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IN VITRO AND IN VIVO NF-KB INHIBITORY EFFECTS OF THE CELL-PENETRATING PENETRATIN PEPTIDE

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Running title: Penetratin inhibits NF-κB activity

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List of Abbreviations:

nuclear localization signal (NLS); fluorescein isothiocyanate (FITC); phosphate buffered saline (PBS); intercellular adhesion molecule-1 (ICAM-1); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS); electrophoretic mobility shift assay (EMSA); enzyme-linked immunosorbent assay (ELISA); real-time quantitative polymerase chain reaction (RT-qPCR); pancreatitis-associated protein (PAP); ferric reducing ability of plasma (FRAP); reactive oxygen species (ROS).

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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 NF-κB in TNF-stimulated L929 fibroblasts and LPS-activated RAW 264.7 macrophages. Penetratin also inhibited TNF-induced ICAM-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 of 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; Derossi et al., 1996). Intracellular delivery with penetratin has opened up new possibilities to alter various cellular processes with novel hidrophilic biocompounds like oligonucleotides and (poly)peptides (Derossi et al., 1998; Dupont et al., 2002; Jarver and Langel, 2004; Dietz and Bdeltahr, 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*. injected fluorescently labeled penetratin directly into rat brain and assessed the brain response to the peptide. Immunohistochemistry following intrastriatal injection showed that injection of 10 μg penetratin caused neurotoxic cell death and triggered recruitment of inflammatory cells in a dose-dependent fashion (Bolton et al., 2000). Considering these findings one have to note 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 al., 2005a). 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. Since luciferase activity can be easily and very precisely measured by a luminometer, therefore 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 (SRTFs) 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; Chen et al., 2002; Rakonczay et al., 2003; Gukovsky et al., 2003). Severity of pancreatitis can be quantified by monitoring the characteristic parameters of pancreatic inflammation including serum amylase (that informs about the severity of pancreatic damage), pancreatic weight/body weight ratio (that informs about the extent of pancreatic edema), intrapancreatic concentrations of proinflammatory cytokines (IL-1, IL-6 and TNF-α) and 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). Due to 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 paper will summarize the results of the above-mentioned inflammatory studies that shed light on the formerly unidentified biological activity of penetratin and demonstrate the effects of the peptide on the NF-κB-dependent inflammatory responses both *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Peptide Synthesis and Labeling — Penetratin (RQIKIWFQNRRMKWKK) and its glycine (Gly) 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., 2005b). For the internalization studies, the peptides were labeled with fluorescein isothiocyanate (FITC, Sigma, distributed by Sigma-Aldrich Hungary Ltd., Budapest, Hungary) as described by Fülöp *et al.* (Fülöp et al., 2001). Cholecystokinin-octapeptide (CCK) was also prepared in our laboratory with the method of Penke *et al.* (Penke et al., 1984).

Cell lines — Murine L929 fibroblasts and RAW 264.7 macrophages were cultured in MIX MEM [1:1 mixture of DMEM (Sigma) and F-12 HAM (Sigma) plus 10 % (v/v) FCS (Sigma)] at 37 °C in a humified 5% CO_2 containing air environment. Human microvascular endothelial HMEC-1 cells [HE-SFM (GIBCO BRL, distributed by Csertex Ltd., Budapest, Hungary) supplemented 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., 2005a).

Luciferase assay — One-day-old cultures of L929 and RAW 264.7 cells, grown on luminoplates (Corning-Costar, distributed by Zenon Biotechnology Ltd**.**, Szeged, Hungary) were used. 3 x 10^4 cells/well (in MIX MEM 10 % FCS) were exposed to various concentrations (1.56 to 25 μM) of penetratin or the Gly 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, distributed by Bio-Science Hungary, Budapest). Substrate was added (20 μl/well; Promega) and luciferase activity was measured in a Luminoskan Ascent (Thermo Labsystems, USA) scanning luminometer. Control cells received only TNF-α (10 U/ml) or LPS (30 ng/ml) treatment and processed as mentioned above. In some of the assays the cells were co-incubated with penetratin and 25 μg/ml of 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 assays were always carried out on viable cells.

ICAM-1 expression — HMEC-1 cells, grown on microplates (Corning-Costar) were used. 3 x 10^4 cells/well in HE-SFM 2 % FCS were exposed to various concentrations (6.25 to 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) 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 (Becton Dickinson/Pharmingen, USA). Control cells received only TNF treatment. To seek the importance of attachment to polyanionic cell surface proteoglycans, some of the cells were co-incubated with penetratin and 25 μg/ml of heparan sulfate (HS) for 30 min before TNF-stimulation. Viability of the cells was determined by concurrent

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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 Gly mutant analog was assessed by using MTS assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega) according to the manufacturer's protocol. Briefly, 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 Labsystem Multiscan MS ELISA reader.

Internalization studies — Uptake of penetratin peptides (at a concentration of 5 μM) into HMEC-1, L929 and RAW cells was investigated as described previously (Letoha et al., 2005b). 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 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 (ip) with 20 nM/kg of fluorescent penetratin (dissolved in 0.5 ml PBS) and other six ones with the same dose (20 nM/kg) of the Gly mutant analog (also in 0.5 ml PBS). Control animals (n=6) received ip injections of 0.5 ml PBS. Rats were anesthetized (with pentobarbital sodium 50 mg/kg ip) and killed 15 min after the injections by exsanguinations through the abdominal aorta. Lung and pancreas

tissues were harvested and frozen in Histo Prep media (Fisher Scientific GmbH, Schwerte, Germany). Sections (10 to 50 μm) were cut on a cryostat and analyzed by fluorescence confocal microscopy.

CCK-induced pancreatitis — In each experimental group 10 rats were used. The rats were fasted for 16 h then acute pancreatitis was induced by injecting 100 μg/kg body weight of CCK (dissolved in PBS) ip twice at an interval of 1 h ("Group CCK"). Penetratin pretreated group ("Group Pen+CCK") received 2 mg/kg body weight of penetratin (in 0.5 ml PBS) ip 30 min before the first injection of CCK. Another group of animals was treated with equimolar dose (1.5 mg/kg) of the Gly mutant analog (in 0.5 ml PBS) 30 min before the induction of pancreatitis ("Group GlyPen+CCK"). Control rats received 3 ip injections of 0.5 ml PBS instead of the peptides. Anesthetized (pentobarbital sodium 50 mg/kg ip) rats were killed by exsanguinations through the abdominal aorta 4 h after the first CCK injection. Lung and pancreas were quickly removed, 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 (EMSA) of NF-κB — The 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 utilized to evaluate the degree of pancreatic

edema. To measure the serum amylase activities, all blood samples were centrifuged at 2,500 x 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 manufacturers' 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*. (Kuebler et al., 1996).

Real time quantitative polymerase chain reaction (RT-qPCR) — RT-qPCR was performed on a RotorGene 3000 instrument (Corbett Research, Australia) with gene-specific primers (designed with the software PrimerExpress, Applied Biosystems, USA) 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 PCR primers used are shown in Table I.

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 (FRAP) — The total antioxidant activity of the plasma was determined with the method of Benzie and Strain (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. FRAP values are obtained by preparing 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 (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*. (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 details in Table II).

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 at 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 (Figs. 1A and B). Meanwhile the mutated analog (GlyPen) could not suppress the NF-κB response.

TNF-α induces ICAM-1 expression in endothelial cells through 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, while the mutant peptide (GlyPen) did not have any effects 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 (Figs. 1A-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 were cytotoxic even at 50 μM (Figs. 2A and B).

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), while the Gly analog was not taken up by any of the cells (Figs. 3A and B). Since our main goal was to examine the effects of the peptides on pancreatic inflammation, therefore 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

fifteen minutes after its ip injection FITC-labeled penetratin entered the cells of these organs (Figs. 3C 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 x 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 (Figs. 4A and C). The nuclear import of NF-κB was also decreased in penetratin-treated animals as revealed by electrophoretic mobility shift assays (EMSAs) (Figs. 4B 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 cold competition experiments, when incubation with increasing doses of the cold 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 the characteristic features of pancreatic inflammation. Thus CCK hyperstimulation resulted in intrapancreatic edema and cellular damage as reflected by increased pancreatic weight/body weight ratio and serum amylase activity (Fig. 5A). Supramaximal doses of CCK increased neutrophil sequestration and thus myeloperoxidase (MPO) activities both within the pancreas and lung (Fig. 5B). Intrapancreatic concentrations

of proinflammatory cytokines TNF-α and IL-6 were significantly elevated compared to controls (Fig. 5C). Furthermore CCK hyperstimulation induced the expression of IL-1β, IL-6, TNF-α and pancreatitis-associated protein (PAP) mRNAs (Figs. 6A and B). Laboratory parameters of oxidative stress revealed increased ROS production due to 2 x 100 μg/kg CCK. Serum and intrapancreatic concentrations of reduced glutathione (GSH) were depleted, while those of malondialdehyde (MDA; the measure of lipid peroxidation) were increased (Figs. 7A and B). The ferric reducing ability of the plasma (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 Gly 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.

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 III.

DISCUSSION

Penetratin is a cell-penetrating peptide that has been frequently used 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., 2005b). Despite of the frequent application of penetratin as a vector of various bioactive compounds, up till 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 recruitment of inflammatory cells and neurotoxicity in a dose-dependent fashion (Bolton et al., 2000). In our recent paper 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., 2005a). 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. As exogenous heparan sulfate abolished the NF-κB suppressing activity of the peptide, we showed that attachment to surface proteoglycans had 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 polysaccharides (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 interferes with their signaling processes. The finding that a Gly 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 non-penetrating Gly analog, fluorescently labeled penetratin was taken up by cells *in vitro* and was rapidly (only fifteen minutes after its ip 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 ROS 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 paper Fotin-Mleczek *et al*. has published their results on the *in vitro* effects of penetratin and other cationic CPPs on TNF-signaling (Fotin-Mleczek et al., 2005). In their paper the authors demonstrated that penetratin and other cationic CPPs inhibited TNF-mediated signaling trasduction by downregulating 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 pro-inflammatory genes (Baud and Karin, 2001; Hanada and Yoshimura, 2002). Thus it is quite feasible that downregulation of proinflammatory cell surface receptors by penetratin might be responsible for the biological effects described in our paper. 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 downregulation 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 (just 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 downregulation of NF-κB-dependent processes. Our observation that penetratin prevents the development of (pancreatic) inflammation represents an important step towards the *in vivo* application of penetratin–based bioactive

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molecules. However future studies should be conducted to reveal the yet undetermined biological effects of penetratin.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Penetratin inhibits NF-κB transcriptional activity *in vitro*. *A-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 of TNF-α (*A*) or 30 ng/ml LPS (*B*). Peptide-treated cells were incubated with various concentrations of penetratin (Pen) with or without heparan sulfate (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 peptid-treated cells compared 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 to controls (treated with TNF only). Means ± S.E. of three independent experiments are shown. Statistical significance was assessed by analysis of variance (ANOVA). $*p < 0.05$ *vs* controls; $**p < 0.01$ *vs* controls .

Figure 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 following a standard MTS assay procedure. Results of three independent assays were normalized and plotted as percent of viable cells. Data points represent mean ± S.E.

Figure 3. Cellular internalization of penetratin peptides. *A-B: In vitro* experiments show that fluorescently labeled penetratin (*A*) is internalized by cells (RAW

macrophages), while the Gly analog (*B*) is not. *C-D*: In the *in vivo* uptake studies male Wistar rats (weighing 250-280 g) were injected ip with 20 nM/kg of FITC-labeled penetratin and its mutant. *C* shows pancreas, *D* shows lung tissue sections isolated from rats 15 min after the injection of fluorescent penetratin.

Figure 4. Penetratin prevents IκB degradation and subsequent NF-κB activation in acute pancreatitis. *A:* The level IκB-α in the pancreas analyzed by Western blot. β-Actin was used as loading control. Data for each group are representative of 4 independent experiments. Nuclear protein from these same tissue samples was subjected to NF-κB EMSA. *B*: The figure shows 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. The striped white bar represents controls (receiving 3 x 0.5 ml of PBS ip), black bar represents Group CCK (animals receiving 2 x 100 μg/kg of CCK ip), light gray bar represents Group Pen+CCK (animals treated with 2 mg/kg of penetratin ip 30 min before the first CCK injection) and dark gray bar represents Group GlyPen+CCK (animals treated with 1.5 mg/kg of the mutant peptide ip 30 min before pancreatitis induction). Values presented are means ± S.E., n=6-8 animals/group. Statistical significance was assessed by analysis of variance (ANOVA). *****$p < 0.05$ *vs* Group CCK; ******$p < 0.01$ *vs* Group CCK.

Figure 5. Penetratin ameliorates the parameters of acute pancreatitis *in vivo*. Acute pancreatitis was induced by injecting of 100 μg/kg body weight of CCK ip twice at an interval of 1 h into male Wistar rats. *A* shows the effect of penetratin (2 mg/kg ip 30 min before the first CCK injection) on pancreatic weight/body weight

ratio and serum amylase activity, *B* shows pancreatic and lung myeloperoxidase (MPO) activity and *C* shows TNF-α and IL-6 levels in CCK-induced acute pancreatitis. Striped white bars represent controls (receiving 3 x 0.5 ml of PBS ip), black bars represent Group CCK (animals receiving 2 x 100 μg/kg of CCK ip), light gray bars represent Group Pen+CCK (animals treated with 2 mg/kg of penetratin ip 30 min before the first injection of CCK) and dark gray bar represents Group GlyPen+CCK (animals treated with 1.5 mg/kg of the mutant peptide ip 30 min before pancreatitis induction). Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by analysis of variance (ANOVA). *****$p < 0.05$ *vs* Group CCK; ******$p < 0.01$ *vs* Group CCK.

Figure 6. Effects of penetratin on the mRNA expression of proinflammatory genes in acute pancreatitis. The figures show 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. *A* shows the expression of interleukin (IL)-1β and interleukin (IL)-6; *B* shows the expression of tumor necrosis factor (TNF)-α and pancreatitis-associated protein (PAP) in the pancreatic tissue. Bars represent relative expression ratios normalized to those of the housekeeping gene (cyclophilin) in the same samples. Black bars represent Group CCK (animals receiving 2 x 100 μg/kg of CCK ip), light gray bars represent Group Pen+CCK (animals treated with 2 mg/kg of penetratin ip 30 min before the first injection of CCK) and dark gray bar represents Group GlyPen+CCK (animals treated with 1.5 mg/kg of the mutant peptide ip 30 min before pancreatitis induction). Values are means ± SE for 3-6 animals/group and are given relative to the control group.

Statistical significance was assessed by analysis of variance (ANOVA). ***p* < 0.05 *vs* Group CCK; ****p* < 0.01 *vs* Group CCK.

Figure 7. Penetratin improves the parameters of oxidative stress in acute pancreatitis. *A* and *B* shows the effect penetratin pretreatment (2 mg/kg ip 30 min before the induction of pancreatitis) on pancreatic and serum levels of MDA (*A*) and GSH (*B*), *C* shows pancreatic SOD activity and ferric reducing ability of plasma (FRAP) in CCK-induced acute pancreatitis. Striped white bars represent controls (receiving 3 x 0.5 ml of PBS ip), black bars represent Group CCK (animals receiving 2 x 100 μg/kg of CCK ip), light gray bars represent Group Pen+CCK (animals treated with 2 mg/kg of penetratin ip 30 min before the first injection of CCK) and dark gray bar represents Group GlyPen+CCK (animals treated with 1.5 mg/kg of the mutant peptide ip 30 min before pancreatitis induction). Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by analysis of variance (ANOVA). ***p* < 0.05 *vs* Group CCK; ****p* < 0.01 *vs* Group CCK.

Figure 8. Penetratin attenuates the morphological damage of pancreas in CCK-induced pancreatitis. *A* shows a pancreatic sample from Group CCK (animals receiving 2 x 100 μg/kg of CCK ip): lobular necrosis with edema, extravasation and neutrophil infiltration (HE x 250). *B* shows a pancreatic sample from Group Pen+CCK (animals treated with 2 mg/kg of penetratin ip 30 min before the first injection of CCK): mild acinar degeneration and vacuolization (HE x 250).

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Table I

Sequences of gene-specific primers used for RT-qPCR

Gene name	**Forward primer**	**Reverse primer**
Cyclophilin	TCTCTTCAAGGGACAAGGCTG	TGGCAAATCGGCTGACG
IL-1 ß	CTTCCCCAGGACATGCTAGG	CAAAGGCTTCCCCTGGAGAC
IL-6	TGTCTCGAGCCCACCAGG	TGCGGAGAGAAACTTCATAGCTG
TNF-α	TCTCTTCAAGGGACAAGGCTG	TGGCAAATCGGCTGACG
PAP	CCTCTGCACGCATTAGTTGC	TGAAACAGGGCATAGCAGTAGG

The primers were designed with the software PrimerExpress, Applied Biosystems, USA.

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Table II

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 Changes	absent	congestion	focal haemorrhage	diffuse haemorrhage	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. 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.* (Hughes et al., 1996)

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Table III

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

	Controls	*Group CCK*	*Group Pen+CCK*	*Group GlyPen+CCK*
Edema	$0.1 \pm 0.07^{**}$	0.95 ± 0.05	$0.7 \pm 0.08^{*}$	0.85 ± 0.07
Vascular Changes	$0.15 \pm 0.08^{**}$	0.8 ± 0.08	$0.45 \pm 0.12^{*}$	0.75 ± 0.11
Inflammation	$0.05 \pm 0.05^{**}$	0.75 ± 0.08	$0.5 \pm 0.07^{*}$	0.8 ± 0.11
Acinar necrosis	0^{**}	0.85 ± 0.08	$0.6 \pm 0.07^{*}$	0.8 ± 0.08
Calcification	0	0.05 ± 0.05	0	0
Fat necrosis	0	0.1 ± 0.07	0	0.1 ± 0.07

Histological evaluation of acute pancreatitis was done according to the scoring system shown in Table II. Means ± S.E. of 10 animals in each group are shown. Statistical significance was assessed by analysis of variance (ANOVA). *$p < 0.05$ *vs* Group CCK; **$p < 0.01$ *vs* Group CCK.

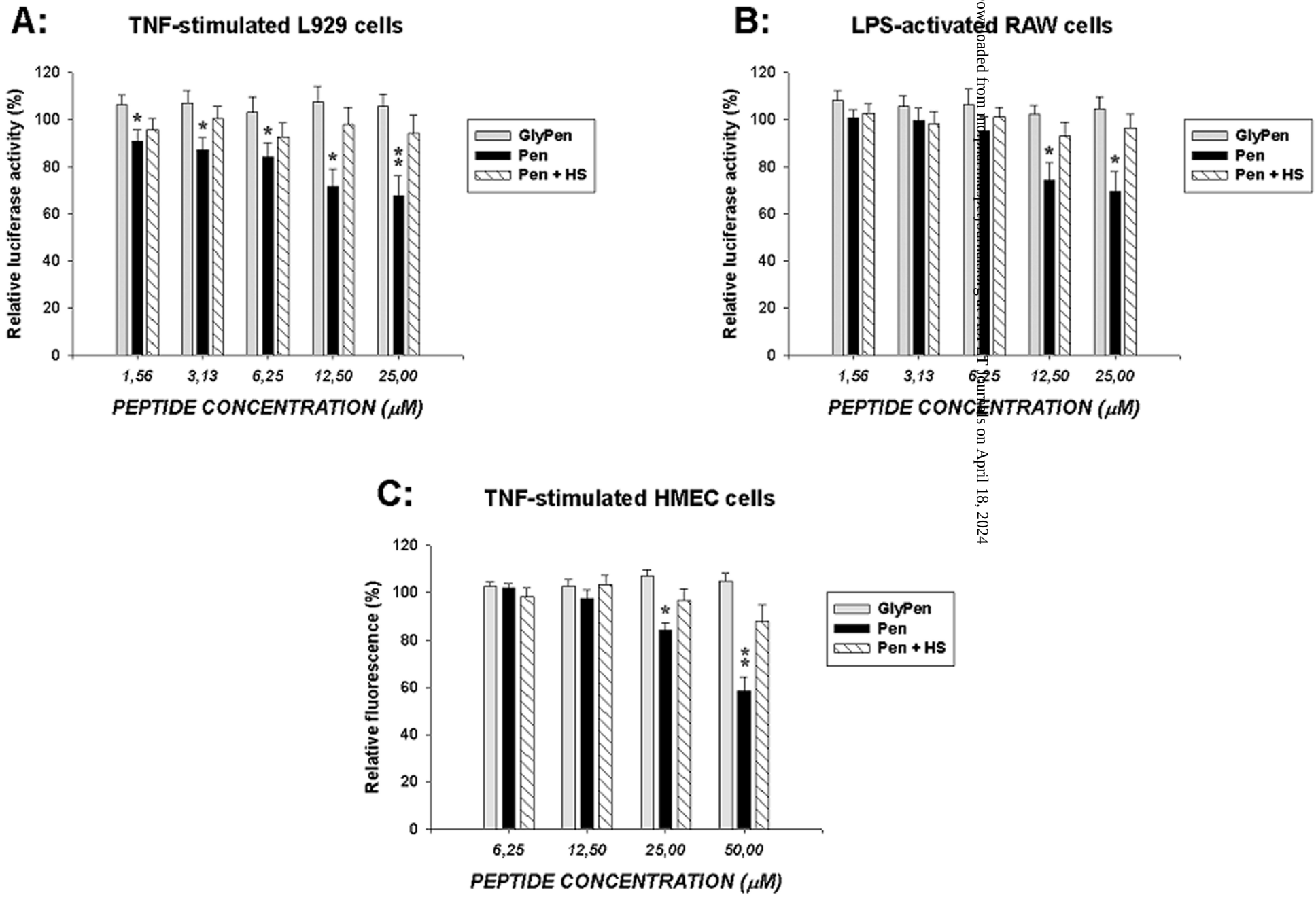

FIGURE 1

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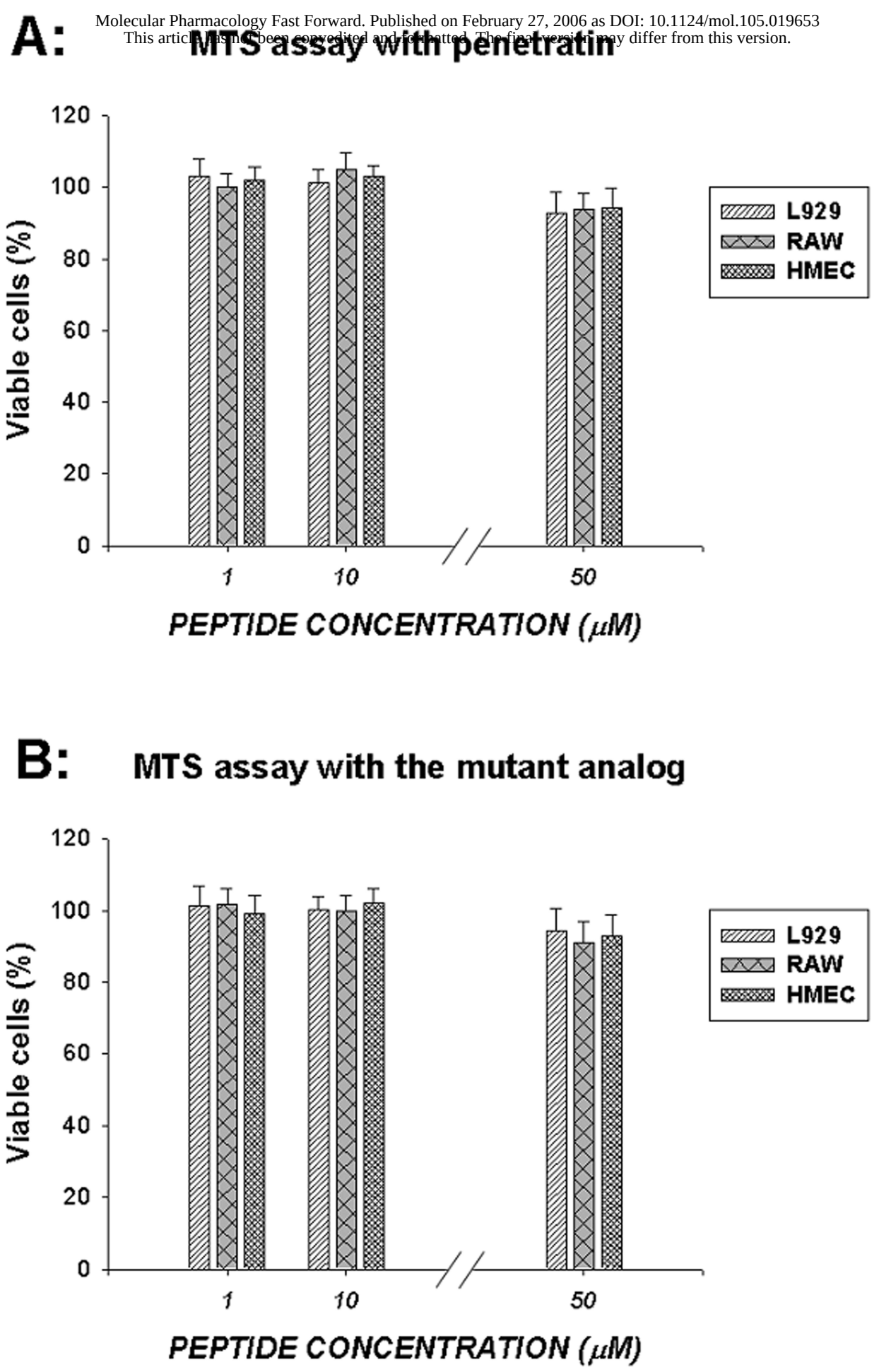

FIGURE 2

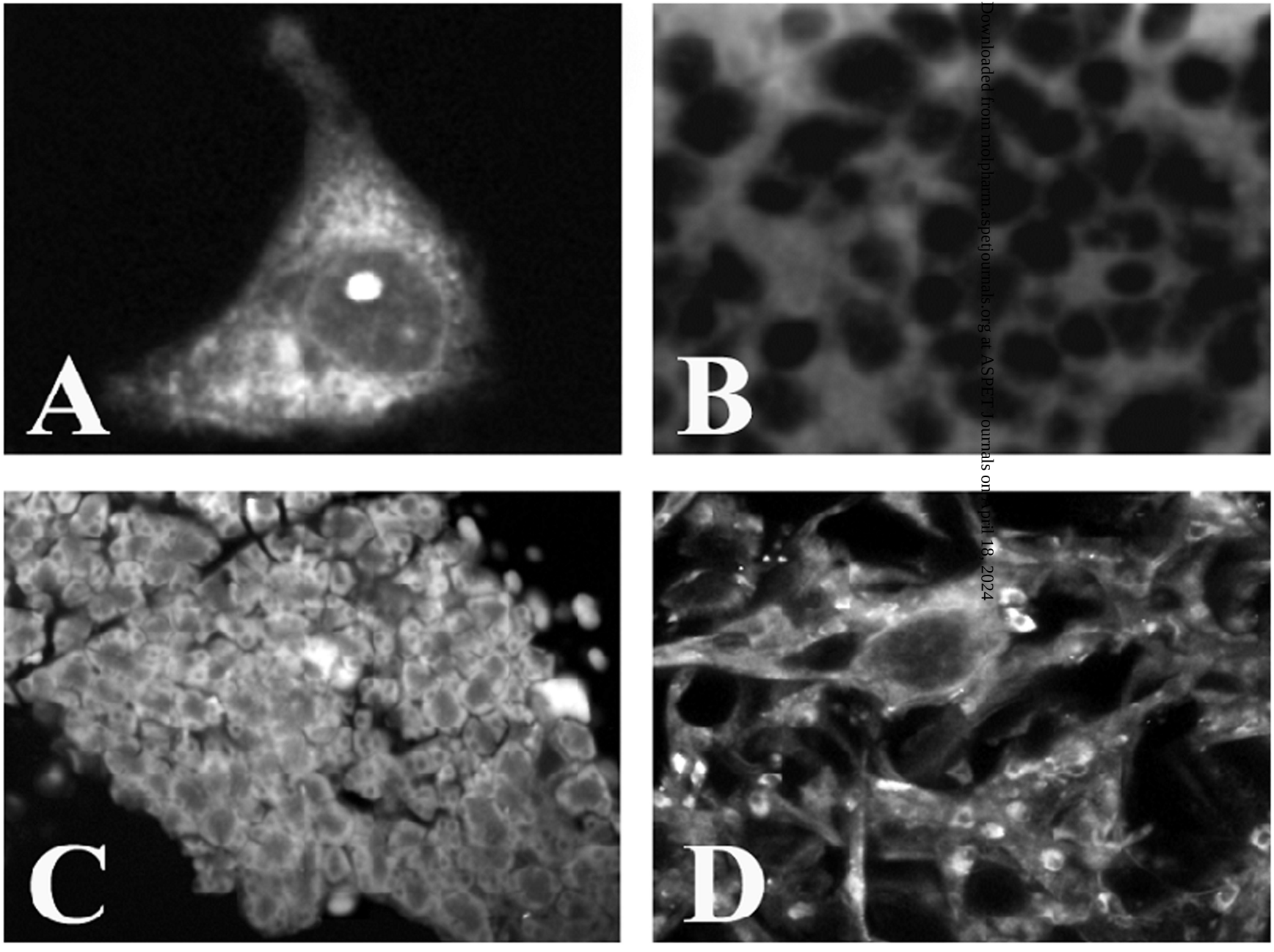

FIGURE 3

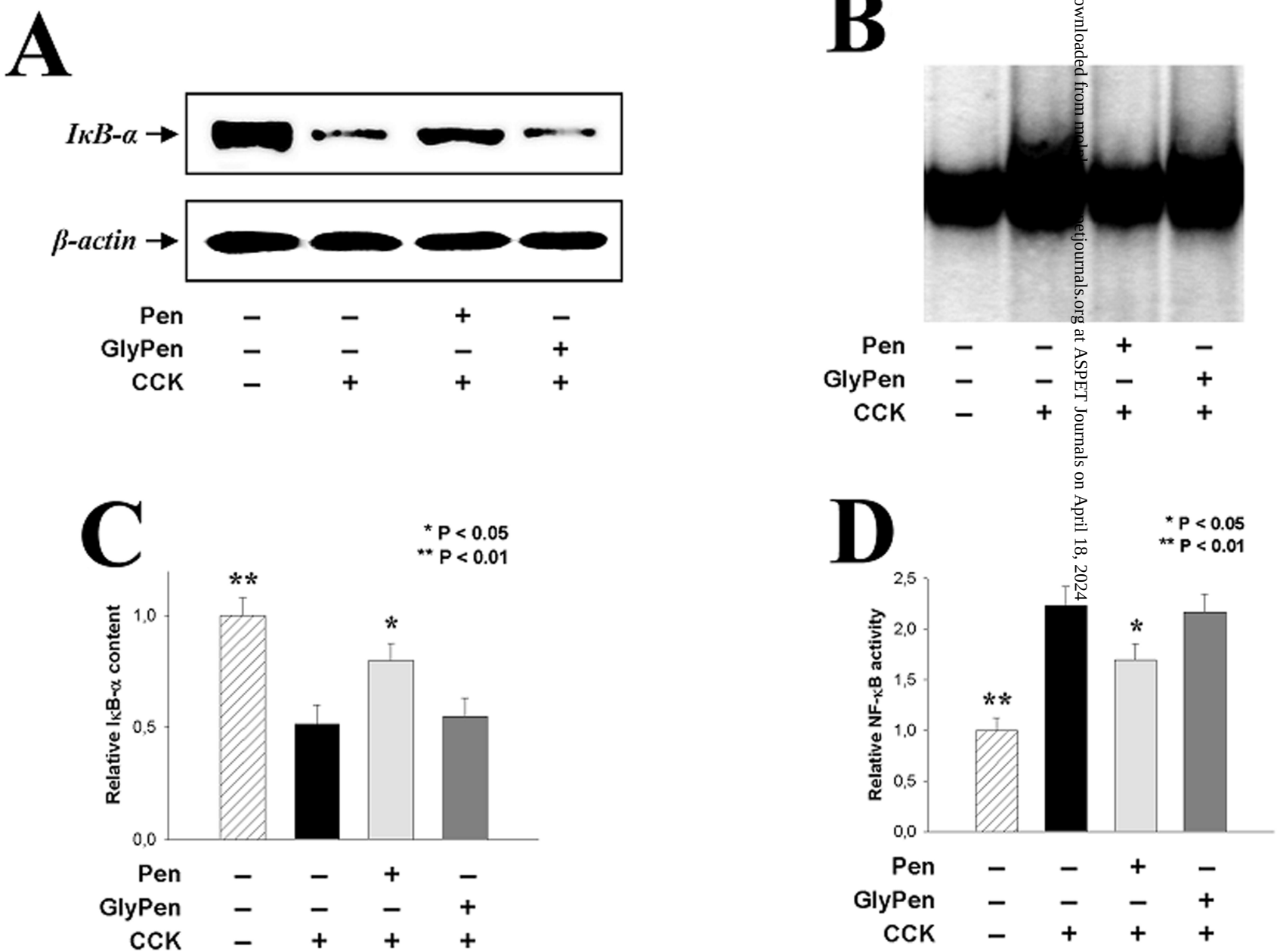

FIGURE 4

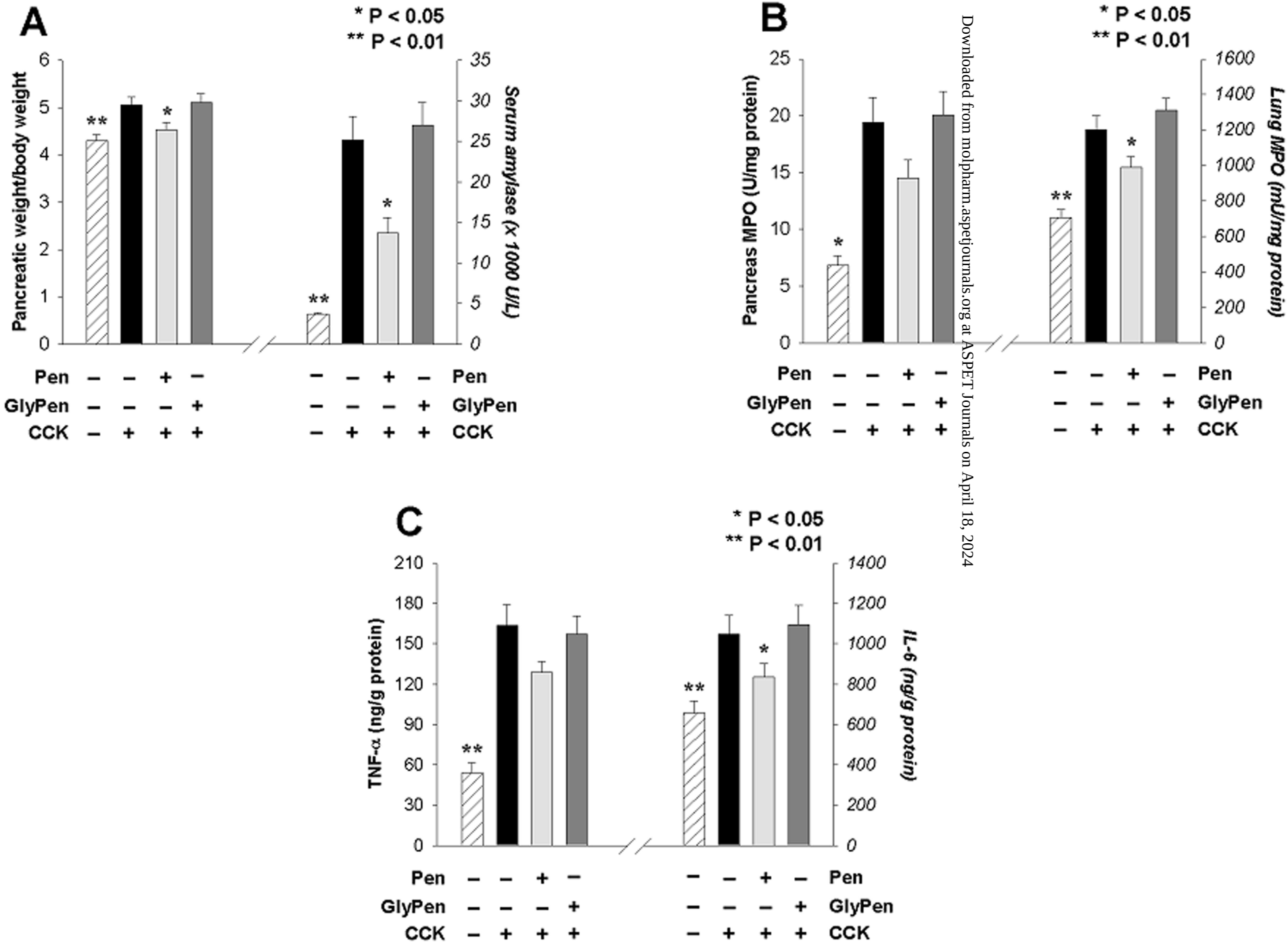

FIGURE 5

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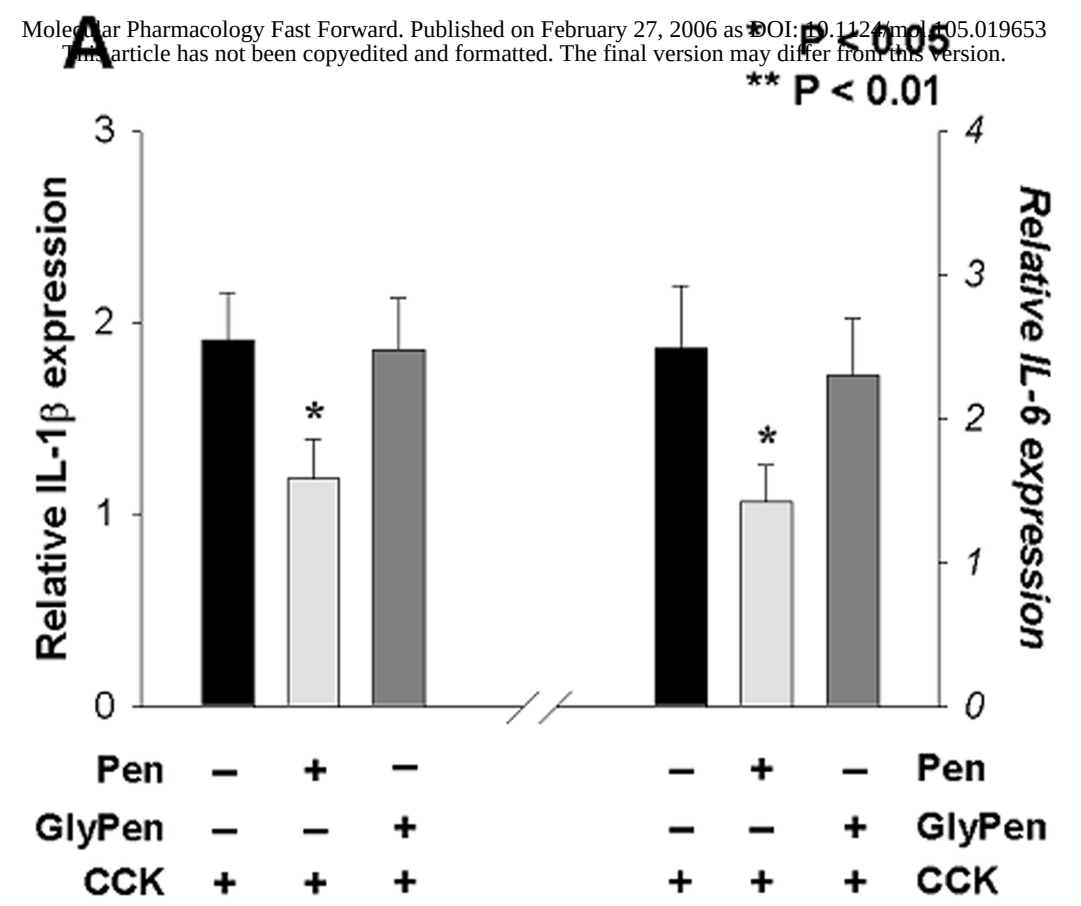

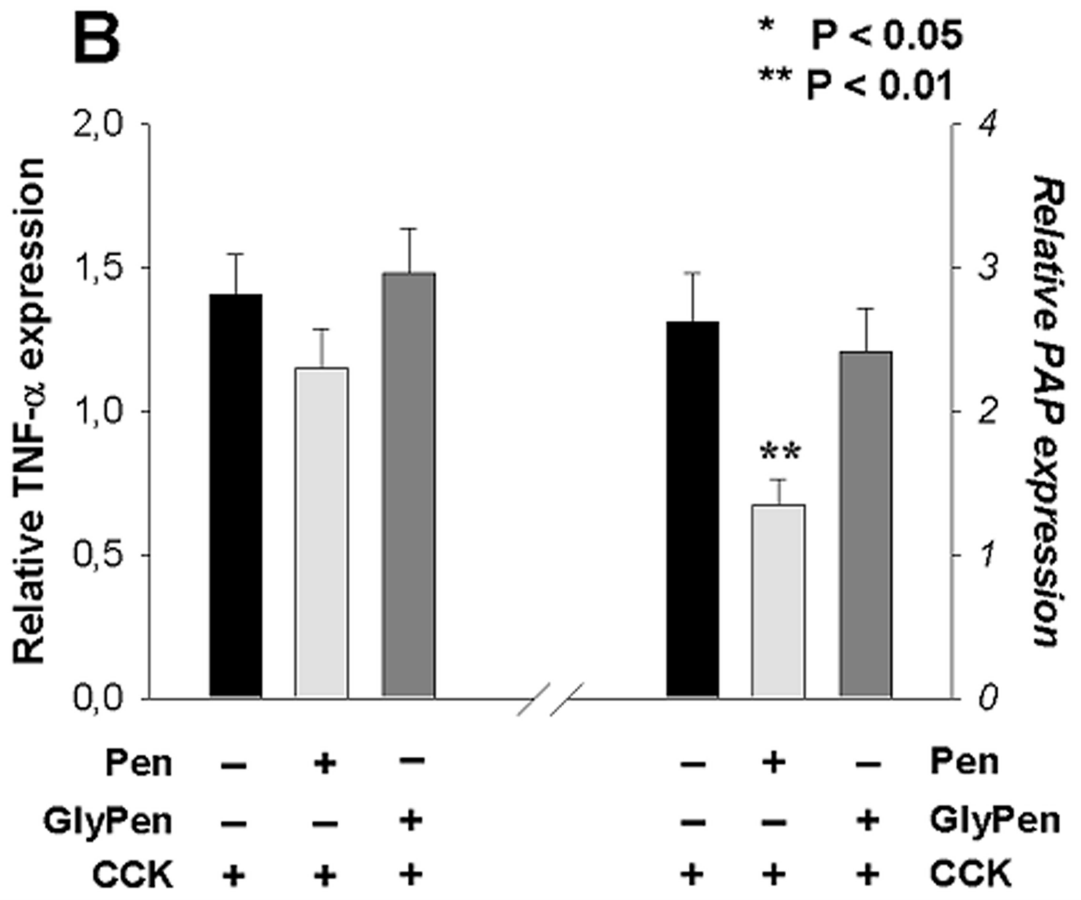

FIGURE 6

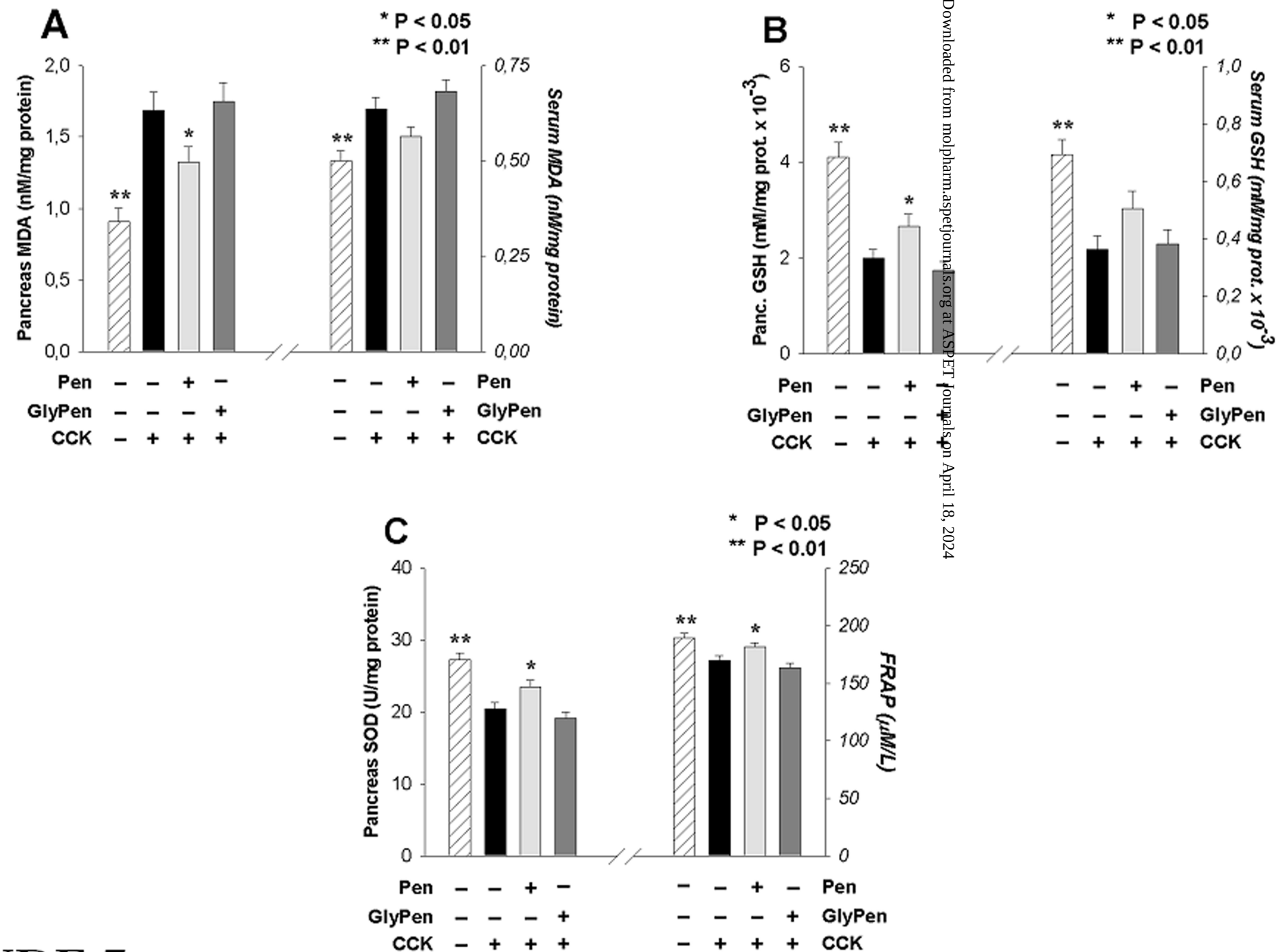

FIGURE 7

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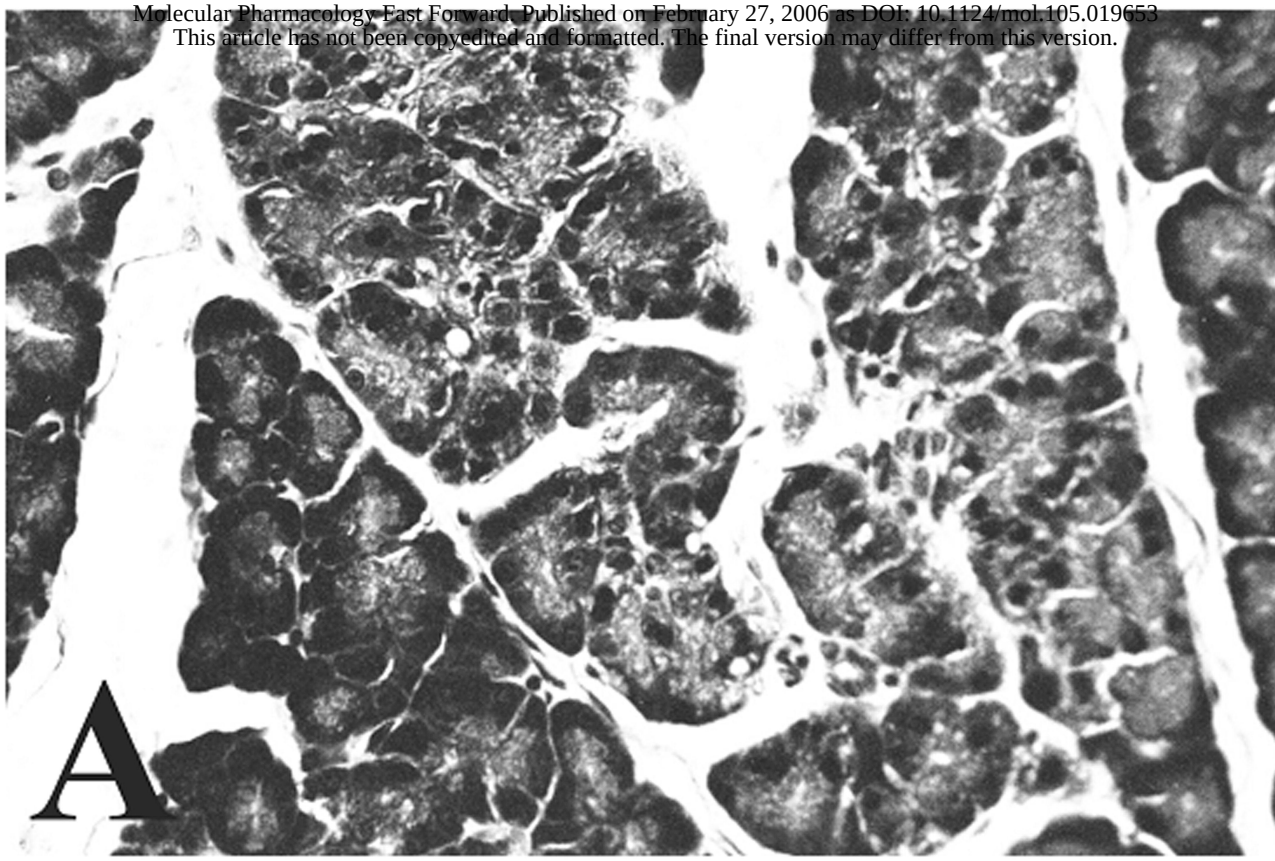

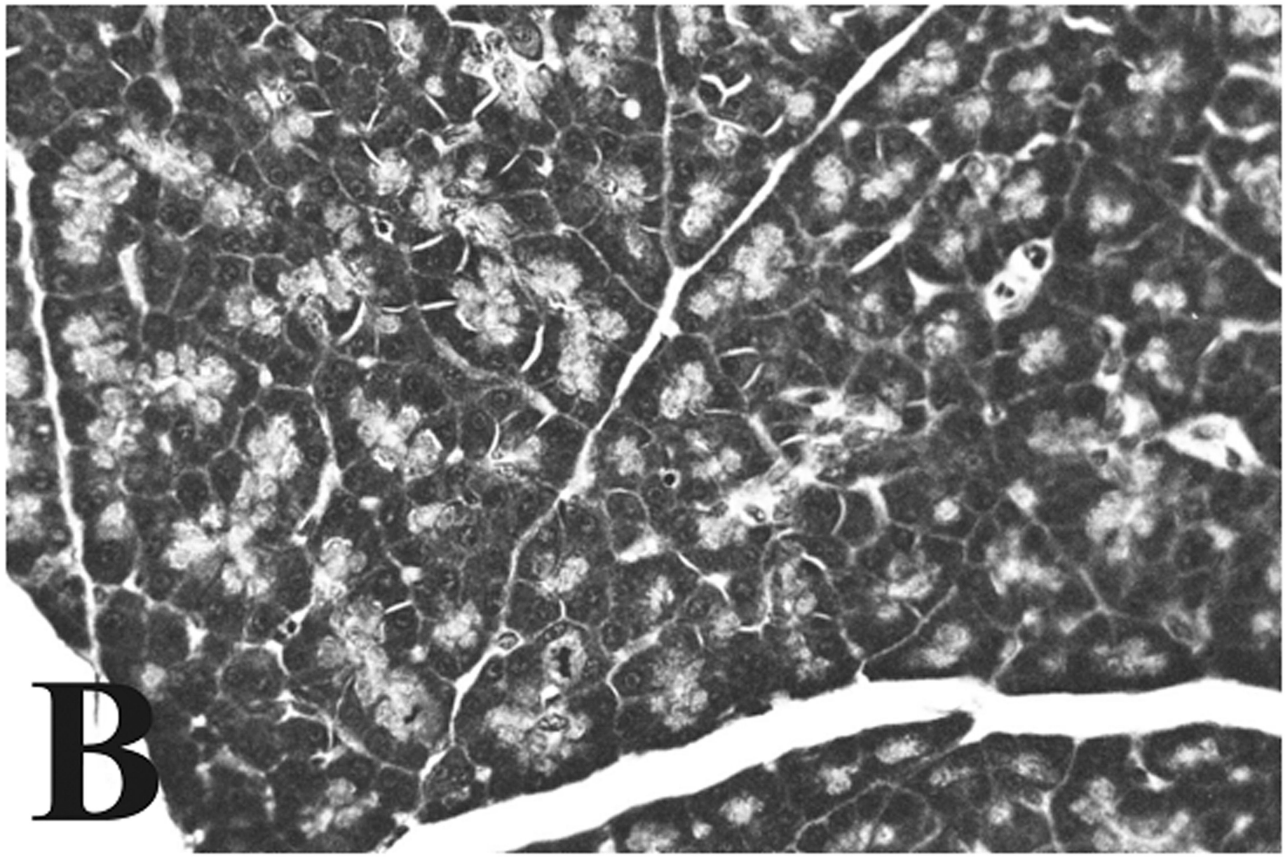

FIGURE 8