	Forward	AGACAAAGTTCCAAAGACAG
	Reverse	GAGAGCAGAGATTACAGGG
Rat IL-1β	Forward	CTTCAAATCTCACAGCAGCATC
	Reverse	GCTGTCTAATGGGAACATCACA
Rat IL-6	Forward	TCCGTTTCTACCTGGAGTTTGT
	Reverse	GTTGGATGGTCTTGGTCCTTAG
Rat TNF-α	Forward	CATGGATCTCAAAGACAACCAA
	Reverse	CTCCTGGTATGAAATGGCAAAT
Rat CXCL-1	Forward	AAACCGAAGTCATAGCCACACTC
	Reverse	ACACCCTTTAGCATCTTTTGGAC
Rat β-actin	Forward	CACCCGCGAGTACAACCTTC
	Reverse	CCCATACCCACCATCACACC

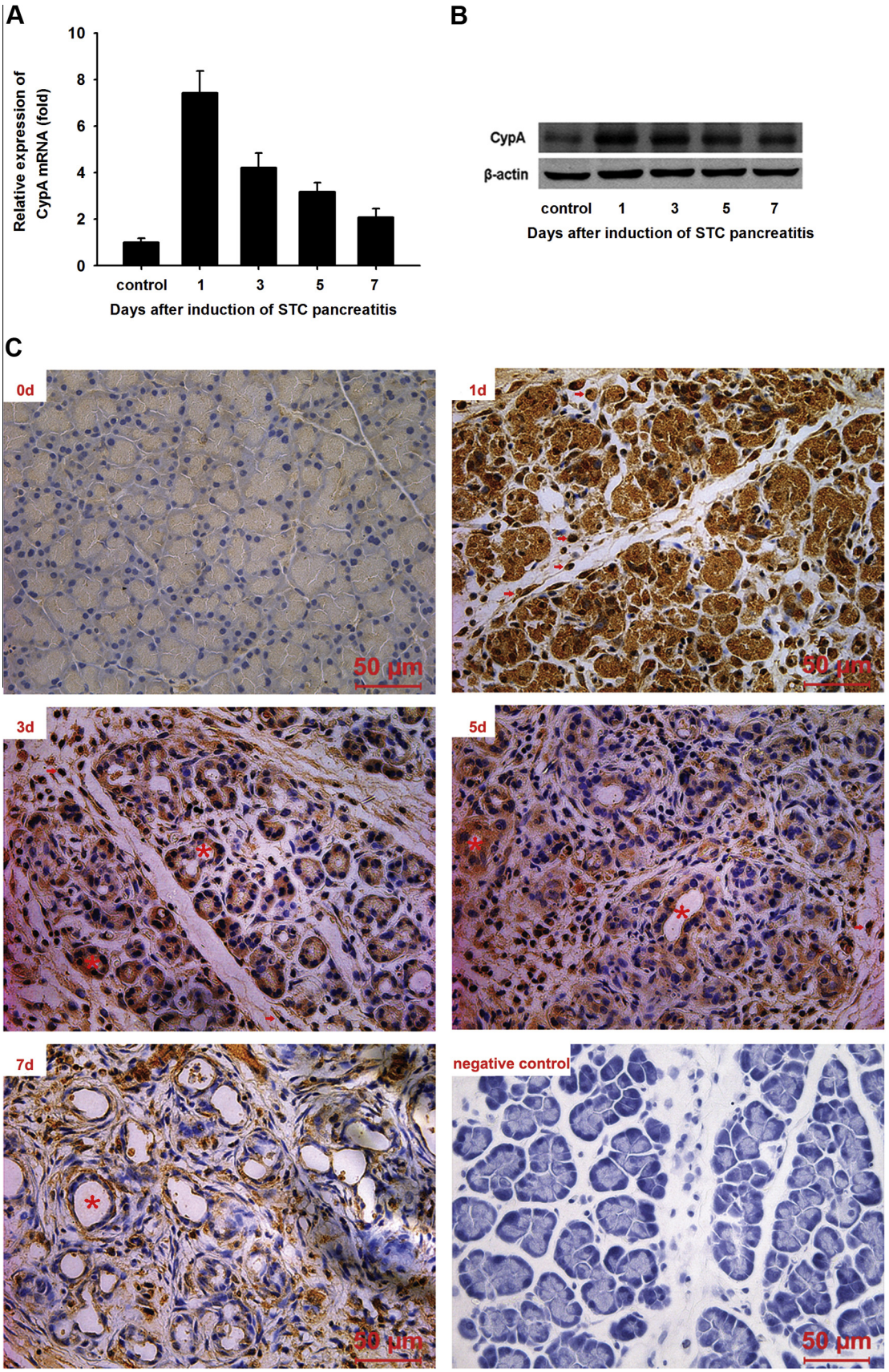

Fig. 1. CypA is upregulated in STC-induced pancreatitis. (A) Real-time qRT-PCR confirmed the increased mRNA levels of CypA in pancreatic tissues. (B) The level of CypA protein was measured by Western blotting. (C) Immunohistochemical staining of CypA in rat pancreas during STC pancreatitis. In control pancreas, CypA was expressed evenly at a low level. On day 1, intense immunoreactivity for CypA was observed in both disrupted acinar cells and infiltrated inflammatory cells (*arrows*). On days 3 and 5, CypA was mainly detected in tubular complexes (*asterisk*) and infiltrated inflammatory cells (*arrows*), but became weaker on day 7. For negative controls, the antibody against CypA was substituted with isotype-matched primary antibody.

China). The biotinylated oligonucleotides were annealed by denaturing at 90 °C for 1 min and cooled to room temperature for 1 h. The EMSA binding reactions were performed by utilizing a LightShift chemiluminescent EMSA kit (Pierce, Rockford, USA). Specifically, 5 μg nuclear extract was incubated in 1 × binding buffer containing 2.5% glycerol, 0.05% NP-40, 50 mM KCl, 5 mM $MgCl_2$, 50 ng poly (dI–dC) and biotinylated probe with or without protein extract for 30 min at room temperature. The complexes were separated on a 5% polyacrylamide-0.5 × Tris-borate-EDTA gel and transferred to a positively charged nylon membrane. After the transfer was completed, the membrane was crosslinked and biotin-labeled DNA was detected by using a chemiluminescent detection kit (Pierce, Rockford, USA).

2.9. Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls test as a post hoc test. The Kruskal–Wallis test was used to evaluate the differences in categorical values followed by Mann–Whitney U test as a post hoc test. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. CypA is upregulated in experimental pancreatitis

In STC pancreatitis, RT-PCR and Western blotting showed that CypA mRNA and protein expression were markedly increased in STC pancreatitis (Fig. 1A and B). Maximal expression was observed on day 1 after induction of pancreatitis, which decreased progressively on days 3 and 5, and was still higher than normal by day 7. We confirmed the increased expression and localization of CypA using immunohistochemistry. As shown in Fig. 1C, CypA was expressed evenly in normal acinar cells. On post-pancreatitis day 1, intense immunoreactivity for CypA was observed in both disrupted acinar cells and infiltrated inflammatory cells, consisting of macrophages, neutrophils, lymphocytes and fibroblast-like cells, in areas of prominent pancreatic injury. On days 3 and 5, CypA was mainly detected in tubular complexes and infiltrated inflammatory cells. Immunoreactivity for CypA in tubular complexes was significantly weaker on day 7. Specificity of the immunoreactivity for CypA on day 1 was confirmed by incubation of the tissue with PBS, which yielded no significant staining for CypA.

3.2. CCK induces expression and release of CypA in acinar cell in vitro

ATP depletion is associated with acinar cell necrosis [18], therefore, the viability of isolated acinar cells incubated with CCK (200 nM) for 0, 6, 12 and 24 h was analyzed using the CellTiter-Glo Luminescent Cell Viability Assay. As expected, CCK induced acinar cell death in a time-dependent manner (Fig. 2A). In CCK pancreatitis *in vitro*, intracellular CypA protein expression was persistently increased, with maximal expression observed after 6 h (Fig. 2B). To determine whether CypA was released from acinar cells, cell culture supernatants were centrifuged after incubation with 200 nM CCK for 24 h. Western blotting demonstrated that CypA protein was released from the cells and was detected in the supernatant (Fig. 2C).

3.3. rCypA aggravates CCK-induced acinar cell death and inflammatory cytokine production

To elucidate further the role of CypA in the regulation of cell death responses, we examined the effects of rCypA on CCK-induced

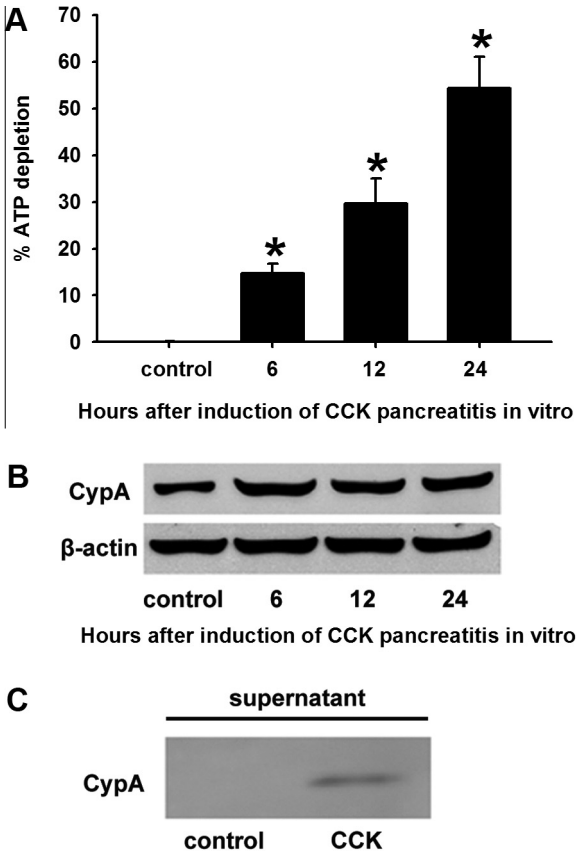

Fig. 2. CCK induces expression and release of CypA in acinar cells *in vitro*. Rat pancreatic acinar cells were cultured for 24 h in DMEM containing 10% FBS, and then incubated for 6, 12 and 24 h in DMEM without and with 200 nM CCK. (A) Acinar cell necrosis was determined by measuring ATP levels using luciferin/luciferase-based ATP determination. CCK induced time-dependent depletion of ATP in acinar cells *in vitro*. Data are presented as mean ± SD from three independent experiments. (B) Western blot analysis showed an increase of CypA at the protein level in acinar cells in CCK pancreatitis *in vitro*. (C) After treatment with 200 nM CCK for 24 h, CypA protein in culture supernatant was detected by Western blotting.

acinar cell death *in vitro*. Addition of rCypA (0, 50, 100, 200 and 500 ng/ml) to the culture medium induced dose-dependent depletion of cellular ATP in rat pancreatic acinar cells with CCK stimulation (Fig. 3A). By quantifying acinar cell necrosis and apoptosis using Hoechst33342/PI double staining, we also confirmed that combination treatment of CCK and rCyPA (200 ng/ml) for 12 h induced more serious acinar cell death than CCK alone (Fig. 3C). Acinar cells seemed more likely to undergo necrosis rather than apoptosis. Definitive treatment of rCyPA without CCK did not cause ATP depletion or changes in Hoechst33342/PI staining (data not shown). The data suggest that CypA cannot directly cause acinar cells necrosis.

As a proinflammatory cytokine, rCypA stimulates expression of IL-6 in human THP-1 monocytes [19]. In addition, acinar cells can synthesize and secrete inflammatory mediators such as cytokines and chemokines in response to injury or stress. Therefore, we investigated mRNA expression of various inflammatory mediators, including IL-1β, IL-6, TNF-α and chemokine CXC ligand (CXCL)-1. Our results showed that IL-1β, IL-6, TNF-α and CXCL-1 mRNA expression was significantly upregulated in acinar cells in the rCypA and rCypA + CCK treatment groups (Fig. 3B). These results showed that CypA may directly trigger an inflammatory response as a proinflammatory cytokine, which is different from that of cell death.

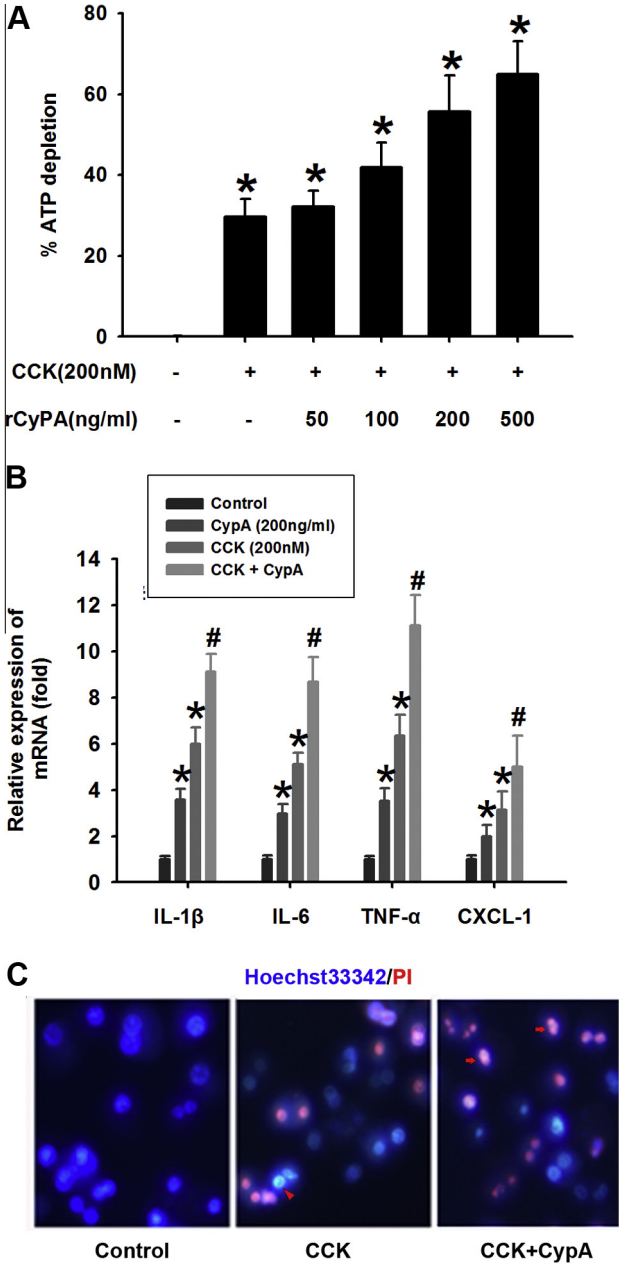

Fig. 3. rCypA aggravates CCK-induced acinar cell necrosis and induces inflammatory cytokine production *in vitro*. Rat acinar cells were incubated with 200 nM CCK and increasing doses of rCypA (0–500 ng/ml) for 12 h, and necrosis was determined by measuring ATP levels (A). Acinar cells were incubated with 200 nM CCK and 200 ng/ml rCypA for 12 h, and mRNA levels of IL-1β, IL-6, TNF-α and CXCL-1 were measured by real-time qRT-PCR (B). Acinar cells were stained with Hoechst 33342 and PI, and examined by fluorescent microscopy (C). Arrowheads indicate apoptotic cells and arrows indicate necrotic cells (400×). Data are presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared with PBS-treated group. $^{\#}P < 0.05$ compared with CCK-treated group.

3.4. rCypA activates the NF-κB pathway in acinar cells in vitro

NF-κB activation plays a key role in the induction of several proinflammatory mediators [4]. Nuclear translocation of the NF-κB transcription factor is preceded by the degradation of IκB. To determine the effect of rCypA on NF-κB activity in acinar cells, we examined the expression level of NF-κB p65, IκB-α by Western blot analysis. As expected, administration of rCypA increased the nuclear level of NF-κB p65, and reduced IκB-α level in cytoplasm during CCK-induced cell injury *in vitro* (Fig. 4A). Furthermore,

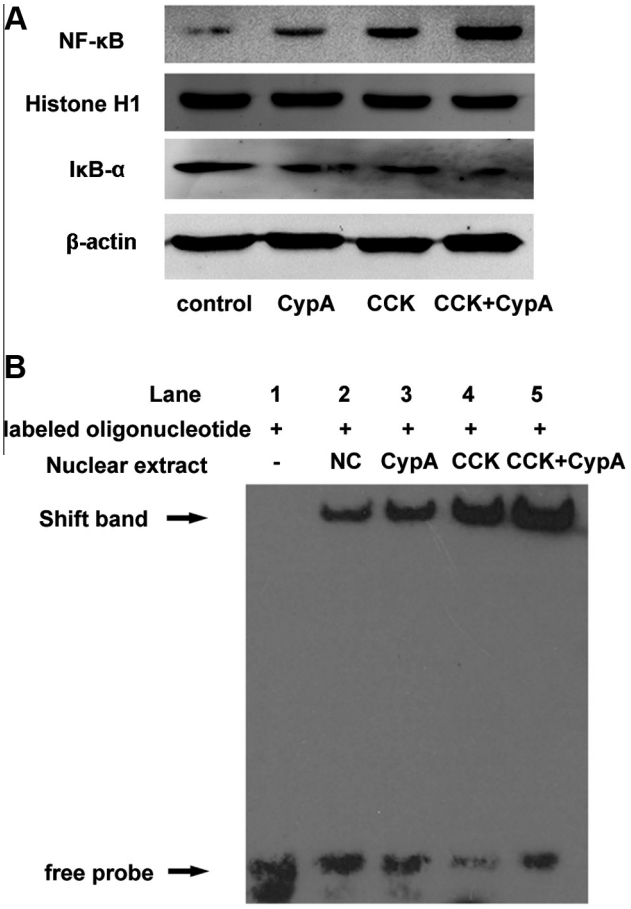

Fig. 4. rCypA activated NF-κB pathway in acinar cells *in vitro*. Mouse pancreatic acinar cells were incubated with CCK (200 nM) and rCypA (200 ng/ml) for 3 h. (A) The expression level of NF-κB p65 in nucleus, IκB-α in cytoplasm of pancreatic acinar cells were detected by Western blotting. Histone H1 and β-actin were used as the internal reference for nuclear proteins and cytoplasmic proteins respectively. (B) The NF-κB p65 nuclear translocation was determined by EMSA.

EMSA showed that rCypA activated NF-κB and increased NF-κB activation induced by CCK (Fig. 4B).

4. Discussion

CypA is a highly inflammatory cytokine that is involved in leukocyte chemotaxis *in vitro* and has been implicated as a driving force in many inflammatory diseases [12]. A growing body of evidence demonstrates that CypA is involved in the drug-induced injury, ischemia/reperfusion injury, and immunological damage in the liver, heart, lung, joints and brain [8,20–24]. However, its role in AP is not well understood.

In the present study, we showed for the first time that both CypA mRNA and protein expression were markedly upregulated in the pancreas in STC pancreatitis. It should be noted that expression of CypA was prominent in injured acinar cells in experimental pancreatitis. Besides, we also detected the expression of CypA protein in acinar cells in CCK pancreatitis *in vitro*. Unlike *in vivo* pancreatitis, the maximal expression was observed at 6 h, and was not in direct proportion to the severity of acinar cell death. This may have been due to stress-induced high intracellular expression of CypA, which protects cells against oxidative stress and ischemic injury [25,26]. It still needs further investigation. In the *in vitro* model, we found that CypA protein was released from the cells and was present in the culture supernatant. This confirms that the release of CypA may be used as a biomarker for necroptosis

and other cell death processes when the integrity of the plasma membrane is compromised [27]. As mentioned above, we speculate that increased expression of CypA was involved in pancreatic damage, which may play an important role in the systemic inflammatory response and remote organ injury during experimental pancreatitis.

It was previously reported that the high expression of CypA in macrophages, lymphocytes or other infiltrating inflammatory cells positively correlated with the severity of the inflammatory diseases [28,29]. In STC pancreatitis, CypA protein immunostaining was found in disrupted acinar cells and the infiltrated inflammatory cells, consisting of macrophages, neutrophils, lymphocytes and fibroblast-like cells. Furthermore, intense immunoreactivity of CypA in tubular complexes was observed on days 3 and 5, while it was still detectable but became weaker on day 7, indicating that CypA plays a role in chronic inflammation of the pancreas. Further research is needed to elucidate the role of CypA in chronic pancreatitis.

AP is characterized by early activation of intracellular proteases followed by acinar cell death and inflammation. The immune system is alerted to cell death when intracellular molecules are released into the extracellular space as a result of damage to the cell membrane. These molecules are referred to as damage-associated molecular patterns (DAMPs) [8,28]. We demonstrated that CypA was released from damaged acinar cells *in vivo* and *in vitro*. Pancreatic acinar cells have the ability to produce inflammatory mediators including IL-1β, IL-6 and TNF-α [2]. Afterwards, we focused on the role of CypA in mediating pancreatic acinar cell damage after induction of AP. rCypA can directly trigger an inflammatory response as an alarmin that induces acinar cells to secrete IL-1β, IL-6,TNF-α and CXCL-1. It also acts synergistically with CCK to enhance inflammatory cytokine release. Despite the fact that CypA cannot directly induce acinar cells death, it can aggravate CCK-induced acinar cell death. Furthermore, necrosis was the major form of pancreatic acinar cell death caused by rCypA. Our results suggest that CypA may enhance susceptibility to AP. Activation of NF-κB in acinar cells is known to play an important role in the pathogenesis of caerulein-induced pancreatitis [4]. NF-κB is a pleiotropic regulator of many genes in stress and inflammatory reactions. In most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm, but it can be activated by phosphorylation-induced degradation of the IκB inhibitor (e.g., IκB-α, IκB-β, IκB-γ, IκB-ε and Drosophila Cactus), which enables the NF-κB dimers to enter the nucleus and activate specific target gene expression [29]. As mentioned above, we found that rCypA directly promoted NF-κB p65 nuclear translocation by decreasing the level of the inhibitory subunit IκB-α in isolated acinar cells. These data enhance the current understanding of proinflammatory mechanisms of CypA and provide a plausible explanation for the damage effect of CypA in AP. Further research should be done to investigate the upstream pathway leading to NF-κB activation by CypA.

In summary, our results showed that CypA contributes to pancreatic damage by increasing the expression of proinflammatory cytokines via activating the NF-κB pathway and aggravating acinar cell necrosis during AP. Thus, interventions that inhibit CypA activity may be effective in settings of AP to minimize pancreatic damage and may be useful in other clinical settings that are associated with inflammation and cellular necrosis.

Acknowledgments

This work was supported in part by National Natural Science Foundation of China (Nos. 81100317, 81370568 and 81270543), Foundation for Shanghai Science and Technology Committee (Nos. 12QA1402600, 114119a6800, 12411950600 and 11411950601) and outstanding Young Scholars Program of Shanghai Health System (No. XYQ2011004). No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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