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• *BASIC RESEARCH* •

A nuclear import inhibitory peptide ameliorates the severity of cholecystokinin-induced acute pancreatitis

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Abstract

AIM: To assess the effect of our novel cell-permeable nuclear factor-kappaB (NF-κB) inhibitor peptide PN50 in an experimental model of acute pancreatitis. PN50 was produced by conjugating the cell-penetrating penetratin peptide with the nuclear localization signal of the NF-κB p50 subunit.

METHODS: Pancreatitis was induced in male Wistar rats by administering 2×100 μg/kg body weight of cholecystokinin-octapeptide (CCK) intraperitoneally (IP) at an interval of 1 h. PN50-treated animals received 1 mg/kg of PN50 IP 30 min before or after the CCK injections. The animals were sacrificed 4 h after the first injection of CCK.

RESULTS: All the examined laboratory (the pancreatic weight/body weight ratio, serum amylase activity, pancreatic levels of TNF-α and IL-6, degree of lipid peroxidation, reduced glutathione levels, NF-κB binding activity, pancreatic and lung myeloperoxidase activity) and morphological parameters of the disease were improved before and after treatment with the PN50 peptide. According to the histological findings, PN50 protected the animals against acute pancreatitis by favoring the induction of apoptotic, as opposed to necrotic acinar cell death associated with severe acute pancreatitis.

CONCLUSION: Our study implies that reversible inhibitors of stress-responsive transcription factors like NF-κB might be clinically useful for the suppression of the severity of acute pancreatitis.

Key words: Acute pancreatitis; Peptide delivery; Penetratin; NF-κB inhibition

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INTRODUCTION

Acute pancreatitis is a disease with high mortality. In the earliest stages, the inflammation is limited to the pancreas. Due to systemic action of diverse inflammatory mediators (such as cytokines, reactive oxygen species (ROS), proteolytic enzymes, lipids, *etc.*), this locally limited inflammation quickly overspreads and develops into systemic inflammatory response syndrome (SIRS) and eventually into multiple organ failure (MOF), the latter is responsible for most pancreatitis-associated mortality and morbidity[1,2]. Nuclear factor-kappaB (NF-κB) plays a pivotal role in the onset of acute pancreatitis not only in pancreatic acinar cells[3-7] and leukocytes[8-12] but also in specific distant organs, such as the lung[13-16]. After activation, NF-κB translocates into the nuclei and induces the expression of mediators of localized and systemic inflammatory responses[17-19]. It is well established that transcription activity of NF-κB correlates with the severity of acute pancreatitis[4,8,19-21]. Various studies have demonstrated the therapeutic benefits of selective NF-κB inhibition in acute pancreatitis[22-24].

Inhibition of NF-κB has been a controversial issue in the treatment of experimental acute pancreatitis[25]. This controversy stems from the rather elusive and unspecified effects of PDTC used by Steinle *et al*[26]. However, this controversy also attracts attention to the well-known fact that genes encoding proinflammatory mediators are also activated by other stress-responsive transcription factors (SRTFs) besides NF-κB[27-30]. Recent evidence strongly supports the crucial role of other SRTFs in the initiation and propagation of acute pancreatitis, e.g., activator protein-1 (AP-1)[31-35] and signal transducers and activators of transcription (STATs)[36]. The observation that T lymphocytes

have a central role in the tissue injury during acute pancreatitis[37,38] implies the importance of a T cell-related SRTF in the pathogenesis of acute pancreatitis, namely the nuclear factor of activated T cells (NFAT). So development of acute pancreatitis involves a multiplex signaling process that is triggered by mobilization of NF-κB and other SRTFs to their nuclear sites of action and by systemic expression of proinflammatory cytokine mediators. Thus, inhibition of a broader range of SRTFs would imply a more favorable clinical outcome in the treatment of acute pancreatitis. Torgerson *et al*[39] have demonstrated that noninvasive intracellular delivery of peptides bearing the NF-κB p50 nuclear localization signal (NLS) inhibits the signal-dependent nuclear import of NF-κB and other SRTFs like AP-1, STAT1 and NFAT. In these experiments, the hydrophobic domain of the Kaposi fibroblast growth factor signal sequence is used to transport the NF-κB p50 NLS into the cells. This cell-permeable p50 NLS construct named SN50 suppresses systemic inflammatory responses *in vivo*[40,41] too. The broad inhibitory range of the SN50 peptide offers an advantage, because genes of cytokines and other proinflammatory mediators are regulated by multiple SRTFs. In order to reduce the production of cytokines and prevent LPS-induced lethal shock in mice, the SN50 peptide has to be applied in quite high doses (1.5 mg given in 7 injections for an animal weighing only 20 g). The necessity to use such a high dosage can be contributed partly to the insufficient intracellular delivery of the otherwise very potent p50 NLS cargo.

To enhance the efficacy of SN50, we replaced the cell-permeable motif with the more efficient cell-transporter peptide penetratin (Table 1). Penetratin has already proven its superior abilities to transport bioactive molecules intracellularly both *in vitro* and *in vivo*[42-45]. We coupled penetratin to the NF-κB p50 NLS through a disulfide bridge, thus enabling easy cleavage of the cargo NLS from its transporter penetratin in the reductive intracellular milieu. We named this peptide PN50, where P indicates the vector penetratin and the rest of the term refers to its parent SN50 peptide. We reasoned that our new peptide PN50 with its enhanced cell permeability and broad inhibitory range exerts both prophylactic and therapeutic effects on acute pancreatitis.

Table 1 Sequence of the cell-permeable PN50 peptide

NF-κB p50 NLS-Cell-Permeable Motif	
VQRKRQKLMPC-CRQIKIWFQNRRMKWKK	

Sequences are given in single letter amino acid code. Underlined residues constitute the cell-permeable peptide penetratin.

Before applying PN50 in an animal model of acute pancreatitis, we analyzed the NF-κB inhibitory activity of the substance with *in vitro* luciferase reporter gene assays. Then, we assessed whether PN50 could enter the pancreas and lung, the organs most affected by acute pancreatitis. In the final and most important stage we investigated the effect of PN50 on CCK-induced acute pancreatitis. The results of the above-mentioned *in vitro* and *in vivo* experiments are reported here.

MATERIALS AND METHODS

Materials and animals

Cholecystokinin-octapeptide (CCK) was prepared in our laboratory at the Department of Medical Chemistry, Szeged, Hungary with the method of Penke *et al*[46]. The NF-κB p50 NLS peptide (VQRKRQKLMPC) was synthesized in our laboratory on solid phase standard methodology. This peptide was coupled with a disulfide bridge to the cell transporter peptide Cys-penetratin (CRQIKIWFQNRRMKWKK) synthesized also on solid phase in our laboratory. For the *in vivo* uptake experiments, the cell permeable conjugate, PN50 was labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich, Munich, Germany) with the method of Fülöp *et al*[47]. A 21-basepair oligonucleotide 5'-GGCAGAGGGG-ACTTTCCGAGA-3'containing the NF-κB consensus sequence (underlined), its mutated form (in which the NF-κB motif was changed to GccACTaaC) and their complementary pairs were synthesized in our laboratory with a DNA-synthesizer. For the *in vivo* studies male Wistar rats (provided by the Animal Center of the University of Szeged) weighing 250-300 g were used. The animals were kept at a constant room temperature with a 12-h light-dark cycle and allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). All animal experiments performed in this study were approved by the Animal Care Committee of the University and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Transformant cell lines

Mouse L929 cells (5×10^5/60 mm plate) were transformed with pNF-κB-luc4 and pSV-2/neo plasmids (coding for firefly luciferase under the regulation of 5 NF-κB-responsive elements and the neo^r gene controlled by the SV40 enhancer/promoter respectively) using the DMIRIE-C cationic lipid transfection agent (GIBCO BRL) one day after trypsinization. Selection started 48 h later, the cells were exposed to Geneticin G418 400 mg/L for two weeks and the medium was refreshed twice weekly. Clones were isolated and tested for the intensity of their TNF-α-elicited NF-κB induction (50-100 U/mL recombinant TNF-α, 6-10 h of induction).

RAW 264.7 cells (5×10^5/60 mm plate) subcultured the previous day were transformed overnight with the above plasmids complexed with polyethylene-imine (jetPEI, Qbiogen, Illkirch, France). Geneticin-containing medium (400 mg/L) was replaced daily from d 2; clones were isolated after 12 d. Clones showing the highest response to LPS activation (0.1 to 10 μg/mL, 6-10 h of incubation) were used for the assays.

Media: Both cell types were grown in MIX MEM (1:1 mixture of DMEM, Sigma and F-12 HAM, Sigma) plus 10% FCS (Sigma). Transformation was carried out in OPTI MEM (GIBCO BRL). Geneticin was purchased from Sigma.

Luciferase assay

One-day-old cultures (both of L929 and RAW cells)

grown on luminoplates (Corning Costar) were used and 3×10^4 cells/well (in MIX MEM 10% FCS) were exposed to various concentrations of PN50 peptide (L929 cells: 0.39 to 50 μmol/L; RAW cells: 0.39 to 25 μmol/L). After 30 min, the cells were treated with TNF-α (10 U/mL) or LPS (30 ng/mL) respectively (in 100 μL of the above medium per well). After 6 h of incubation with TNF-α or LPS, the medium was removed and the cells were washed and lysed for 10 min at room temperature in buffer (20 μL/well, Promega). Substrate was added (20 μL/well, Promega) and luciferase activity was measured in a Luminoskan Ascent (Thermo Labsystems) scanning luminometer. Each of the experiments were repeated three times.

In vivo uptake experiments

Six male Wistar rats weighing 250-300 g were injected intraperitoneally (IP) with 15 nmolar of PN50-FITC peptide in 500 mL of phosphate-buffered saline (PBS). Treated rats were killed 15 min after the injections by exsanguinations *via* the abdominal aorta, pancreatic and lung tissues were harvested and frozen in Histo Prep media (Fisher Scientific). Sections (10 to 50 μm) were cut on a cryostat and analyzed by fluorescence confocal microscopy.

CCK-induced pancreatitis

Acute pancreatitis was induced by injecting 100 μg/kg body weight of CCK IP twice at an interval of 1 h (Figure 1). The rats were fasted for 16 h before the induction of acute pancreatitis. In each experimental group 10 rats were used. The PN50 pre-treated group ("group PN50+CCK") received 1 mg/kg body weight of PN50 IP 30 min before the first injection of CCK. In the post-treatment group ("group CCK+PN50") the animals were injected with 1 mg/kg body weight of PN50 IP 30 min after the second CCK injection. Rats in "group CCK" were injected with 0.5 mL of physiological saline (PS) IP 30 min before the induction of pancreatitis. Animals were killed by exsanguinations *via* the abdominal aorta 4 h after the first CCK injection. The control rats ("group PS") received PS IP instead of CCK and PN50. The pancreas was quickly removed, cleaned of fat and lymph nodes, weighed, frozen in liquid nitrogen and stored at -80 ℃ until use.

Nuclear protein extract

Nuclear protein extracts were prepared essentially as described by Dignam *et al*[48]. A 250-300 mg pancreatic tissue sample was lysed on ice in hypotonic buffer A by 20 strokes in a glass Dounce homogenizer. The hypotonic buffer was supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L benzamidine, 100 IU/mL aprotinin, and 1 mmol/L dithiothreitol. The homogenate was left on ice for 20 min, and Nonidet P-40 was then added to a final concentration of 30-40 mL/L. The samples were briefly vortexed and incubated on ice for an additional 2 min. The nuclear pellet was collected by centrifugation of the lysed tissue for 20 s at 13 000 *g* in a Microfuge. The supernatant (cytosolic fraction) was saved for ELISA. The nuclear pellet was resuspended in buffer C supplemented with 1 mmol/L dithiothreitol, 1.5 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L benzamidine, and 100 IU/mL aprotinin. After rotation at 4 ℃ for 45 min, the nuclear membranes were pelleted by microcentrifugation for 10 min and the supernatant (nuclear extract) was aliquoted and stored at -80 ℃. The protein concentration of the nuclear extract was determined by the method of Goa[49].

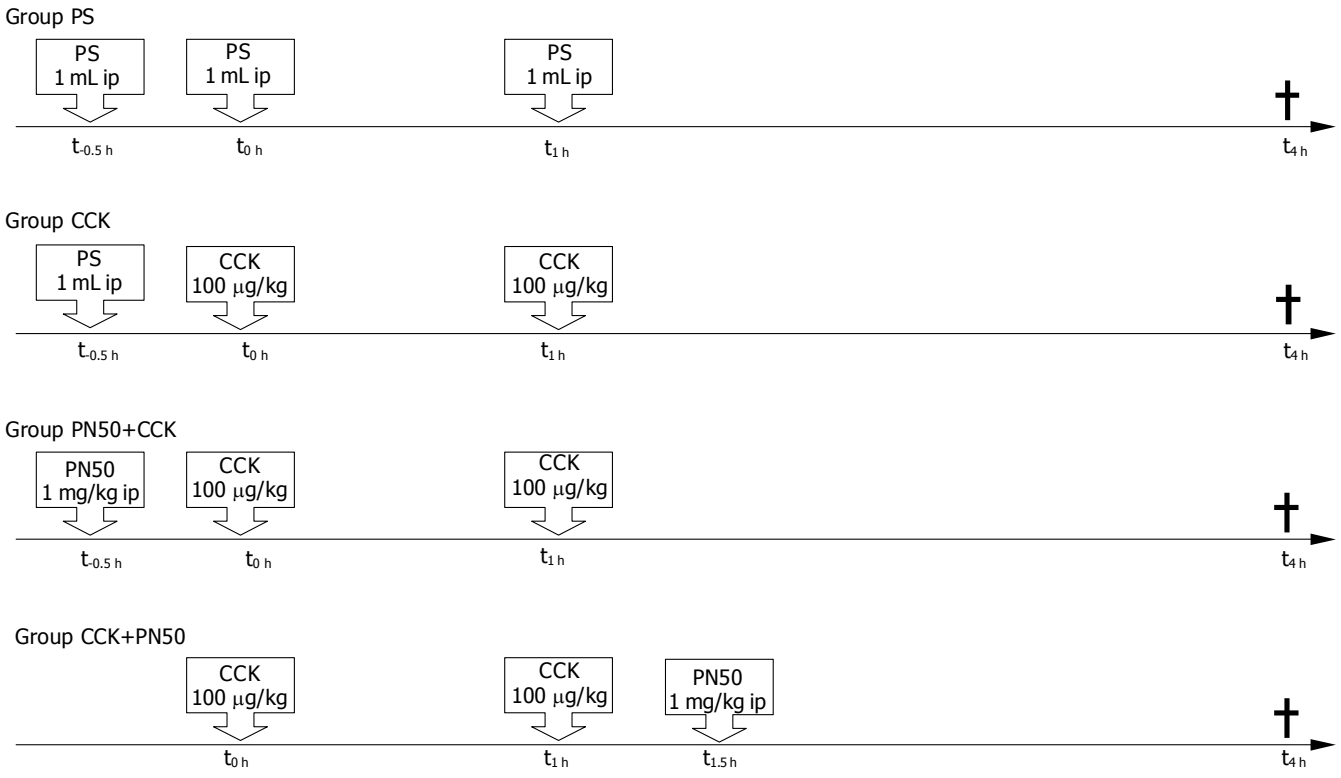

Figure 1 Experimental protocol of CCK-induced acute pancreatitis.

Electrophoretic mobility shift assay (EMSA) of NF-κB

A 21-basepair oligonucleotide 5'-GGCAGAGGGGACTTTCCGAGA-3' containing the NF-κB consensus sequence (underlined) was annealed with its complementary oligonucleotide to generate a double-stranded probe and end-labeled with [γ-^{32}P] by T4 polynucleotide kinase (Fermentas, Lithuania). Labeled oligonucleotides were separated from the unincorporated isotope by PAGE and isolated from a 16% polyacrylamide gel. To determine the NF-κB binding activity, aliquots of nuclear protein (12 μg) were mixed with a buffer containing 10 mmol/L HEPES (pH = 7.9), 50 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 100 mL/L glycerol, and 4.5 μg poly(dI/dC). The binding reaction was started by adding 3 000-5 000 cpm of the radiolabeled double-stranded probe and allowed to proceed for 30-40 min on ice. For cold competition, unlabeled double-stranded wild-type or mutated oligonucleotides were added to the reaction mixture in 20× or 100× molar excess together with the labeled probe. In the mutated oligonucleotide, the NF-κB motif was changed to GccACTaaC. DNA-protein complexes were resolved by PAGE at 4 ℃ on a non-denaturing 4.5% gel in a buffer containing 6.7 mmol/L Tris base, 3.3 mmol/L sodium acetate, and 1 mmol/L EDTA (pH = 7.5). Gels were vacuum-dried and exposed to Fuji RX films (Fuji Tokyo, Japan) with intensifying screens at -80 ℃. The intensities of the bands were quantified by using the ImageJ 1.32j image processing and analysis software (National Institute of Health, USA).

Pancreatic weight/body weight ratio (pw/bw)

This ratio was utilized to evaluate the degree of pancreatic edema.

Serum amylase activity

All blood samples were centrifuged at 2 500 *g* for 20 min. The serum levels of amylase were determined by a Colorimetric-Kinetic method (Dialab, Vienna, Austria).

Pancreatic tumor necrosis factor-α and interleukin-6 levels

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) concentrations were measured in the pancreatic cytosolic fractions with ELISA kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Pancreatic and lung myeloperoxidase activity

Pancreatic and lung myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was assessed by the method of Kuebler *et al*[50].

Pancreatic lipid peroxide and reduced glutathione levels

The malondialdehyde (MDA) level was measured after the reaction with thiobarbituric acid, according to the method of Placer *et al*[51], and was also corrected for the protein content of the tissue. Reduced glutathione (GSH) level was determined spectrophotometrically with Ellman's reagent[52].

Histological examination

A portion of the pancreas was fixed in an 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin (HE). The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol. They used the scoring system of Hughes *et al*[53] for the evaluation of acute pancreatitis. Thus semi-quantitative grading of interstitial edema (0-1), vascular changes (0-2), inflammation (0-1), acinar necrosis (0-2), calcification (0-0.5) and fat necrosis (0-0.5) of the pancreas samples was evaluated in each animal (described in more details in Table 2).

Statistical analysis

Results were expressed as mean±SD. Differences between experimental groups were evaluated by using analysis of variance (ANOVA). Values of $P<0.05$ were accepted as significant.

RESULTS

PN50 suppressed NF-κB transcription activity in vitro

Luciferase reporter gene assay was performed to analyze the endogenous NF-κB transcription activity with transfection of reporter plasmids (pNF-κB-Luc) in L929 and RAW 264.7 cells. First, we measured the transcriptional activity of NF-κB by stimulating L929 cells with 10 U/mL of the inflammatory cytokine TNF-α. NF-κB–driven luciferase activity of TNF-α-stimulated L929 cells markedly increased and reached its maximum at 6 h. PN50 pre-treatment (30 min prior to TNF-α addition) dose-dependently decreased TNF-α-induced luciferase activity at concentrations of 0.39 to 50 μmol/L (Figure 2A).

Table 2 Histological scoring system for the evaluation of CCK-induced acute pancreatitis

	0	0.5	1	1.5	2
Edema	Absent	Focal<50%	Diffuse>50%		
Vascular Change	Absent	Congestion	Focal hemorrhage	Diffuse hemorrhage	Vascular necrosis or thrombosis
Inflammation	Absent	Focal/mild	Diffuse>50%		
Acinar necrosis	Absent	Single acinar cell necrosis/foci of peripheral lobular damage	Lobular necrosis in 10% to 30% of the surface area	Lobular necrosis in 30% to 50% of the surface area	Lobular necrosis in >50% of the surface area/microabscesses
Calcification	Absent	Present			
Fat necrosis	Absent	Present			

The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol. Semi-quantitative grading of interstitial edema, inflammation, hyperemia, and necrosis of acinar cells were evaluated in each animal, with the scoring system of Hughes *et al*[53].

Next we assessed the effect of PN50 peptide on transcriptional activity of NF-κB in cultured murine macrophages. Macrophages are well-known targets for the proinflammatory agonist LPS that can induce synthesis of proinflammatory mediators in these cells. When tested in murine RAW 264.7 macrophages, PN50 suppressed LPS-induced luciferase activity by 72.7% at a concentration of 25 μmol/L (Figure 2B).

PN50 entered the pancreas and lung in vivo

Next we studied the uptake of the PN50 peptide into the pancreas and lung. Fifteen min after the IP injection of the PN50-FITC peptide, tissues were dissected from rats and cryostat sections were prepared. Fluorescence confocal microscopy analysis of the pancreas and lung sections revealed a strong signal from PN50-FITC peptide injected rats (Figure 3). Fluorescence photobleaching was observed (performed?) in sections subjected to prolonged excitation, providing further evidence that the PN50-FITC peptide was present in the pancreas and lung sections.

PN50 decreased the parameters of acute pancreatitis in vivo

After demonstrating the efficient delivery of the NF-κB p50 NLS into the pancreas and lung *in vivo*, we tested PN50 in an animal model of acute pancreatitis. The following results showed the effects of prophylactic and therapeutic administrations of PN50 on the examined parameters of the disease.

Pw/bw ratio and serum amylase activity

Administration of 2×100 μg/kg body weight CCK increased the pw/bw ratio and the serum amylase activity compared to controls. The pw/bw ratio and serum amylase levels significantly decreased both before and after treatment with PN50, reflecting less edema and milder cellular damage within the pancreas (Figure 4).

Pancreatic myeloperoxidase (MPO) activity

Pancreatic MPO activity was significantly higher in group CCK than in group PS (control group). In group PN50+CCK and group CCK+PN50 the PN50 injections significantly reduced pancreatic MPO activity compared to group CCK (Figure 5A).

Lung myeloperoxidase (MPO) activity

The lung MPO activity was significantly elevated in CCK-induced pancreatitis. The administration of PN50 decreased this parameter 4 h after the last CCK injection. However, compared to group CCK, the difference was significant only in the PN50 pre-treated (prophylactic) group (Figure 5B).

Intrapancreatic TNF-α and IL-6

Injection of CCK increased pancreatic TNF-α and IL-6 concentrations after 4 h. This increase was significantly ameliorated in rats treated with PN50 (Figures 5C and D).

Pancreatic lipid peroxidation

In pancreatic tissue, level of malondialdehyde (MDA) was significantly elevated in group CCK compared to group PS. In both of the PN50-treated groups (group PN50+CCK and group CCK+PN50) pancreatic MDA levels were significantly lower than in group CCK (Figure 6A).

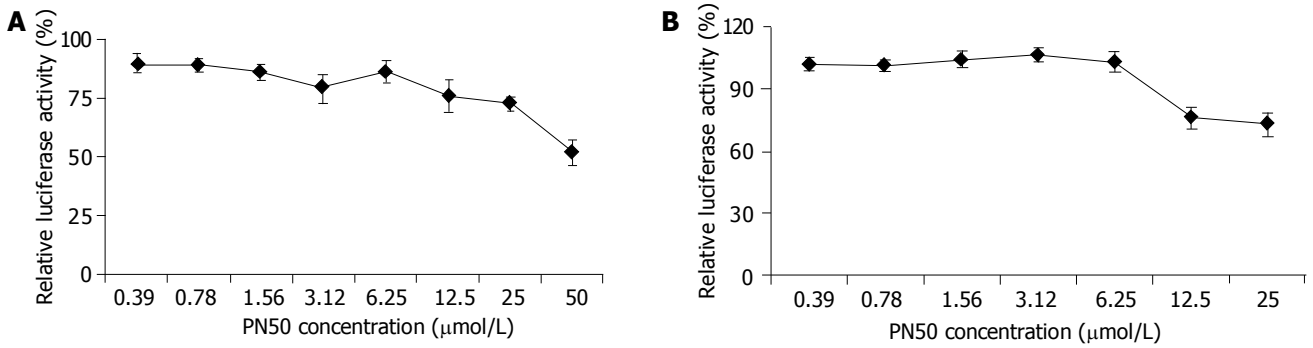

Figure 2 PN50 suppresses transcriptional activity of NF-κB in TNF-α-stimulated L929 fibroblasts (A) and LPS-activated RAW 264.7 macrophages (B) *in vitro*.

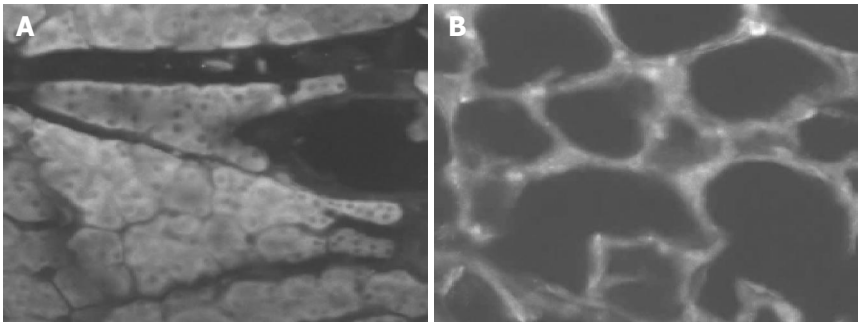

Figure 3 *In vivo* delivery of the NF-κB p50 NLS with penetratin into the pancreas (A) and lung (B).

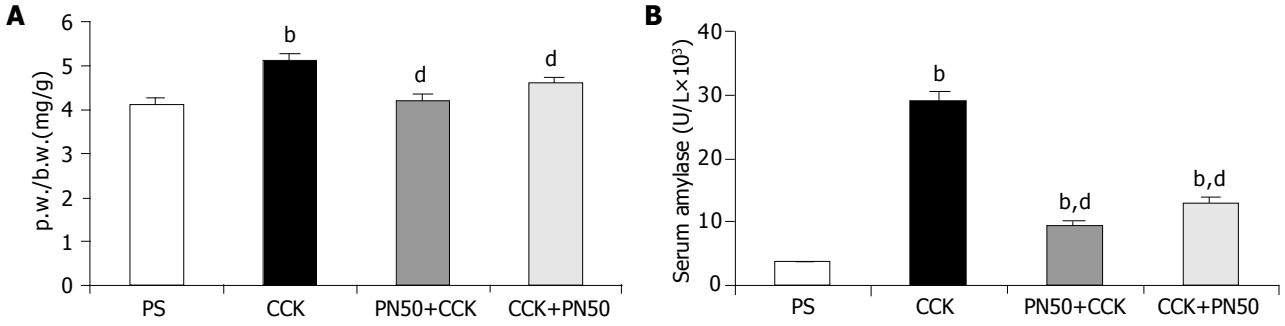

Figure 4 Effect of PN50 on the pancreatic weight/body weight ratio (pw/bw) (A) and serum amylase activity (B) in CCK-induced acute pancreatitis. [b]*P*<0.01 *vs* group PS; [d]*P*<0.01 *vs* group CCK.

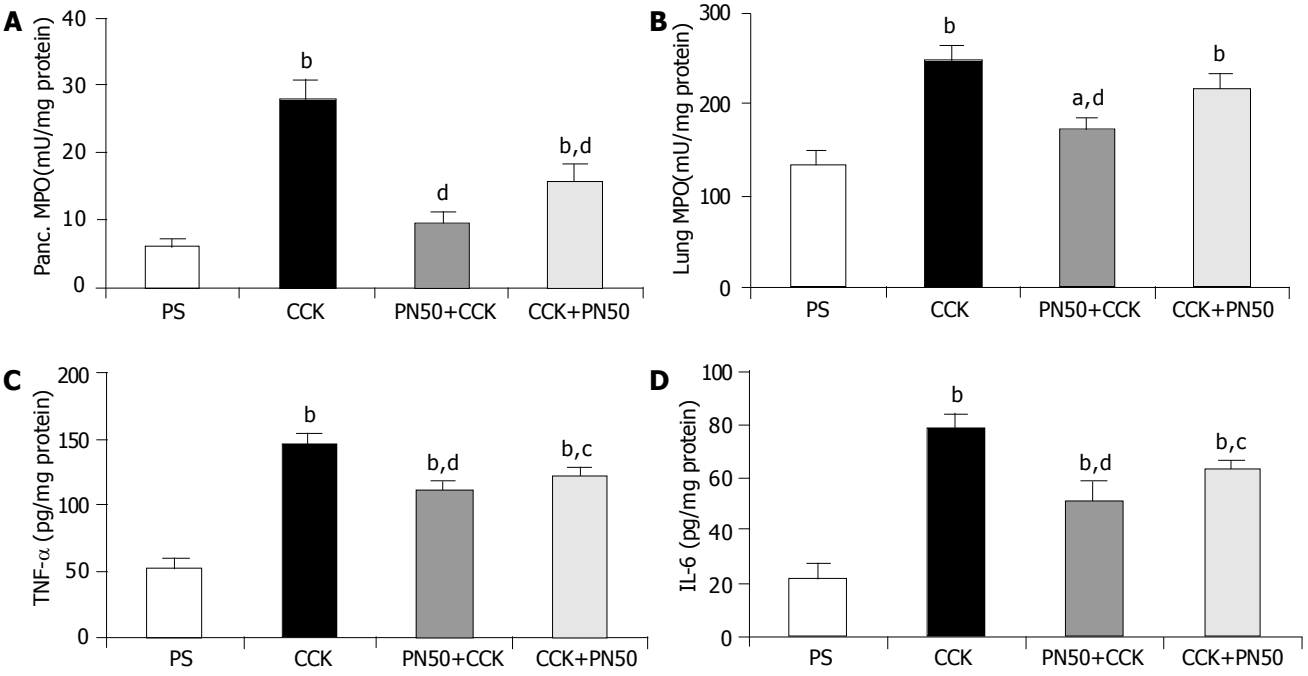

Figure 5 Effect of PN50 on the pancreatic (A) and lung (B) myeloperoxidase (MPO) activity and pancreatic TNF-α (C) and IL-6 (D) levels in CCK-induced acute pancreatitis. [a]*P*<0.05, [b]*P*<0.01 *vs* group PS; [c]*P*<0.05, [d]*P*<0.01 *vs* group CCK.

Pancreatic reduced glutathione (GSH) levels

GSH levels in the pancreas were significantly lower in group CCK than in the control group. Prophylactic PN50 treatment significantly increased the pancreatic GSH level compared to group CCK (Figure 6B).

Electrophoretic mobility shift assay (EMSA) of NF-κB

The level of nuclear import of NF-κB was determined by measuring DNA-binding activity using an EMSA performed on nuclear extracts from the pancreas samples. As shown in Figure 7, NF-κB-binding activity could hardly be detected in the control group. Four hours after the first CCK injection DNA-binding activity of NF-κB increased. Treating animals with PN50 efficiently inhibited NF-κB nuclear import. This inhibitory effect of PN50 was not significant when PN50 was administered 30 min after the second CCK injection. The specificity of NF-κB binding was confirmed in competition experiments. Incubation with increasing doses of the cold unlabeled oligonucleotide led to the inhibition of binding activity. In contrast, incubation with an increased concentration of nonspecific DNA [poly (dI/dC)] did not affect the NF-κB binding (results not shown).

Histological findings

In group CCK, the administration of 2×100 μg/kg body weight CCK induced acute pancreatitis characterized by marked edema, inflammatory activity, and stasis of the pancreas. Microfocal necroses, vacuolar degenerations in acinar cells were also present in the pancreatic samples of this group. Treating the animals with 1 mg/kg PN50 before or after the CCK-injections significantly inhibited the onset of pancreatic morphological damage as compared to group CCK (Figure 8). This inhibition was much more apparent in the prophylactic group (group PN50+CCK). The values for each of the scored parameters are shown in Table 3. Apoptosis was more frequent and acinar cell necrosis was

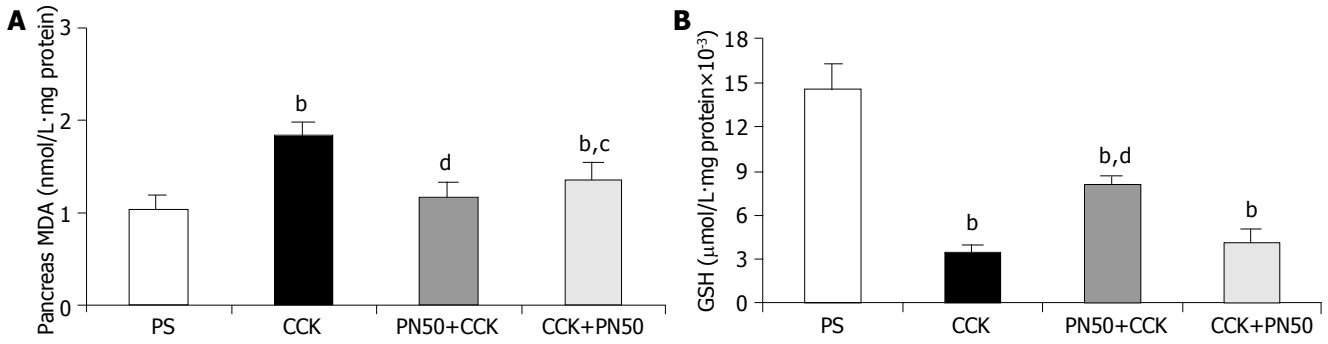

Figure 6 Effect of PN50 treatment on the pancreatic malondialdehyde (MDA) (A) and reduced glutathione (GSH) levels (B) in CCK-induced acute pancreatitis. [b]*P*<0.01 *vs* group PS; [c]*P*<0.05, [d]*P*<0.01 *vs* group CCK.

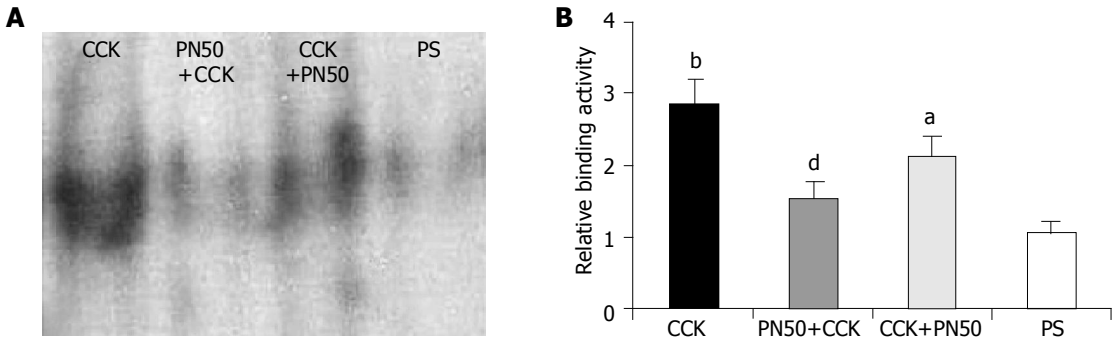

Figure 7 Effect of PN50 on pancreatic NF-κB binding activity in CCK-induced acute pancreatitis. A: a representative EMSA for pancreatic NF-κB DNA binding activity; B: the intensities of NF-κB bands. [a]*P*<0.05, [b]*P*<0.01 *vs* group PS; [d]*P*<0.01 *vs* group CCK.

Table 3 Effects of PN50 before and after treatment on the histologic parameters in CCK-induced acute pancreatitis (mean±SD)

	Group PS	*Group CCK*	*Group PN50+CCK*	*Group CCK+PN50*
Edema	0.1±0.067	0.955±0.045 [b]	0.55±0.117 [b,d]	0.636 ± 0.097 [b,d]
Vascular Change	0.1±0.067	0.773± 0.079 [b]	0.4±0.1 [a,d]	0.55 ± 0.05 [b,c]
Inflammation	0	0.591±0.061 [b]	0.3±0.082 [b,d]	0.35 ± 0.076 [b,c]
Acinar necrosis	0	0.955±0.081 [b]	0.45±0.09 [b,d]	0.65 ± 0.076 [b,c]
Calcification	0	0.125±0.065	0	0
Fat necrosis	0	0.167±0.071 [a]	0.05±0.05	0.05±0.05

[a]*P*<0.05, [b]*P*<0.01 *vs* group PS; [c]*P*<0.05, [d]*P*<0.01 *vs* group CCK.

almost absent in the pancreas of the PN50-treated animals. In group CCK, where pancreatitis was much more severe, necrosis was more pronounced than apoptosis in the pancreatic specimens.

DISCUSSION

A number of well-carried out studies have revealed the importance and beneficial effect of NF-κB inhibition in experimental acute pancreatitis[8,22-24,35]. Ethridge *et al*[23] have demonstrated that a novel peptide that binds the NF-κB essential modifier binding domain (NBD) could attenuate the severity of acute pancreatitis. Other SRTFs also play a key role in the onset and development of acute pancreatitis[31-35]. It is well- established that the acinar cell is a source of increased inflammatory molecule expression through the activation of NF-κB and AP-1 at the initiation of pancreatitis. STAT activation could also occur in acute pancreatitis model of acute lung injury[36]. NFAT and STATs play a key role in T lymphocyte activation and these cells have a central role in proinflammatory cytokine production[27,30]. *In vivo* delivery of the NLS of the NF-κB p50 subunit can block the production of proinflammatory cytokines and significantly reduce the lethality associated with sepsis or endotoxic shock by inhibiting the nuclear translocation of SRTFs like NF-κB, AP1, STAT1 and NFAT[40,41]. We wanted to test our hypothesis that inhibiting a broad range of SRTFs with the NF-κB p50 NLS provides an advantage over selective NF-κB inhibition in acute pancreatitis. We coupled the p50 NLS to one of the most efficient vector peptide penetratin. Our novel cell-permeant conjugate is named PN50.

First, we investigated the effect of PN50 delivery on endogenous NF-κB transcription activity in cultured cells. The *in vitro* luciferase reporter gene assay in TNF-α stimulated

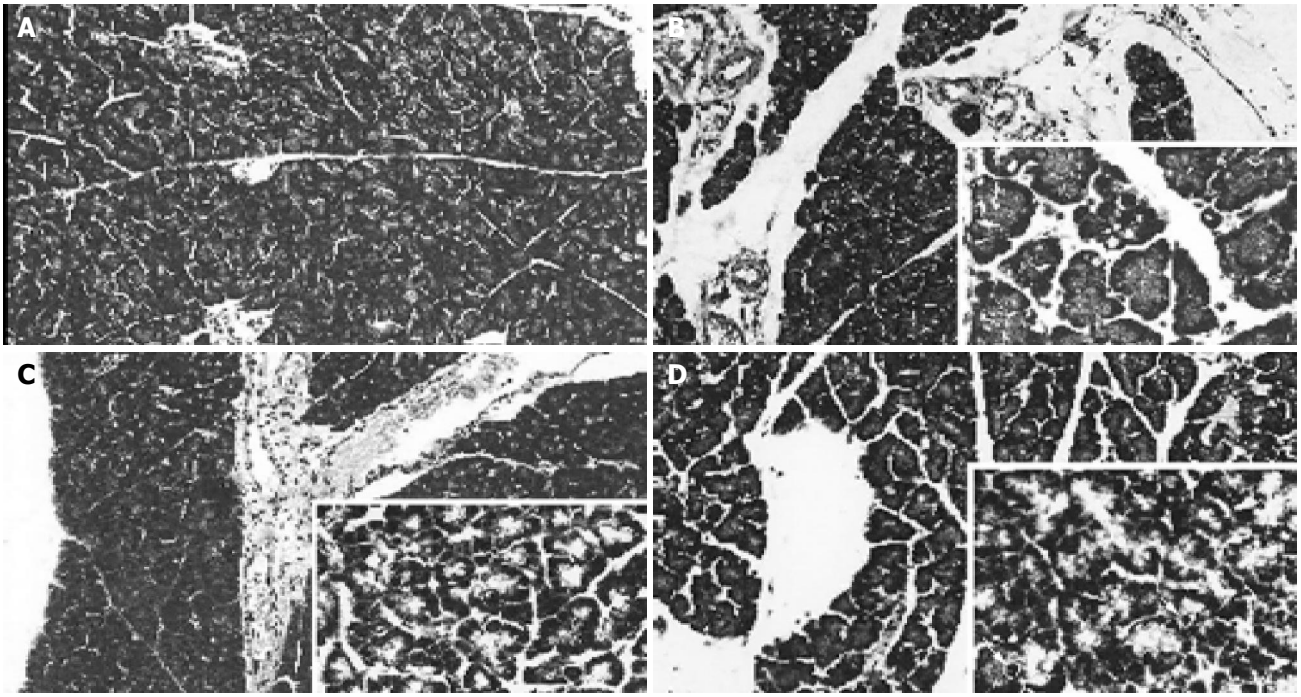

Figure 8 Effect of PN50 on pancreatic morphological damage in CCK-induced pancreatitis. A: group PS (control group): normal pancreas (HE x100); B: group CCK: marked edema, inflammatory activity, stasis (HE x100); microfocal necroses, vacuolar degenerations in the acinar cells (insert: HE x250); C: group PN50+CCK: mild inflammation, mild edema, fairly maintained parenchymal structure (HE x100); mild degenerative changes in the acini (insert: HE x250); D: group CCK+PN50: mild edema, minimal inflammatory activity (HE x 100); degenerative changes with apoptotic cells (arrows) in the acini (insert: HE ×250).

L929 fibroblasts showed that PN50 dose-dependently prevented the increase of NF-κB-driven luciferase activity at concentrations of 0.39 to 50 μmol/L. In LPS-treated RAW 264.7 macrophages PN50 decreased NF-κB transcriptional activity by 76% and 72.7% at 12.5 and 25 μmol/L respectively. The following *in vivo* uptake experiments with fluorescently labeled PN50 showed that the peptide could enter the cells of the pancreas and lung 15 min after its IP injection. Based on these promising results, we moved on to test PN50 in the *in vivo* model of CCK-induced acute pancreatitis. Injection of supramaximal doses of CCK produced acute pancreatitis assessed by the alterations in typical laboratory and morphological parameters of the disease. Thus supramaximal doses of CCK caused pancreas edema, cellular damage, increase of neutrophil sequestration, lipid pero-xidation, oxidative stress and cytokine production, both within the pancreas and lung. PN50 pretreatment (prophylactic treatment) at the very low dose of 1 mg/kg decreased and almost completely prevented the onset of pancreatitis. Adding PN50 30 min after the second CCK injection (therapeutic treatment) also improved the severity of the disease, but not as much as pre-treatment. The EMSA of NF-κB revealed that PN50 suppressed the DNA-binding activity of NF-κB especially when administered 30 min before the CCK injections. Considering the observation that DNA-binding activity of NF-κB peaks already at 30 min of cerulein hyperstimulation[4], our finding that PN50 inhibits the DNA-binding activity of NF-κB to a smaller extent in the post-treated group (group CCK+PN50) is not surprising. The double-blind histological examination revealed that PN50 (either administered before or after induction of acute pancreatitis) protected the pancreas against edema and cellular damage. Our observation that PN50 favored the induction of apoptosis (as opposed to the necrotic cell death observed in group CCK) supports the results of several experimental studies claiming that apoptosis protects against acute pancreatitis[55-59]. It is already proven that TNF-α induces concomitantly proapoptotic and antiapoptotic mechanisms in pancreatic acinar cells and the antiapoptotic mechanism of TNF-α is mediated by NF-κB[60]. By blocking the nuclear import of NF-κB with PN50, we also inhibited the antiapoptotic mechanisms of TNF-α mediated by NF-κB.

Our findings that inhibiting SRTFs like NF-κB attenuates the severity of acute pancreatitis are in accordance with the results of previous studies[8,22-24,35]. The capacity of NF-κB p50 NLS to block the nuclear translocation and transcriptional activity of a broad range of SRTFs (including NF-κB, AP-1, STAT1 and NFAT) makes this peptide preferable over other NF-κB inhibitors. Given their coordinated involvement in regulation of genes encoding the key inflammatory mediators of systemic inflammation, broad inhibition of SRTFs nuclear import becomes superior as a treatment strategy over inhibiting only a single SRTF signaling pathway[41]. The striking fact that a very low dose of 1 mg/kg of PN50 reduces the severity of CCK-induced acute pancreatitis *in vivo*, could be explained by the broad inhibitory range of its p50 NLS subunit observed by Torgerson *et al*[39].

In summary, our study shows the *in vivo* efficacy of a cell-permeable NF-κB NLS peptide for treatment of experimental acute pancreatitis. Given the broad inhibitory range of its bioactive subunit, nuclear import inhibitors like PN50 may provide a better therapeutic solution for the treatment of this disease.

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