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In Vitro and in Vivo Nuclear Factor-κB Inhibitory Effects of the Cell-Penetrating Penetratin Peptide

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ABSTRACT

Penetratin is a cationic cell-penetrating peptide that has been frequently used for the intracellular delivery of polar bioactive compounds. Recent studies have just revealed the major role of polyanionic membrane proteoglycans and cholesterol-enriched lipid rafts in the uptake of the peptide. Both proteoglycans and lipid-rafts influence inflammatory processes by binding a wide array of proinflammatory mediators; thus, we decided to analyze the effect of penetratin on in vitro and in vivo inflammatory responses. Our in vitro luciferase gene assays demonstrated that penetratin decreased transcriptional activity of nuclear factor-κB (NF-κB) in tumor necrosis factor (TNF)-stimulated L929 fibroblasts and lipopolysaccharide-activated RAW 264.7 macrophages. Penetratin also inhibited TNF-induced intercellular adhesion molecule-1 expression in human endothelial HMEC-1 cells. Exogenous heparan sulfate abolished the in vitro NF-κB inhibitory effects of the peptide. Uptake experiments showed that penetratin was internalized by all of the above-mentioned cell lines in vitro and rapidly entered the cells of the lung and pancreas in vivo. In an in vivo rat model of acute pancreatitis, a disease induced by elevated activities of stress-responsive transcription factors like NF-κB, pretreatment with only 2 mg/kg penetratin attenuated the severity of pancreatic inflammation by interfering with IκB degradation and subsequent nuclear import of NF-κB, inhibiting the expression of proinflammatory genes and improving the monitored laboratory and histological parameters of pancreatitis and associated oxidative stress.

Penetratin is a 16 amino acid-long cationic cell-penetrating peptide derived from the third helix of Antennapedia homeodomain (Derossi et al., 1994, 1996). Intracellular delivery with penetratin has opened new possibilities to alter various cellular processes with novel hydrophilic biocompounds like oligonucleotides and (poly)peptides (Derossi et al., 1998; Dupont et al., 2002; Dietz and Bdeltahr, 2004; Jarver and Langel, 2004). In the early delivery studies, penetratin was used as an inert cell-transporter peptide without any biological activity. Only one study aimed to reveal the biological effects of penetratin. In that particular study, Bolton et al. (2000) injected fluorescently labeled penetratin directly into rat brain and assessed the brain response to the peptide. Immunohistochemistry conducted after intrastriatal injection showed that injection of 10 μg of penetratin caused neurotoxic cell death and triggered the recruitment of inflammatory cells in a dose-dependent fashion (Bolton et al., 2000). Considering these findings, it is noteworthy that the brain is an extremely sensitive organ, and direct intrastriatal injection itself promotes enough mechanical stress that can trigger the inflammatory response in the brain.

In our previous work, we used penetratin to deliver an anti-inflammatory nuclear localization signal (NLS) peptide into the cells to suppress acute pancreatitis in vivo (Letoha et

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ABBREVIATIONS: NLS, nuclear localization signal; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; ICAM-1, intercellular adhesion molecule-1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; EMSA, electrophoretic mobility shift assay; RT-qPCR, real-time quantitative polymerase chain reaction; PAP, pancreatitis-associated protein; FRAP, ferric reducing ability of plasma; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; CCK, cholecystokinin; IL, interleukin; MIX MEM, Dulbecco's modified Eagle's medium, Ham's F-12, and fetal calf serum; FCS, fetal calf serum; GlyPen, glycine mutant analog; LPS, lipopolysaccharide; HS, heparan sulfate; MPO, myeloperoxidase; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; H&E, hematoxylin and eosin; ANOVA, analysis of variance; CPP, cell-penetrating peptide; Luc, luciferase; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

al., 2005b). Taking the severity of pancreatic inflammation into account, it was crucial for us that the transporter penetratin peptide itself would not enhance the inflammatory response. Therefore, we decided to investigate the effects of penetratin on NF-κB, a transcriptional factor responsible for the induction of inflammatory processes, in the same experimental settings as the penetratin-NLS conjugate. Thus, in vitro effects of penetratin on NF-κB were studied in NF-κB luciferase gene assays with genetically modified L929 fibroblasts and RAW macrophages, cells in which activated NF-κB induces the expression of the firefly luciferase enzyme. Because luciferase activity can be easily and very precisely measured by a luminometer, these assays give very accurate information on the transcriptional activity of NF-κB. In vivo anti-inflammatory activity of the peptide was studied in cholecystokinin (CCK)-induced model of acute pancreatitis, a disease induced by enhanced transcriptional activity of stress-responsive transcription factors like NF-κB (Algul et al., 2002; Bhatia et al., 2005). In this model, CCK hyperstimulation activates NF-κB and triggers intracellular events, leading to pancreatic and then generalized inflammation that affects other organs, including the lung (Gukovsky et al., 1998, 2003; Chen et al., 2002; Rakonczay et al., 2003). Severity of pancreatitis can be quantified by monitoring the characteristic parameters of pancreatic inflammation, including serum amylase (which informs about the severity of pancreatic damage), pancreatic weight/body weight ratio (which informs about the extent of pancreatic edema), intrapancreatic concentrations of proinflammatory cytokines [interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α], activity of neutrophil-derived myeloperoxidase (as the marker of neutrophil sequestration), and other parameters of the pancreatitis-associated oxidative stress (reduced glutathione, malondialdehyde, superoxide dismutase, etc.) (Takacs et al., 1996; Ranson, 1997; Schulz et al., 1999; Makhija and Kingsnorth, 2002). As a result of accurately measurable laboratory markers and the major role of NF-κB in the disease, CCK-induced acute pancreatitis is an excellent model to study the anti-inflammatory and NF-κB inhibitory activities of compounds in vivo. This article summarizes the results of the above-mentioned inflammatory studies that shed light on the formerly unidentified biological activity of penetratin and demonstrates the effects of the peptide on the NF-κB-dependent inflammatory responses both in vitro and in vivo.

Materials and Methods

Peptide Synthesis and Labeling. Penetratin (RQIKIWFQNRRMKWKK) and its glycine mutant analog (GQIGIWFQNGGMGWGG) were synthesized in our laboratory (at the Department of Medical Chemistry, Szeged, Hungary) in solid phase by standard methodology as described previously (Letoha et al., 2005a). For the internalization studies, the peptides were labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich Hungary Ltd., Budapest, Hungary) as described by Fülöp et al. (2001). Cholecystokinin-octapeptide (CCK) was also prepared in our laboratory by method of Penke et al. (1984).

Cell Lines. Murine L929 fibroblasts and RAW 264.7 macrophages were cultured in MIX MEM [1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 plus 10% (v/v) FCS (all from Sigma)] at 37°C in a humidified 5% CO_2-containing air environment. Human microvascular endothelial HMEC-1 cells [HE-SFM (Csertex Ltd., Budapest, Hungary) supplemented with 2% FCS, 1 μg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Sigma), and antibiotics] were cultured similarly.

Transformation of Cell Lines. Mouse L929 fibroblasts and RAW 264.7 macrophages were transformed with pNF-κB-luc4 and pSV-2/neo plasmids as described previously (Letoha et al., 2005b).

Luciferase Assay. One-day-old cultures of L929 and RAW 264.7 cells, grown on luminoplates (Corning-Costar; Zenon Biotechnology Ltd., Szeged, Hungary) were used. Cells (3×10^4 cells/well in MIX MEM, 10% FCS) were exposed to various concentrations (1.56–25 μM) of penetratin or the glycine mutant analog (GlyPen) for 30 min. Thirty minutes later, the cells were treated with TNF-α (5 pg, 10 U/ml) or LPS (30 ng/ml) in 100 μl of the above medium per well. After 6 h of incubation with TNF or LPS, the medium was removed, and the cells were washed and lysed for 10 min at room temperature in Reporter Lysis Buffer (20 μl/well; Promega Bio-Science Hungary,

TABLE 1
Sequences of gene-specific primers used for RT-qPCR
The primers were designed with the software PrimerExpress (Applied Biosystems).

Gene Name	Forward Primer	Reverse Primer
Cyclophilin	TCTCTTCAAGGGACAAGGCTG	TGGCAAATCGGCTGACG
IL-1β	CTTCCCCAGGACATGCTAGG	CAAAGGCTTCCCCTGGAGAC
IL-6	TGTCTCGAGCCCACCAGG	TGCGGAGAGAAACTTCATAGCTG
TNF-α	TCTCTTCAAGGGACAAGGCTG	TGGCAAATCGGCTGACG
PAP	CCTCTGCACGCATTAGTTGC	TGAAACAGGGCATAGCAGTAGG

TABLE 2
Histological scoring system for the evaluation of CCK-induced acute pancreatitis
The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blinded to the experimental protocol. Semiquantitative grading of interstitial edema, vascular changes, inflammation, necrosis of acinar cells, calcification, and fat necrosis was evaluated in each animal with the scoring system of Hughes et al. (1996).

	0	0.5	1	1.5	2
Edema	Absent	Focal <50%	Diffuse >50%		
Vascular changes	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			

Budapest, Budapest). Substrate was added (20 μl/well; Promega), and luciferase activity was measured in a Luminoskan Ascent (Thermo Electron Corporation, Waltham, MA) scanning luminometer. Control cells received only TNF-α (10 U/ml) or LPS (30 ng/ml) treatment and were processed as mentioned above. In some of the assays, the cells were coincubated with penetratin and 25 μg/ml heparan sulfate (HS) at 37°C and then treated as mentioned above. Cell viability was routinely determined using trypan blue exclusion test during the assays to make sure that assays were always carried out on viable cells.

ICAM-1 Expression. HMEC-1 cells grown on microplates (Corning-Costar) were used. Cells (3×10^4 cells/well) in HE-SFM 2% FCS were exposed to various concentrations (6.25–50 μM) of penetratin and its mutant analog for 30 min. Thirty minutes later, the cells were treated with TNF-α (100 U/ml in 100 μl of the above medium per well). After 6 h of incubation with TNF-α, the cells were trypsinized, washed, and resuspended in 10% FCS, then vortexed for 5 min at 2000 rpm, resuspended in PBS, and vortexed again for 5 min. Then the medium was removed, and the cells were incubated in PBS with the FITC-conjugated monoclonal mouse anti-human ICAM-1 antibody (10 μg/ml; Dako North America, Inc., Carpinteria, CA) for 30 min on ice. After two washes and fixation with 2% paraformaldehyde while being vortexed, the samples were analyzed with flow cytometry using the FACScan flow cytometer and the CellQuest analysis program (BD PharMingen, San Diego, CA). Control cells received only TNF treatment. To seek the importance of attachment to polyanionic cell surface proteoglycans, some of the cells were coincubated with penetratin and 25 μg/ml HS for 30 min before TNF stimulation. Viability of the cells was determined by concurrent propidium iodide (0.2 μg/ml; Sigma) staining. Cells stained with propidium iodide were excluded from the analyses for ICAM-1 expression.

Cell Viability Assays. Cytotoxicity of penetratin and its glycine mutant analog was assessed by using MTS assay (CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay; Promega) according to the manufacturer's protocol. In brief, L929, RAW 264.7, and HMEC-1 cells (10^5/well) were cultured in 96-well microtiter plates in MIX MEM supplemented with 10% FCS in the presence of the peptides at different concentrations (1, 10, and 50 μM, respectively). Control cells did not receive any peptide treatment. Twenty-four hours later, the cells were incubated with the MTS assay reagents, and absorbance was measured at 492 nm by a Multiscan mass spectrometry enzyme-linked immunosorbent assay reader (Thermo Electron Corporation).

Internalization Studies. Uptake of penetratin peptides (at a concentration of 5 μM) into HMEC-1, L929, and RAW cells was

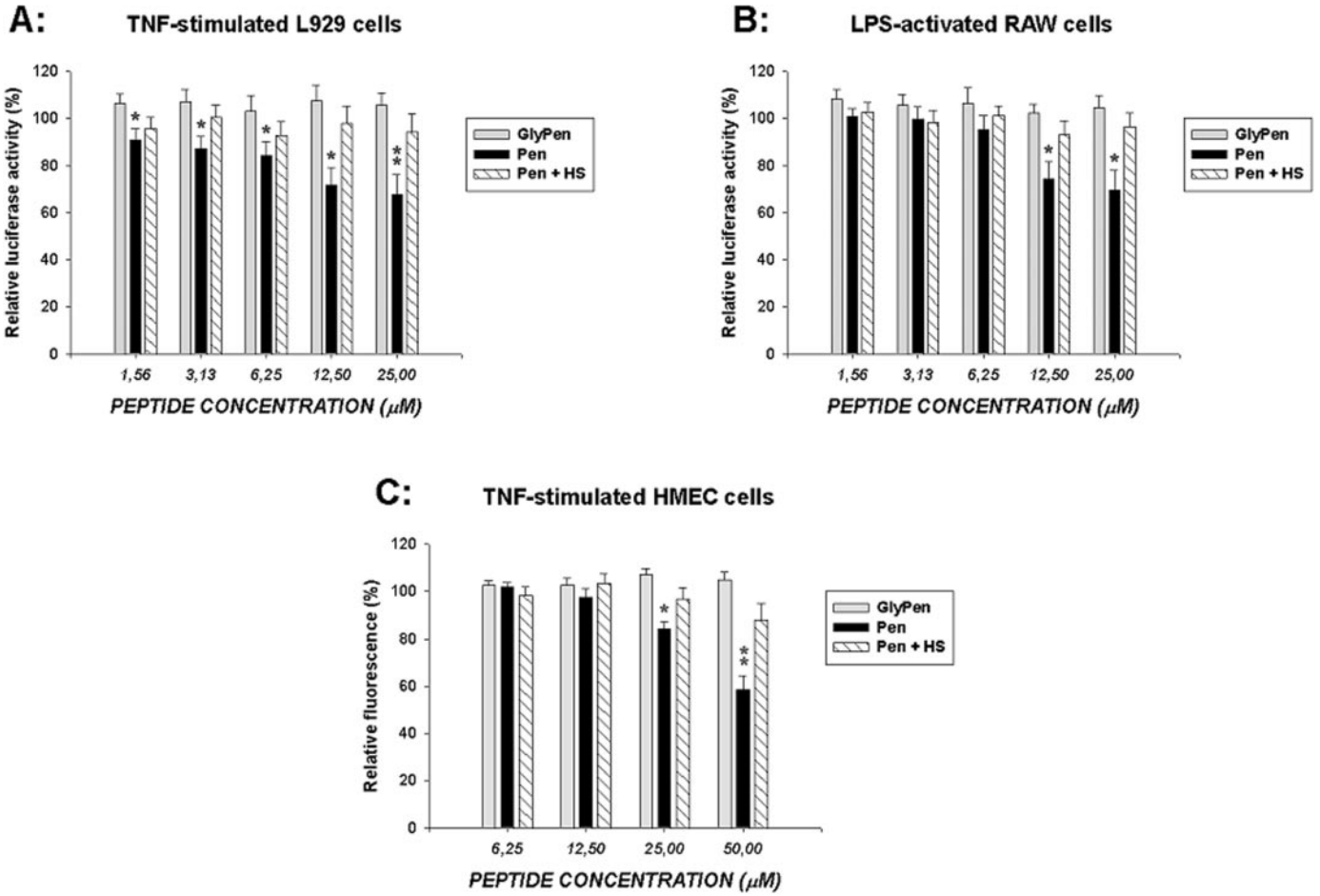

Fig. 1. Penetratin inhibits NF-κB transcriptional activity in vitro. A and B, luciferase reporter assays of TNF-stimulated L929 fibroblasts (A) and LPS-activated RAW 264.7 macrophages (B) with pNF-κB-Luc are shown. Controls were treated with 10 U/ml TNF-α (A) or 30 ng/ml LPS (B). Peptide-treated cells were incubated with various concentrations of penetratin (Pen) with or without HS and the mutant peptide (GlyPen) for 30 min before TNF or LPS was added. Luciferase activity was measured 6 h later. Error bars represent relative luciferase activity of the peptide-treated cells compared with controls (treated with TNF or LPS only). Means ± S.E. of four independent experiments are shown. C, surface ICAM-1 expression as detected by flow cytometric analysis on HMEC-1 cells pretreated with penetratin (Pen) with or without HS and the mutant peptide (GlyPen) for 30 min before TNF (100 U/ml) stimulation for 6 h. Error bars represent relative fluorescence of peptide-treated cells compared with controls (treated with TNF only). Means ± S.E. of three independent experiments are shown. Statistical significance was assessed by ANOVA. *, $p < 0.05$ versus controls; **, $p < 0.01$ versus controls.

investigated as described previously (Letoha et al., 2005a). For the in vivo studies, male Wistar rats (provided by the Animal Center of the University of Szeged) weighing 250 to 300 g were used. The animals were kept at a constant room temperature with a 12-h light/dark cycle and were 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). Six animals were injected intraperitoneally with 20 nM/kg fluorescent penetratin (dissolved in 0.5 ml of PBS), and the other six were injected with the same dose (20 nmol/kg) of the glycine mutant analog (also in 0.5 ml of PBS). Control animals ($n = 6$) received intraperitoneal injections of 0.5 ml of PBS. Rats were anesthetized (with pentobarbital sodium, 50 mg/kg i.p.) and killed 15 min after the injections by exsanguination through the abdominal aorta. Lung and pancreas tissues were harvested and frozen in Histo Prep media (Fisher Scientific GmbH, Schwerte, Germany). Sections (10–50 μm) were cut on a cryostat and were analyzed by fluorescence confocal microscopy.

CCK-Induced Pancreatitis. In each experimental group, 10 rats were used. The rats were fasted for 16 h, and then acute pancreatitis was induced by injecting 100 μg/kg body weight CCK (dissolved in PBS) intraperitoneally twice at an interval of 1 h (group CCK). The penetratin-pretreated group (Group Pen+CCK) received 2 mg/kg body weight penetratin (in 0.5 ml of PBS) intraperitoneally 30 min before the first injection of CCK. Another group of animals was treated with an equimolar dose (1.5 mg/kg) of the glycine mutant analog (in 0.5 ml of PBS) 30 min before the induction of pancreatitis (Group GlyPen+CCK). Control rats received three intraperitoneal injections of 0.5 ml of PBS instead of the peptides. Anesthetized (pentobarbital sodium, 50 mg/kg i.p.) rats were killed by exsanguination through the abdominal aorta 4 h after the first CCK injection. Lung and pancreas were quickly removed, and the latter was cleaned of fat and lymph nodes, weighed, frozen in liquid nitrogen, and stored at −80°C until use.

Nuclear Protein Extract. Nuclear protein extracts from pancreatic tissues were prepared as described previously (Rakonczay et al., 2003).

Electrophoretic Mobility Shift Assay of NF-κB. The electrophoretic mobility shift assay (EMSA) was carried out as described previously (Rakonczay et al., 2003). Intensities of the bands were quantified by using the Scanpack Image Analysis Program (Biometra, Goettingen, Germany).

Western Blotting. Western blot analysis of pancreatic IκB-α was performed as described previously (Rakonczay et al., 2003).

The Pancreatic Weight/Body Weight Ratio and Serum Amylase Activity. The pancreatic weight/body weight ratio was used to evaluate the degree of pancreatic edema. To measure the serum amylase activities, all blood samples were centrifuged at 2500*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. TNF-α and IL-6 concentrations were measured in the pancreatic cytosolic fractions with enzyme-linked immunosorbent assay 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. (1996).

Real-Time Quantitative Polymerase Chain Reaction. Real-time quantitative polymerase chain reaction (RT-qPCR) was per-

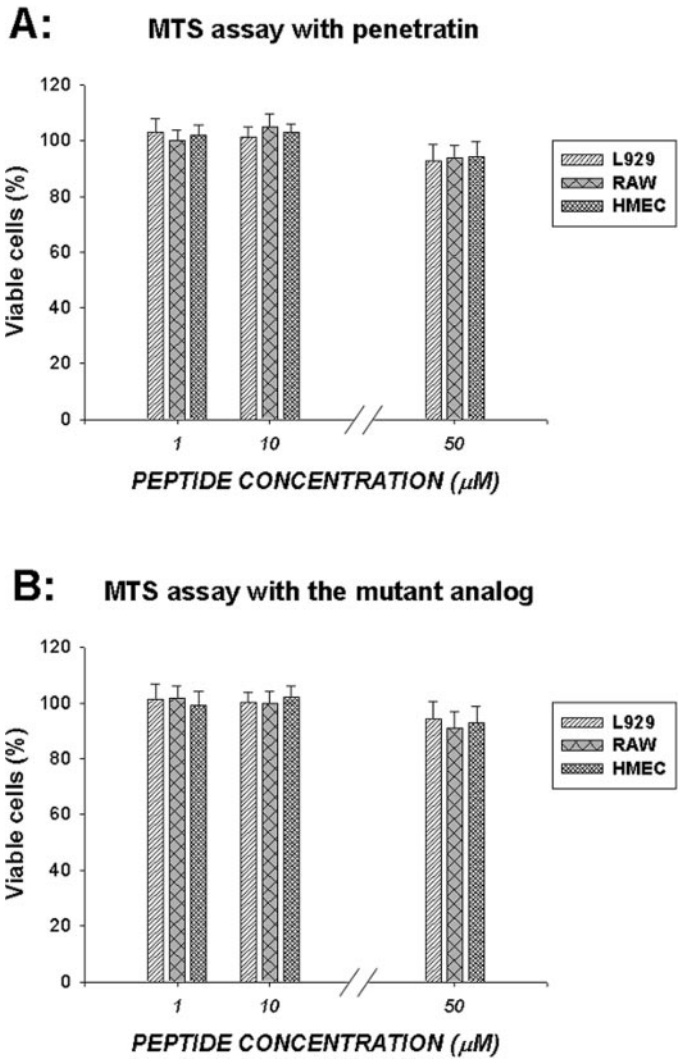

Fig. 2. Effects of penetratin and its mutant on cell-viability. L929, RAW, and HMEC-1 cells were incubated for 24 h with increasing concentrations of penetratin and the mutant peptide. Cell viability was measured after a standard MTS assay procedure. Results of three independent assays were normalized and plotted as the percentage of viable cells. Data points represent mean ± S.E.

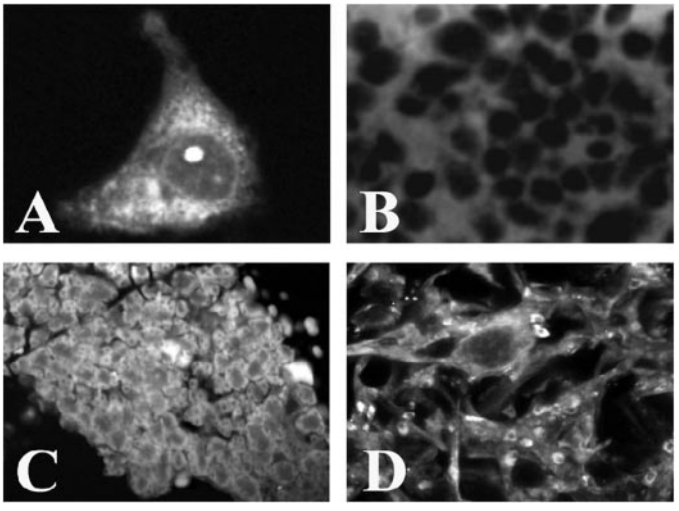

Fig. 3. Cellular internalization of penetratin peptides. A and B, in vitro experiments show that fluorescently labeled penetratin (A) is internalized by cells (RAW macrophages), whereas the glycine analog (B) is not. C and D, in the in vivo uptake studies, male Wistar rats (weighing 250–280 g) were injected intraperitoneally with 20 nmol/kg FITC-labeled penetratin and its mutant. C shows pancreas, and D shows lung tissue sections isolated from rats 15 min after the injection of fluorescent penetratin.

formed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers (designed with the software PrimerExpress; Applied Biosystems, Foster City, CA) and SYBRGreen I protocol to follow gene expression changes during treatment as described previously (Letoha et al., 2005c). Relative expression ratios were normalized to cyclophilin and calculated with the Pfaffl method (Nagy et al., 2005). The polymerase chain reaction primers used are shown in Table 1.

Pancreatic Lipid Peroxide and Reduced Glutathione Levels. Lipid peroxides may undergo metal- or enzyme-catalyzed decomposition to form multiple products, including malondialdehyde (MDA). Pancreatic and serum MDA, reduced glutathione (GSH) levels, and pancreatic total superoxide dismutase (SOD) activity were measured as described previously (Letoha et al., 2005c).

Ferric Reducing Ability of Plasma. The total antioxidant activity of the plasma was determined with use of the method of Benzie and Strain (1996). Ferric to ferrous ion reduction—in a complex with tripyridyl-triazine—at low pH causes the development of an intense blue color, which has absorption maximum at 593 nm. Ferric reducing ability of plasma (FRAP) values are obtained by preparing a calibration curve with a solution of known Fe(II) concentration.

Histological Evaluation of CCK-Induced Acute Pancreatitis. A portion of the pancreas was fixed in 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin (H&E). The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blinded to the experimental protocol. They used the scoring system of Hughes et al. (1996) for the evaluation of acute pancreatitis. Thus, semiquantitative 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 detail in Table 2).

Statistical Analysis. Results are expressed as means ± S.E. Differences between experimental groups were evaluated by using analysis of variance (ANOVA). Values of $p < 0.05$ were accepted as significant.

Results

Penetratin Suppresses TNF- or LPS-Induced NF-κB Activation In Vitro. First, we examined the effects of penetratin on NF-κB activity in vitro. Therefore, we transfected L929 and RAW 264.7 cells with plasmids coding for firefly luciferase under the control of five NF-κB-responsive elements. NF-κB-driven luciferase activity peaked 6 h after the addition of TNF-α or LPS to L929 fibroblasts or RAW macrophages. Pretreating these cells with penetratin 30 min before TNF or LPS stimulation significantly inhibited NF-κB transcriptional activity (Fig. 1, A and B). Meanwhile, the mutated analog (GlyPen) could not suppress the NF-κB response.

TNF-α induces ICAM-1 expression in endothelial cells through an NF-κB-dependent mechanism (True et al., 2000). In our experiment, 100 U/ml TNF-α increased the expression of ICAM-1 in HMEC-1 cells 6 h after its administration. Penetratin pretreatment inhibited this ICAM-inducing effect of TNF-α, whereas the mutant peptide (GlyPen) did not have any effect on ICAM-1 expression in HMEC-1 cells (Fig. 1C).

Exogenous heparan sulfate abolished all of the above-mentioned effects of penetratin on NF-κB activity, suggesting that attachment of the cationic peptide to polyanionic proteoglycans is necessary for its biological activity (Fig. 1, A–C).

Cell-Viability Assays. Effects of penetratin and its mutant on cell viability were determined on L929, RAW, and HMEC-1 cells by standard MTS assay. Cells were incubated with the peptide for 24 h at various concentrations (1, 10, and 50 μM, respectively) at 37°C. The results of three independent MTS cell-viability assays revealed that neither penetratin nor its mutant was cytotoxic, even at 50 μM (Fig. 2, A and B).

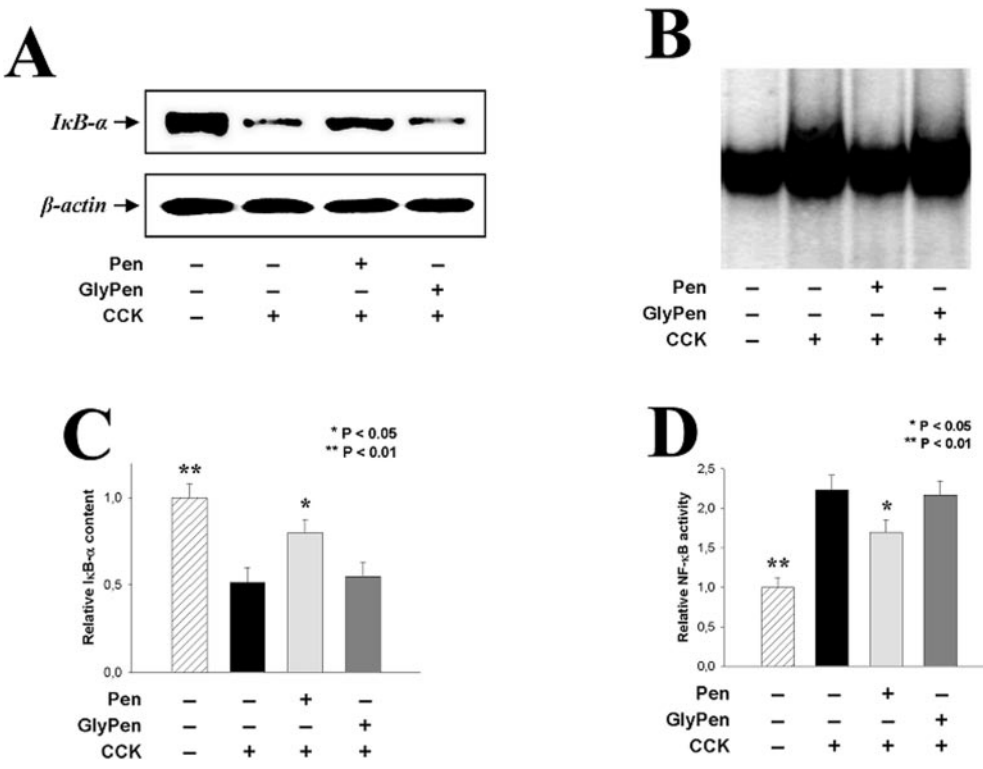

Fig. 4. Penetratin prevents IκB degradation and subsequent NF-κB activation in acute pancreatitis. A, the level of IκB-α in the pancreas was analyzed by Western blot. β-Actin was used as loading control. Data for each group are representative of four independent experiments. Nuclear protein from these same tissue samples was subjected to NF-κB EMSA. B, a representative EMSA for pancreatic NF-κB DNA-binding activity. C and D, intensities of IκB-α and NF-κB bands were densitometrically quantified relative to control pancreases. ▨, controls (receiving 3 × 0.5 ml of PBS i.p.); ■, group CCK (animals receiving 2 × 100 μg/kg CCK i.p.); □, group Pen+CCK (animals treated with 2 mg/kg penetratin i.p. 30 min before the first CCK injection); ▩, group GlyPen+CCK (animals treated with 1.5 mg/kg i.p. of the mutant peptide 30 min before pancreatitis induction). Values presented are means ± S.E., $n = 6$ to 8 animals/group. Statistical significance was assessed by ANOVA. *, $p < 0.05$ versus group CCK; **, $p < 0.01$ versus group CCK.

Internalization of Penetratins. After demonstrating the in vitro NF-κB-suppressing ability of penetratin, we moved on to test cellular uptake of the peptides. After 180 min of incubation, penetratin was in the cytoplasm (and nuclei) of all three cell lines (HMEC-1, L929, and RAW), whereas the glycine analog was not taken up by any of the cells (Fig. 3, A and B). Because our main goal was to examine the effects of the peptides on pancreatic inflammation, we also studied their in vivo internalization into the pancreas and lung, the two organs most affected by acute pancreatitis. Penetratin was internalized very rapidly into the pancreas and lung; only 15 min after its intraperitoneal injection, FITC-labeled penetratin entered the cells of these organs (Fig. 3, C and D). Apart from background autofluorescence of the tissues, we could not detect any fluorescent signal from tissues of animals injected with the mutant peptide.

Prophylactic Treatment with Penetratin Inhibits NF-κB and Improves the Parameters of Acute Pancreatitis in Vivo. After revealing the in vitro NF-κB-suppressing activity and efficient in vivo internalization of penetratin, we analyzed the in vivo NF-κB inhibitory effects of the peptide. In CCK-induced acute pancreatitis, supramaximal doses (2 × 100 μg/kg body weight) of the cholecystokinin octapeptide (CCK) induced the activation of NF-κB. Western blots performed on pancreas samples showed that penetratin pretreatment prevented degradation of IκB-α (Fig. 4, A and C). The nuclear import of NF-κB was also decreased in penetratin-treated animals, as revealed by EMSAs (Fig. 4, B and D). The mutant analog did not have these effects on IκB or NF-κB. (In our EMSAs, the specificity of NF-κB binding was confirmed by nonradioactive competition experiments, when incubation with increasing doses of the unlabeled oligonucleotide led to inhibition of the binding activity. In contrast, incubation with an increased concentration of nonspecific DNA [poly(dI/dC)] did not affect NF-κB binding (results not shown).

Besides activating NF-κB, CCK injections induced all of the characteristic features of pancreatic inflammation. Thus, CCK hyperstimulation resulted in intrapancreatic edema and cellular damage, as reflected by increased pancreatic

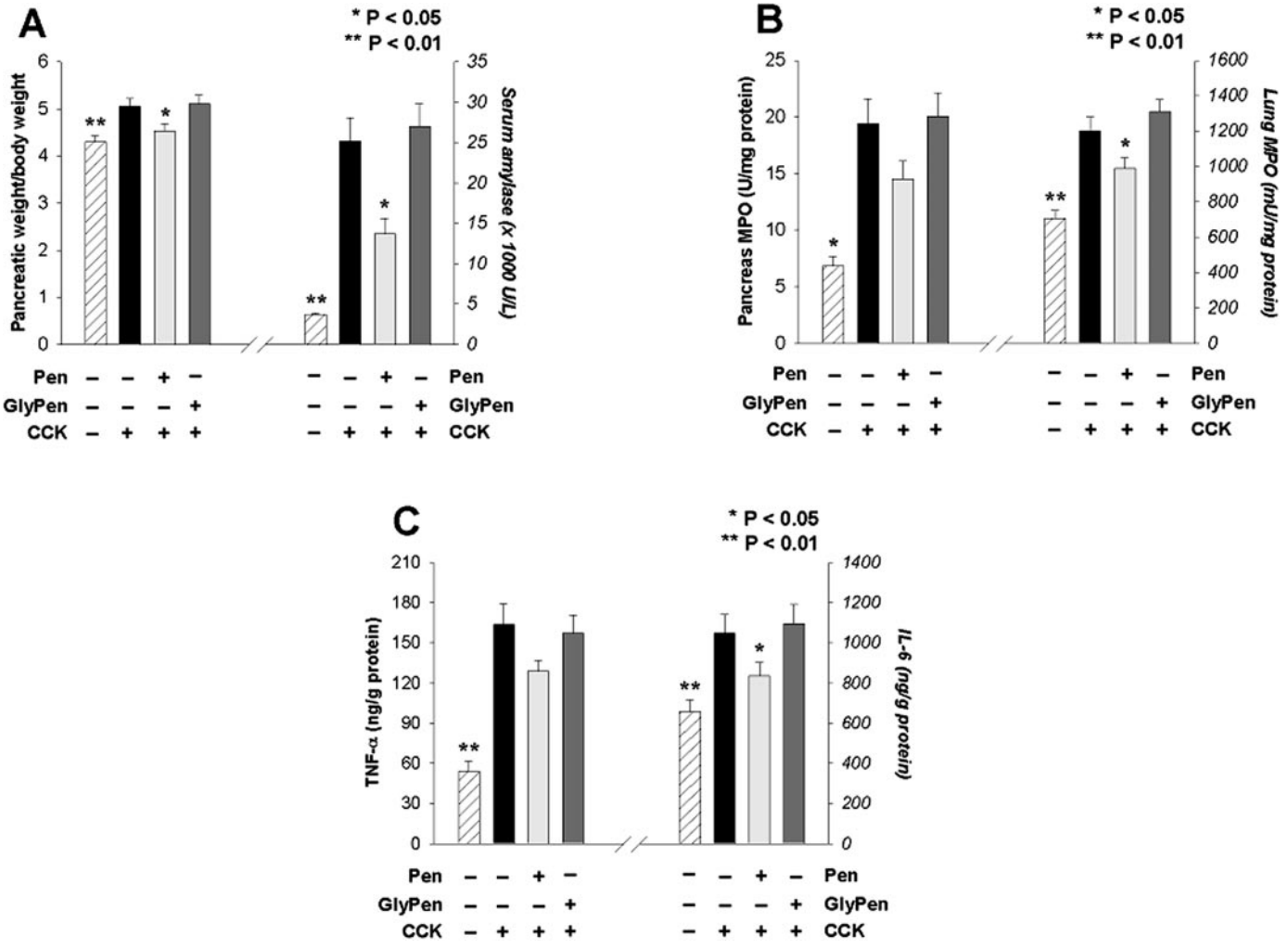

Fig. 5. Penetratin ameliorates the parameters of acute pancreatitis in vivo. Acute pancreatitis was induced by injecting 100 μg/kg body weight CCK i.p. twice at an interval of 1 h into male Wistar rats. A, the effect of penetratin (2 mg/kg i.p. 30 min before the first CCK injection) on pancreatic weight/body weight ratio and serum amylase activity. B, pancreatic and lung MPO activity. C, TNF-α and IL-6 levels in CCK-induced acute pancreatitis. ▨, controls (receiving 3 × 0.5 ml of PBS i.p.); ■, group CCK (animals receiving 2 × 100 μg/kg CCK i.p.); □, group Pen+CCK (animals treated with 2 mg/kg penetratin i.p. 30 min before the first injection of CCK); ▩, group GlyPen+CCK (animals treated with 1.5 mg/kg i.p. of the mutant peptide 30 min before pancreatitis induction). Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by ANOVA. *, $p < 0.05$ versus group CCK; **, $p < 0.01$ versus group CCK.

weight/body weight ratio and serum amylase activity (Fig. 5A). Supramaximal doses of CCK increased neutrophil sequestration and thus MPO activities within both the pancreas and lung (Fig. 5B). Intrapancreatic concentrations of proinflammatory cytokines TNF-α and IL-6 were significantly elevated compared with controls (Fig. 5C). Furthermore, CCK hyperstimulation induced the expression of IL-1β, IL-6, TNF-α, and pancreatitis-associated protein (PAP) mRNAs (Fig. 6, A and B). Laboratory parameters of oxidative stress revealed increased reactive oxygen species production as a result of 2 $\times$ 100 μg/kg CCK. Serum and intrapancreatic concentrations of reduced GSH were depleted, whereas those of MDA (the measure of lipid peroxidation) were increased (Fig. 7, A and B). The FRAP was reduced, reflecting decreased antioxidant capacity of the plasma. Intrapancreatic SOD levels were also depleted (Fig. 7C). Most of these effects of CCK were significantly inhibited by pretreating the animals with 2 mg/kg penetratin 30 min before the first CCK injection (Figs. 5–7). Thus, penetratin could decrease the inflammatory response and oxidative stress associated with acute pancreatitis. Administration of equimolar dose of the glycine mutant peptide did not have any significant effects on the above-mentioned parameters of pancreatitis; thus, the neutral penetratin analog could not prevent the onset of pancreatic inflammation.

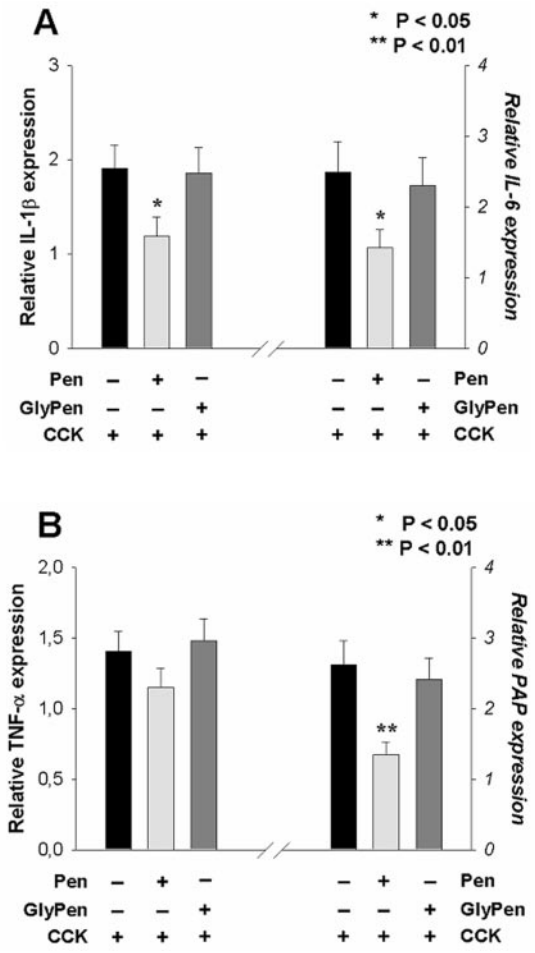

Fig. 6. Effects of penetratin on the mRNA expression of proinflammatory genes in acute pancreatitis. Relative real-time RT-qPCR analysis of mRNA expression for cytokines (IL-1β, IL-6, TNF-α) and PAP in pancreatic samples of rats treated with or without penetratin in CCK-induced acute pancreatitis is shown. A, expression of IL-1β and IL-6. B, expression of TNF-α and PAP in the pancreatic tissue. Bars represent the relative expression ratios normalized to those of the housekeeping gene (cyclophilin) in the same samples. ■, group CCK (animals receiving 2 $\times$ 100 μg/kg CCK i.p.); □, group Pen+CCK (animals treated with 2 mg/kg penetratin i.p. 30 min before the first injection of CCK); ▨, group GlyPen+CCK (animals treated with 1.5 mg/kg i.p. of the mutant peptide 30 min before pancreatitis induction). Values are means ± S.E. for three to six animals per group and are given relative to the control group. Statistical significance was assessed by ANOVA. *, $p < 0.05$ versus group CCK; **, $p < 0.01$ versus group CCK.

Histological examination also revealed the pancreatitis-inducing effects of CCK. Pancreas samples of animals treated with supramaximal doses of CCK showed the characteristic signs of edematous pancreatitis. Thus, edema, inflammatory activity (neutrophil infiltration), stasis, vacuolar degeneration, and foci of necrosis were present in samples of animals treated with CCK (Fig. 8A). Pretreating the animals with 2 mg/kg penetratin could inhibit these effects of CCK, as revealed by the milder degenerative changes of pancreatic samples from penetratin-pretreated animals (Fig. 8B). Values for each of the scored parameters are shown in Table 3.

Discussion

Penetratin is a cell-penetrating peptide that has been used frequently for the intracellular delivery of various bioactive agents. After many years of intense research and debate, recent studies clearly demonstrated the importance of lipid raft- and proteoglycan-mediated pathways in the membrane translocation of penetratin (Jones et al., 2005; Letoha et al., 2005a). Despite the frequent application of penetratin as a vector of various bioactive compounds, until now, only a few studies aimed to examine its biological activity. However, exploring the effects of cell-penetrating peptides (CPPs) on cellular processes has crucial importance. Once we are aware of the cellular effects of penetratin and other CPPs, we will be able to choose the most suitable peptide vector for a given biological application.

In a previous publication, direct injection of penetratin into rat brain was reported to induce the recruitment of inflammatory cells and neurotoxicity in a dose-dependent fashion (Bolton et al., 2000). In our recent article, we demonstrated that a conjugate of penetratin and the NF-κB p50 NLS inhibited NF-κB transcriptional activity and thus suppressed the inflammatory response in various in vitro and in vivo inflammatory models (Letoha et al., 2005b). It was well-established that intracellular delivery of the NF-κB p50 NLS blocks stress-responsive gene expression and inflammation (Torgerson et al., 1998; Yan Liu et al., 2000; Liu et al., 2004); however, penetratin (without any bioactive cargo attached) also proved to be active in the same experimental setting. Our in vitro luciferase gene assays clearly demonstrated that penetratin pretreatment could prevent TNF- or LPS-induced NF-κB activation. Because exogenous heparan sulfate abolished the NF-κB suppressing activity of the peptide, we showed that attachment to surface proteoglycans had a major role in the cellular effects of the peptide. It has been well documented that the cationic penetratin specifically interacts with polyanionic heparin and heparan sulfate moieties of membrane proteoglycans (Console et al., 2003; Ghibaudi et al., 2005). Proteoglycans encompass a heterogeneous group of proteins that are substituted with linear polysulfated and, thereby highly negatively charged, glycosaminoglycan poly-

saccharides (e.g., heparan sulfate) that surround almost every eukaryotic cell type (Iozzo, 2001). These surface proteoglycans bind a multitude of ligands and influence a vast array of cellular processes, including cytokine signaling and inflammation (Belting, 2003; Götte, 2003). Thus, it is quite feasible that cationic peptides binding to these polyanions can competitively inhibit the attachment of proinflammatory mediators and interfere with their signaling processes. The finding that a glycine mutant devoid of positive charges did not have any effect on NF-κB activity emphasized the importance of basic residues in the biological activity of penetratin.

Internalization studies showed that contrary to the nonpenetrating glycine analog, fluorescently labeled penetratin was taken up by cells in vitro and was rapidly (only 15 min after its intraperitoneal injection) internalized into the lung and pancreas in vivo. In CCK-induced acute pancreatitis, a model in which activation and nuclear translocation of NF-κB peaks already at 30 min after CCK administration (Gukovsky et al., 1998; Rakonczay et al., 2003), penetratin prevented IκB degradation and nuclear translocation of NF-κB. NF-κB inhibitory activity of the peptide resulted in improved histological and laboratory parameters of pancreatitis. Penetratin also decreased the expression of NF-κB-dependent proinflammatory genes and inhibited reactive oxygen species production. Thus, the cationic peptide prevented the onset of pancreatic inflammation in vivo by interfering with IκB degradation and NF-κB nuclear translocation.

During the preparation of our article, Fotin-Mleczek et al. (2005) published their results on the in vitro effects of penetratin and other cationic CPPs on TNF signaling. In their article, the authors demonstrated that penetratin and other cationic CPPs inhibited TNF-mediated signaling transduction by down-regulating TNF receptors at the cell surface. It is well known that ligation of the TNF receptor induces inflammation by triggering the degradation of IκB and subsequent transport of NF-κB into the nucleus, inducing proinflammatory genes (Baud and Karin, 2001; Hanada and Yoshimura, 2002). Thus, it is quite feasible that down-regulation of proinflammatory cell surface receptors by

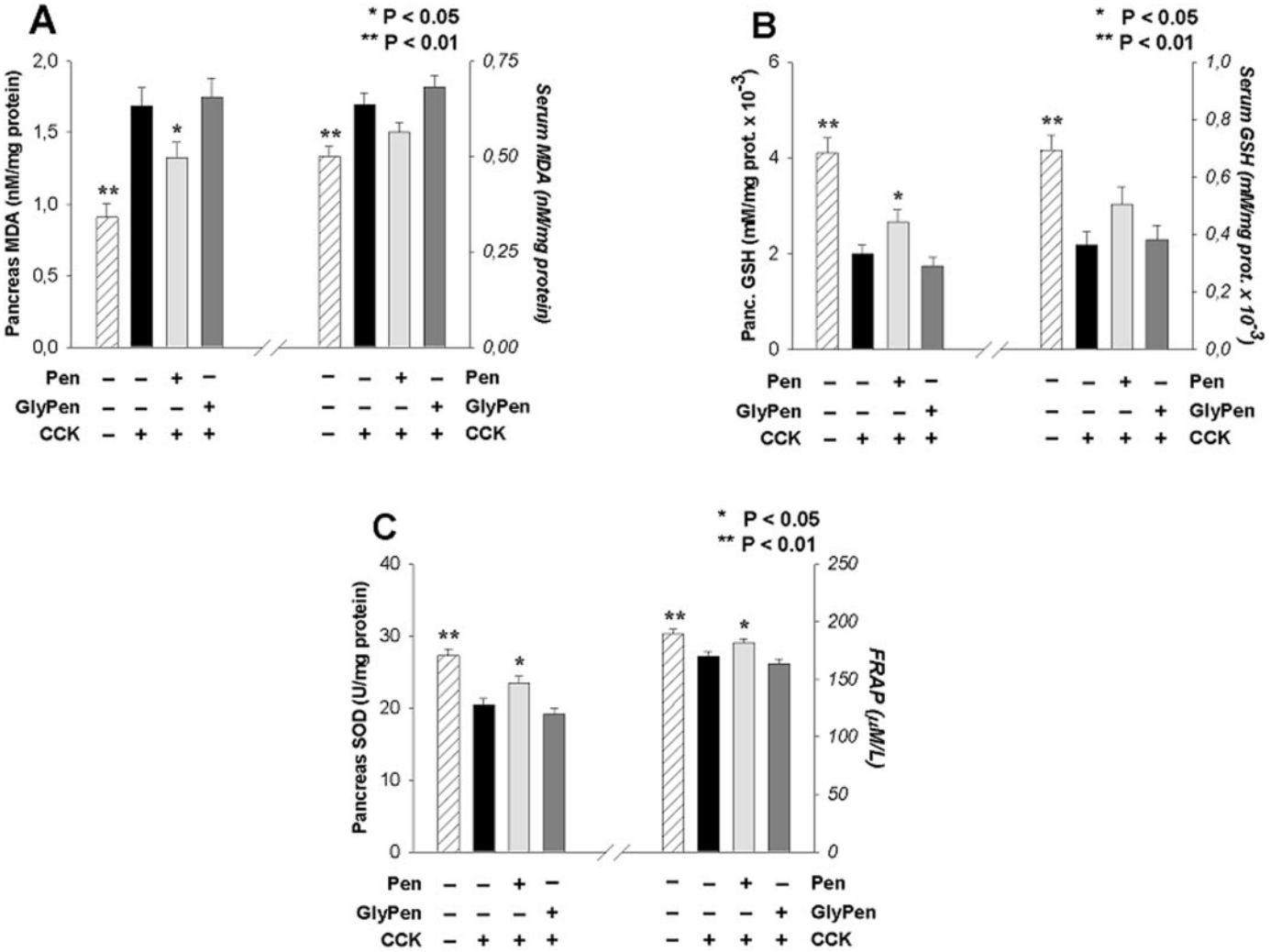

Fig. 7. Penetratin improves the parameters of oxidative stress in acute pancreatitis. A and B, the effect of penetratin pretreatment (2 mg/kg i.p. 30 min before the induction of pancreatitis) on pancreatic and serum levels of MDA (A) and GSH (B). C, pancreatic SOD activity and FRAP in CCK-induced acute pancreatitis. ▨, controls (receiving 3 × 0.5 ml of PBS i.p.); ■, group CCK (animals receiving 2 × 100 μg/kg CCK i.p.); □, group Pen+CCK (animals treated with 2 mg/kg penetratin i.p. 30 min before the first injection of CCK); ▨, group GlyPen+CCK (animals treated with 1.5 mg/kg i.p. of the mutant peptide 30 min before pancreatitis induction). Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by ANOVA. *, $p < 0.05$ versus group CCK; **, $p < 0.01$ versus group CCK.

TABLE 3

Effects of penetratin on the histological parameters in CCK-induced acute pancreatitis

Histological evaluation of acute pancreatitis was done according to the scoring system shown in Table 2. Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by ANOVA.

	Controls	Group CCK	Group Pen+CCK	Group GlyPen+CCK
Edema	0.1 ± 0.07**	0.95 ± 0.05	0.7 ± 0.08*	0.85 ± 0.07
Vascular changes	0.15 ± 0.08**	0.8 ± 0.08	0.45 ± 0.12*	0.75 ± 0.11
Inflammation	0.05 ± 0.05**	0.75 ± 0.08	0.5 ± 0.07*	0.8 ± 0.11
Acinar necrosis	0**	0.85 ± 0.08	0.6 ± 0.07*	0.8 ± 0.08
Calcification	0	0.05 ± 0.05	0	0
Fat necrosis	0	0.1 ± 0.07	0	0.1 ± 0.07

* $p < 0.05$ versus group CCK.
** $p < 0.01$ versus group CCK.

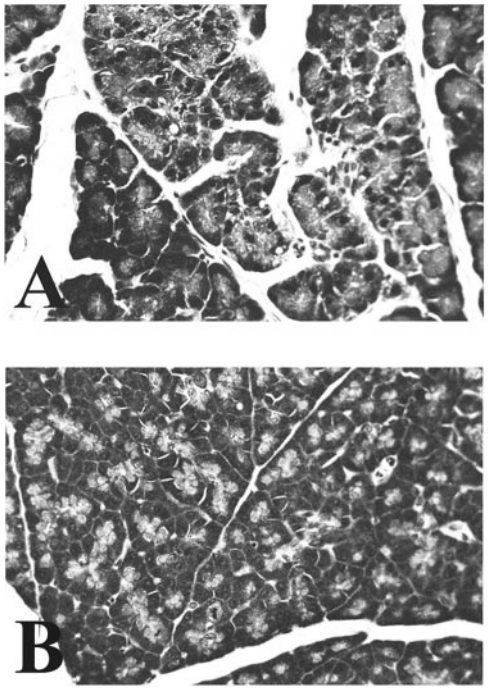

Fig. 8. Penetratin attenuates the morphological damage of pancreas in CCK-induced pancreatitis. A, a pancreatic sample from group CCK (animals receiving 2 × 100 μg/kg CCK i.p.): lobular necrosis with edema, extravasation, and neutrophil infiltration (H&E ×250). B, a pancreatic sample from group Pen+CCK (animals treated with 2 mg/kg penetratin i.p. 30 min before the first injection of CCK): mild acinar degeneration and vacuolization (H&E ×250).

penetratin might be responsible for the biological effects described in our article. On the other hand, a cationic peptide that enters cells via lipid rafts and attaches to a wide array of polyanionic cellular components can interfere with a wide spectrum of cellular signals. Experiments are under way in our laboratory to test this hypothesis. However, TNF receptor down-regulation and interference with binding and cellular signaling of other proinflammatory mediators could have an additive or synergistic effect, resulting in the NF-κB-inhibitory actions of penetratin.

In summary, our work shows that the cell-penetrating penetratin peptide can suppress the inflammatory response in vitro and in vivo by inhibiting activation and nuclear translocation of NF-κB. The very low dose of penetratin administered in this study (for the sake of comparison: in our recent study, the proteosome inhibitor MG132 peptide had to be administered at the dose of 10 mg/kg to prevent the onset of CCK-induced acute pancreatitis; Letoha et al., 2005c) shows the efficiency of the peptide in the down-regulation of NF-κB-dependent processes. Our observation that penetratin prevents the development of (pancreatic) inflammation represents an important step toward the in vivo application of penetratin–based bioactive molecules. However, future studies should be conducted to reveal the yet-undetermined biological effects of penetratin.

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