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Lipid Peroxidation Inhibition Reduces NF-κB Activation and Attenuates Cerulein-induced Pancreatitis

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Increased lipid peroxidation, enhanced nuclear factor kappa-B (NF-κB) activation and augmented tumor necrosis factor-α (TNF-α) production have been implicated in cerulein-induced pancreatitis. We investigated whether lipid peroxidation inhibition might reduce NF-κB activation and the inflammatory response in cerulein-induced pancreatitis.

Male Sprague–Dawley rats of 230–250 g body weight received administration of cerulein (80 μg/kg s.c. for each of four injections at hourly intervals). A control group received four s.c. injections of 0.9% saline at hourly intervals. Animals were randomized to receive either raxofelast, an inhibitor of lipid peroxidation (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of a 10% DMSO/NaCl solution). All these rats were sacrificed 2 h after the last injection of either cerulein or its vehicle.

Raxofelast administration (20 mg/kg i.p. with the first cerulein) significantly reduced malondialdehyde (MDA) levels, an index of lipid peroxidation (CER + DMSO = 3.075 ± 0.54 μmol/g; CER + raxofelast = 0.693 ± 0.18 μmol/g; $p < 0.001$), decreased myeloperoxidase (MPO) activity (CER + DMSO = 22.2 ± 3.54 mU/g; CER + raxofelast = 9.07 ± 2.05 mU/g; $p < 0.01$), increased glutathione levels (GSH) (CER + DMSO = 5.21 ± 1.79 μmol/g; CER + raxofelast = 15.71 ± 2.14 μmol/g; $p < 0.001$), and reduced acinar cell damage evaluated by means of histology and serum levels of both amylase (CER + DMSO = 4063 ± 707.9 U/l; CER + raxofelast = 1198 ± 214.4 U/l; $p < 0.001$), and lipase (CER + DMSO = 1654 ± 330 U/l; CER + raxofelast = 386 ± 118.2 U/l; $p < 0.001$), Furthermore, raxofelast reduced pancreatic NF-κB activation and the TNF-α mRNA levels and tissue content of mature protein in the pancreas.

Indeed, lipid peroxidation inhibition might be considered a potential therapeutic approach to prevent the severe damage in acute pancreatitis.

Keywords: Acute pancreatitis; Lipid peroxidation; Raxofelast; NF-κB; TNF-α

INTRODUCTION

Acute pancreatitis is a disease of variable severity in which patients experience mild or severe attacks: it initially leads to interstitial edema and migration of neutrophils and macrophages into the pancreatic parenchyma, proceeds thereafter to acinar cell damage and finally culminates in hemorrhagic necrotizing pancreatitis and multiple organ failure.[1]

A large body of evidence suggests that up-regulation of inflammatory mediators, including cytokines, chemokines, adhesion molecules and inducible nitric oxide synthase plays a pivotal role in this pathological process.[2–4] The cellular mechanism orchestrating these inflammatory

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mediators involves transcription factors such as nuclear factor kappa-B (NF-κB). NF-κB is an early transcription response complex essential for gene expression of inflammatory molecules.[5–7] NF-κB exists as a complex of homo or heterodimers composed of members of the Rel family of proteins.[5,6] In most resting cells, NF-κB is sequestered within the cytoplasm in an inactive form, complexed with the inhibitory protein I-κBα. After activation, NF-κB complexes translocates into the nucleus and activate transcription from target genes. Activation of NF-κB has been demonstrated in rat cerulein pancreatitis.[8,9] Finally, inhibition of NF-κB activation caused a significant organ protection and a marked decrease in the expression of several inflammatory cytokines.[9]

The role of oxidative stress in the pathogenesis of acute pancreatitis and the potential beneficial effects of antioxidant agents have also been deeply investigated.[10,11] Intracellular levels of glutathione (GSH) are severely reduced, whereas, lipid peroxidation markedly augments in pancreatic tissue during the development of acute pancreatitis.[12] The generation of oxygen radicals with the consequent triggering of lipid peroxidation and depletion of GSH, therefore, plays a key role in the initiation of acute pancreatitis.

Besides causing a direct damage, oxidative stress might also be involved in the triggering of the inflammatory cascade during acute pancreatitis. As a matter of fact it has been shown that the oxidative state of the cell influences the induction of NF-κB.[13] Reactive oxygen intermediates probably induce IκB phosphorylation by influencing the activity of tyrosine kinases.[14] Furthermore, vitamin E-like antioxidants, which block the lipid peroxidation process, can work as strong inhibitors of NF-κB activation.[15,16] However, there is a lack of information whether lipid peroxidation in acute pancreatitis is cause or consequence of NF-κB activation. In order to investigate this aspect, we studied whether raxofelast, a powerful and effective inhibitor of lipid peroxidation[17] might reduce NF-κB activation and the inflammatory response in cerulein-induced pancreatitis.

MATERIALS AND METHODS

Animals and Treatment

Male Sprague–Dawley rats (230–250 g body weight) were used. Animals were fed *ad libitum* on a standard diet and had free access to tap water. They were maintained on a 12-h light/dark cycle at 21°C. Housing conditions and experimental procedures were in strict accordance with the European Community regulations on the use and care of animals for scientific purposes. Acute pancreatitis was induced by administration of cerulein (80 μg/kg s.c. for each of four injections at hourly intervals). A control group received four s.c. injections of 0.9% saline at hourly intervals. Animals were randomized to receive either raxofelast, an inhibitor of lipid peroxidation (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of a 10% DMSO/NaCl solution). All these rats were sacrificed 2 h after the last injection of either cerulein or its vehicle: the blood was drawn for measuring serum amylase and lipase activity and the pancreas was removed for the biochemical and histological evaluations.

Lipid Peroxidation Determination

Determination of pancreas malondialdehyde (MAL) was carried out in order to estimate the extent of lipid peroxidation in the damaged tissue. Samples of pancreas were frozen at −70°C until the assay. On the day of analysis, after the thaw, tissue samples were washed in ice-cold 20 mM Tris–HCl, pH 7.4, blotted on absorbant paper and weighed. Each sample was then minced in ice-cold 20 mM Tris–HCl, pH 7.4 containing butylated hydroxytoluene (BMT) 1 mg/ml and homogenized in a ratio 1:10 (w/v) by using an ultra-turrax homogenizer. After centrifugation at 3000*g* for 10 min at 4°C, the clear homogenate supernatant was used for biochemical assay. The assay was carried out by using a colorimetric commercial kit (Lipid peroxidation assay kit, cat. No. 437634, Calibiochem-Novabiochem Corporation, USA).

We used for our experiments the MAL-586 method; this method is specific to assay free MAL, or, after a hydrolysis step, total MAL. The assay serve to minimize interference from other lipid peroxidation products, such as 4-hydroxy-alkenals.

Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenylindole in acetonitrile were added to 0.2 ml of homogenate supernatant. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well and closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at 586 nm.

A calibration curve of an accurately-prepared standard MAL solution was also run for quantification. MAL standard was obtained by acid hydrolysis of 1,1,3,3 tetramethoxypropane. The MAL concentration was expressed as μmol/mg protein.

Pancreas GSH and Tumor Necrosis Factor-α (TNF-α) Determination and Plasma Amylase and Lipase Activity

GSH activity was evaluated to estimate endogenous defenses against oxidative stress. GSH levels in pancreas were determined as previously described.[18] Samples were frozen at −70°C until the assay. Briefly, tissue samples were homogenized with an ultra-turrax homogenizer in a solution containing 5% trichloroacetic acid and 5 mM ethylenediaminotetracetic acid at 4°C. Then each sample was centrifuged at 15,000*g* for 10 min at 4°C. A 0.4 ml of homogenate supernatant were added in a polyethylene dark tubes containing 1.6 ml Tris–EDTA buffer 0.4 M pH 8.9. After vortexing, 40 μl of 10 mM dithiobisnitrobenzoic acid 10 were added. The samples were vortexed again and the absorbance was read after 5 min at 412 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The amount of pancreatic GSH was expressed as μmol/g protein.

TNF-α levels in pancreas were measured by means of a commercially available immunoElisa specific for rat TNF-α (EuroClone, Milan, Italy).

Plasma amylase and lipase activity were determined using commercially available kits (Sigma Chemical, St Louis, MO). The values of plasma amylase and lipase activity were expressed as units per liter (U/l).

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was evaluated as an index of neutrophil accumulation in the injured tissue because it correlates closely with the number of neutrophils present in the organ.[19] We measured MPO activity in the pancreas of rats by a specific assay for this enzyme.[19] Briefly, pancreas samples were first homogenized in a solution containing 20 mM of potassium phosphate to 1:10 (w/v) and then centrifuged for 30 min at 20,000*g* at 4°C. The supernatants of each sample were then discarded and the resulting pellet was added to a buffer solution consisting of 0.5% hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH = 6) containing 30 ml of protease and phosphatase inhibitor cocktail. Each sample was then sonicated for 1 min and centrifuged for 30 min at 20,000*g* at 4°C. An aliquot of the supernatant was allowed to react with a solution of *o*-dianisidine dihydrochloride (0.167 mg/ml) and 0.0005% H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 405 nm (Micro-spectrophotomer model 340 ATTC, SLT Lab. Instruments, Austria). MPO activity has been defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C and was expressed as U/g protein.

Isolation of Nuclear and Cytoplasmic Proteins

Briefly, pulverized pancreas samples were homogenized in 0.8 ml ice cold hypotonic buffer [10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT); protease inhibitors: 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, pepstatin, leupeptin (10 μg/ml each); and phosphatase inhibitors: 50 mM NaF, 30 mM β-glycerophosphate, 1 mM Na_3VO_4 and 20 mM *p*-nitrophenylphosphate]. The homogenates were centrifuged for 30 s at 2000 rpm at 4°C to eliminate any unbroken tissues. The supernatants were incubated on ice for 20 min, vortexed for 30 s after addition of 50 μl of 10% Nonidet P-40 and then centrifuged for 1 min at 4°C in an Eppendorf centrifuge. Supernatants containing cytoplasmic protein were collected and stored at −80°C. The pellets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total proteins in the samples was determined by a commercially available protein assay reagent (Sigma Chemical, St Louis, MO). To estimate possible contamination of the nuclear extracts with the cytoplasmic extracts, when preparing the nuclear and cytoplasmatic proteins, lactate dehydrogenase (LDH) activity was determined by a commercially available kit (Sigma Chemical, St Louis, MO). Values were expressed as LDH activity units per milligram of protein. To establish that the nuclear extracts contained mainly nuclear proteins, 40 μg of nuclear protein preparations were subjected to Western blot analysis for histone H3, a nuclear protein, with anti-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).

Electrophoretic Mobility Shift Assay

NF-κB binding activity was performed in a 15-μl binding reaction mixture containing 1% binding buffer [50 μg/ml of double-stranded poly (dI–dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$ and 10% glycerol], 15 μg of nuclear proteins, and 35 fmol (50,000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3; Promega, Madison, WI, USA) which was end-labeled with [γ ^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Life Sciences, Arlington Heights, IL) using T4 polynucleotide

kinase. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at −70°C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results for each time point from each group were expressed as relative integrated intensity compared with the control group pancreas measured in the same batch, because the integrated intensity of group samples from different electrophoretic mobility shift essay (EMSA) batches would be affected by the half-life of the isotope, exposure time, and background levels.

Western Blot Analysis of IκBα in Cytoplasm

Cytoplasm proteins (40 μg) from each sample were mixed with 2 × SDS sample buffer [62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue], heated at 95°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis on 12.5% polyacrylamide gels, the separated proteins were transferred from the gels into Hybond electrochemiluminescence membranes (Amersham) using a Bio-Rad semidry transfer system (Bio-Rad) for 2 h. The membranes were blocked with 5% non fat dry milk in TBS-0.05% Tween for 1 h at room temperature, washed three times for 10 min each in TBS-0.05% Tween 20, and incubated with a primary IκBα antibody (Santa Cruz Biotechnology) in TBS-0.05% Tween 20 containing 5% non fat dry milk for 1–2 h at room temperature. After being washed three times for 10 min each in TBS-0.05% Tween 20, the membranes were incubated with a second antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham). The IκBα protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil). The results from each experimental group were expressed as relative integrated intensity compared with control normal pancreas measured with the same batch.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCT)

Total cellular RNA was extracted from pancreas section. The methods used in the current study have been described elsewhere.[22] In brief, approximately 100 mg of liver was homogenized with 800 μl RNAZOL STAT (Teltest, Firendswood, TX) in a microfuge tube, after which 80 μl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at −80°C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in speed vacuum, centrifuged a second time and then dissolved in 20 μl of buffer. A 2 μg portion of total RNA was subjected to first strand cDNA synthesis in a 20 μl reaction mixture containing the AMV reverse transcriptase (Superscript II; BRL, USA), each dNTP, the specific primers, Tris–HCl and $MgCl_2$.

After dilution of the product with distilled water, 5 μl were used for each polymerase chain reaction (PCR) which contained the Taq polymerase (Perkin Elmer), the buffer as supplied with the enzyme, each dNTP and the specific primers, designed to cross introns and to avoid confusion between mRNA expression and genomic contamination.

The following oligonucleotide pairs were used (5′ oligo/3′ oligo), each sequence as 5′ to 3′: TNF-α: CACGCTCTTCTGTCTTACTGA/GGACTCCGTGATGTCTAAGTGAPDH: ACCACCATGGAGAAGGTCGG/CTCAGTGTAGCCCAGGATGGC. The optimal cycle number for TNF-α was 25 and we used a PCR negative and a PCR positive control without cDNA or with a known cDNA, respectively.

A portion of the PCR product was electrophoresed and transferred to a nylon membrane which is prehybridized with oligonucleotide probes, radiolabeled with [^{32}P] ATP by a T4 oligonucleotide kinase.

After an overnight hybridization at 55°C, filters underwent the autoradiography in a dark-room with a fixed camera.

The captured image, sent to an image analysis software (Bio-Profil) was subjected to densitometric analysis.

Histological Studies and Evaluation of Pancreas Edema

For light microscopy, a piece from the central body of the pancreas was rapidly removed and fixed in 10% buffered formalin. Subsequently, it was embedded in paraffin, cut, and stained with hematoxylin and eosin.

Assessment of tissue changes was carried out by an experienced pathologist who was unaware of the treatments. Histologic grading of edema and infiltrate of inflammatory cells was based on the following scale: 0, absent; 1, mild; 2, moderate; 3, severe. Grading of vacuolization was based on the approximate fraction of cells involved 0, 0–25%; 1, 25–50%; 2, 50–75%; 3, 75–100%. The statistical analysis of the results was performed by applying the statistical method of the "mode" which identifies the predominant value in each group.

To measure pancreas edema a piece from the body of the pancreas was rapidly removed, weighed and the blotted dry on filter paper. The extent of pancreatic edema was calculated by measuring tissue water content: pancreatic tissue was weighed before and after desiccation at 95° for 24 h. The difference between the wet and dry tissue weights was calculated and the degree of edema was expressed as a percentage of the tissue wet weight.

Drugs

Raxofelast (IRFI 016: 2,3-dihydro-5-hydroxy-4,6,7-trimethyl-2-benzofuranacetic acid) was supplied by Biomedica Foscama Reserch Center, Ferentino, (FR), Italy. The compound was administered i.p. in dimethylsulphoxide/NaCl 0.9% (1:10 (v/v)) Cerulein was obtained from Bachem AG laboratories (Bubendorf, Switzerland). All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

Statistical Analysis

Data are expressed as means ± SD and were analyzed by ANOVA for multiple comparison of results. Duncan's multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. The analysis of the histological grading was performed by applying the statistical method of the "mode" which identifies the predominant value in each experimental group.

RESULTS

Pancreatic MAL and GSH

Lipid peroxidation was evaluated by determination of pancreatic MAL 2 h after the last cerulein injection. Very low MAL levels were measured in tissues obtained from control animals treated with vehicle or raxofelast (Fig. 1A). By contrast, cerulein caused a marked increase in pancreatic MAL, thus suggesting the presence of a strong lipid peroxidation process. Administration of raxofelast reduced pancreatic MAL (Fig. 1A).

GSH levels were not modified by the administration of either vehicle or raxofelast in control animals (Fig. 1B). Cerulein produced a severe depletion of GSH in the pancreas. The administration of raxofelast significantly protected from the depletion of GSH (Fig. 1B).

Pancreatic TNF-α mRNA Expression

The top of Fig. 2 depicts representative autoradiograms highlighting mRNA expression for

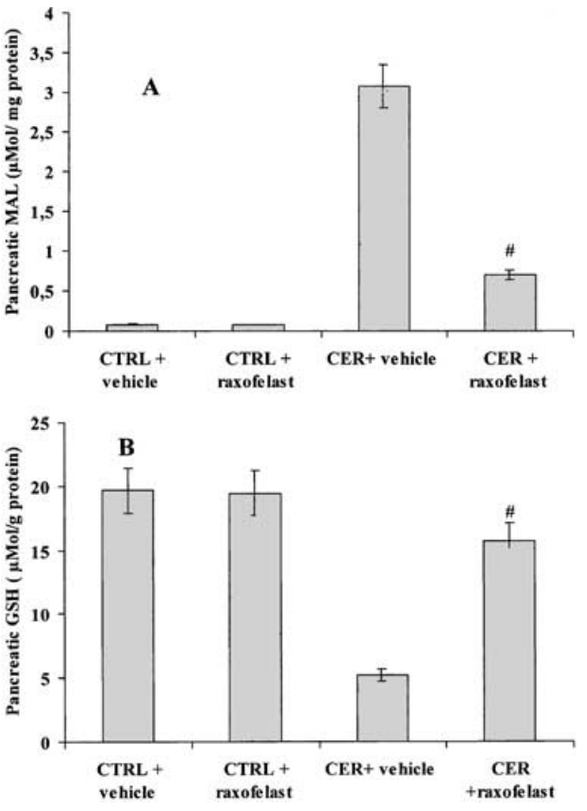

FIGURE 1 (A) Pancreatic content of malondialdehyde (MDA) levels in rats injected with cerulein (CER; 80 µg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.01$ vs. CER + vehicle. (B) Pancreatic glutathione (GSH) levels in rats injected with cerulein (CER; 80 µg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.01$ vs. CER + vehicle.

TNF-α in the several experimental groups. The bottom of the same figure indicates quantitative data. Control pancreas treated with either vehicle or raxofelast showed a low constitutive presence of the message for the inflammatory cytokine. Induction of acute pancreatitis by cerulein resulted in a strong increase of TNF-α message. This message was markedly attenuated in the pancreas obtained from cerulein-injected rats treated with raxofelast.

TNF-α Levels and Leukocyte Accumulation in the Pancreas

Control rats treated with either vehicle or raxofelast showed a constitutive presence of the TNF-α protein (Fig. 3A). Administration of cerulein markedly

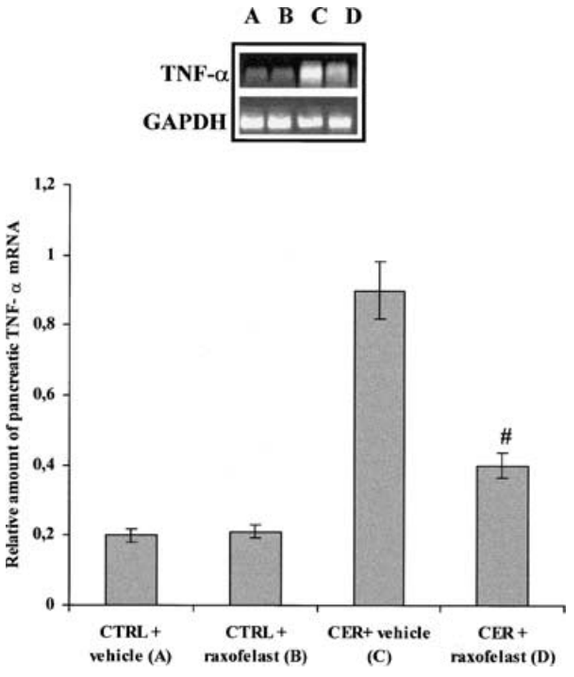

FIGURE 2 Pancreatic TNF-α mRNA expression in samples obtained from rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.001$ vs. CER + vehicle.

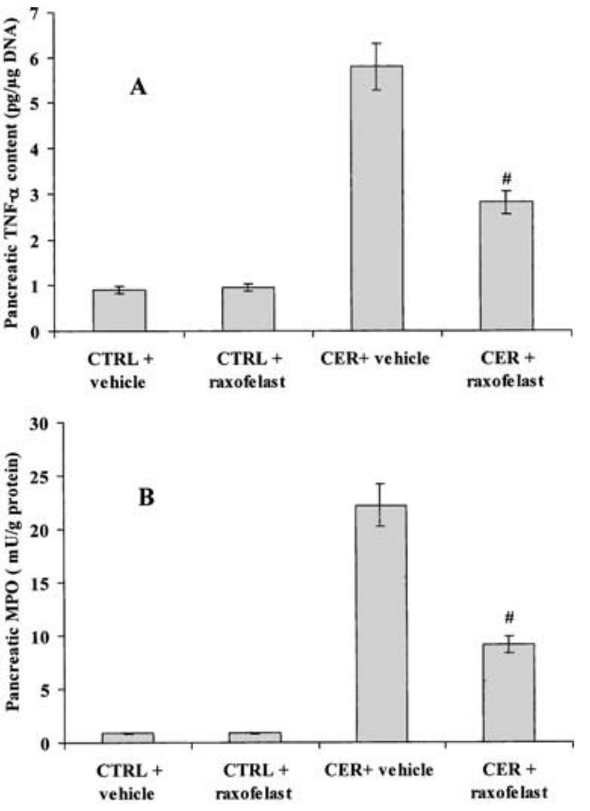

FIGURE 3 (A) Pancreatic TNF-α levels in rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.001$ vs. CER + vehicle. (B) Pancreatic myeloperoxidase activity (MPO) in rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.005$ vs. CER + vehicle.

increased the pancreatic content of the inflammatory cytokine. Treatment with raxofelast resulted in a marked reduction of the inflammatory cytokine (Fig. 3A).

Leukocyte accumulation in the pancreas was investigated by means of the MPO activity. Very low MPO activities were measured in the pancreas of control rats treated with either vehicle or raxofelast (Fig. 3B). By contrast, cerulein injection caused a marked leukocyte accumulation in the pancreas. The administration of raxofelast resulted in blunted pancreatic MPO levels (Fig. 3B).

Activation of NF-κB

NF-κB activation in nuclear extracts of pancreas was determined by EMSA. The top of Fig. 4 shows representative EMSA picture indicating NF-κB activity in the several experimental groups. The bottom of the figure shows quantitative data. NF-κB binding activity was present at very low levels in control rats treated with either vehicle or raxofelast. Pancreas harvested from cerulein-injected rats showed a significant increase in NF-κB binding activity. The administration of raxofelast markedly decreased NF-κB binding activity in cerulein injected rats.

Loss of IκBα Protein in the Cytoplasm

The top of Fig. 5 shows representative Western Blot analysis indicating IκBα protein in the cytoplasma of pancreas obtained from the several experimental groups. The bottom of the figure indicates quantitative data. IκBα protein levels were evidenced in pancreas of control rats treated with vehicle or raxofelast. Pancreas obtained from cerulein-injected rats showed a marked loss of the protein. Treatment with raxofelast blunted the consistent depletion of inhibitor protein.

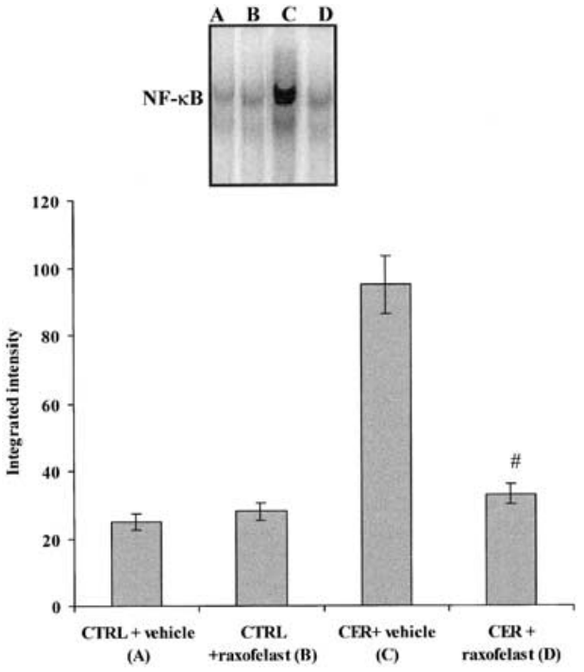

FIGURE 4 Electrophoretic mobility shift assay (EMSA) of NF-κB binding activity in the nucleus of pancreas harvested from rats injected with cerulein (CER; 80 μ/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.01$ vs. CER + vehicle.

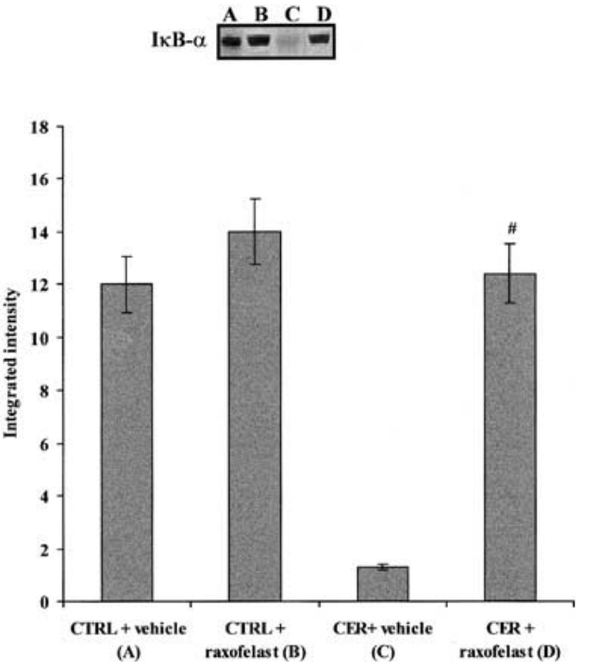

FIGURE 5 Western blot analysis of IκBα protein levels in the cytoplasm of pancreas of rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.01$ vs. CER + vehicle.

Severity of Cerulein-Induced Pancreatitis

Several parameters that characterize the severity of secretagogue-induced pancreatitis were measured. Injection of cerulein enhanced serum lipase and amylase activity (Fig. 6A, B). Cerulein induced pancreatitis also resulted in a significant organ edema (Fig. 6C). Animals injected with cerulein and treated with raxofelast showed a marked reduction in the markers of pancreatitis severity: as a matter of fact, rats treated with raxofelast had blunted increase in serum lipase and amylase activity and reduced pancreas edema (Fig. 6A–C).

Histological Studies

Figure 7A, B shows the normal histology of the control pancreas. Lobules and acini of the pancreas are intact; the acinar cells are intact and the acinar nuclei have a peripheral placement. In contrast, light microscopy histology showed that cerulein-induced pancreatitis caused interstitial oedema, inflammatory cells infiltration and at higher magnification, presence of cytoplasmatic vacuolization (Table I and Fig. 7C, D). The administration of raxofelast markedly reduced these histological alterations in the pancreas (Table I and Fig. 7E, F).

DISCUSSION

It has been shown that preventing the production of either oxygen free radicals and the consequent chain breaking reaction of lipid peroxidation ameliorates cerulein induced pancreatitis.[20,21,22] In the present study co-treatment of animals with raxofelast, an inhibitor of lipid peroxidation, reduced the levels of MAL, prevented the depletion of pancreatic GSH and markedly attenuated the severity of pancreatitis. As a matter of fact, raxofelast caused a reduction in the biochemical signs of pancreatitis, such as the increased serum amylase and lipase activity, improved the histological picture and decreased tissue edema.

Thus, our results are in close agreement with the idea that quenching the oxidative stress represents an interesting and valuable approach for the management of acute pancreatitis. Furthermore for the first time we clearly suggest that raxofelast, an inhibitor of lipid peroxidation previously shown to

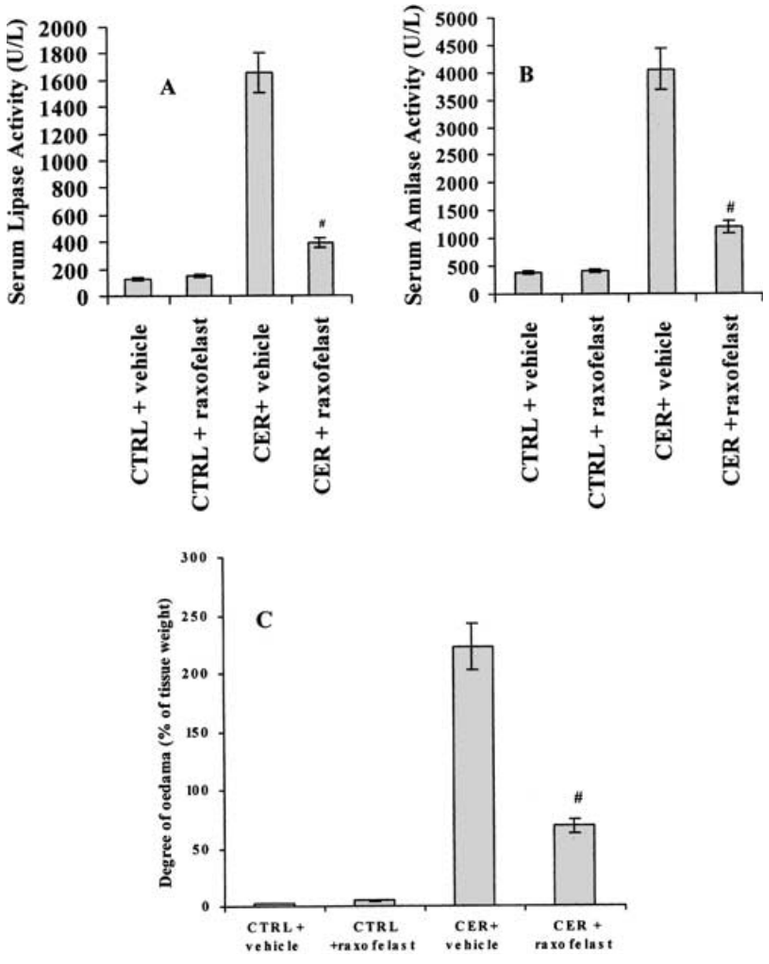

FIGURE 6 (A) Serum lipase activity in rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.001$ vs. CER + vehicle. (B) Serum amylase activity in rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.001$ vs. CER + vehicle. (C) Degree of pancreas oedema in rats injected with cerulein (CER; 80 μg/kg s.c. for each of four injections at hourly intervals) or with four s.c. injections of 0.9% saline at hourly intervals (CTRL). Animals were treated with raxofelast (20 mg/kg i.p. administered with the first cerulein injection) or its vehicle (1 ml/kg of 10% DMSO/NaCl solution). Bars represent the mean ± SD of seven animals. $^{\#}p < 0.005$ vs. CER + vehicle.

have protective effects in the cardiovascular system,[17] may also confer protection against oxidative damage in the gastrointestinal tract.

Indeed, the drug protective effects might solely ascribe to this antioxidant effects. However, inhibition of lipid peroxidation also reduced markers of the inflammatory cascade, thus rising the question whether this event could represent an epiphenomenon or alternatively, be an important component of the protective effects.

Pro-inflammatory cytokines have been also implicated in the pathogenesis of acute pancreatitis.[23,24] TNF-α is detected in plasma early in the course of acute pancreatitis and pretreatment of rats with antibodies raised against the inflammatory cytokine reduces elevated serum amylase in acute pancreatitis.[25]

Nevertheless TNF-α in the serum may underestimate the production of the pleiotropic cytokine because of the short serum half life of TNF-α.

In the present study, we therefore, investigated the pancreatic content of the inflammatory cytokine as well as its mRNA. The results clearly showed that TNF-α was up-regulated in the pancreas following cerulein hyper-stimulation as previously shown.[26]

The mechanism(s) by which TNF-α causes organ injury and damage have been already pointed out. The inflammatory cytokine might promote organ damage by inducing deleterious leukocyte accumulation within the pancreas and therefore, through

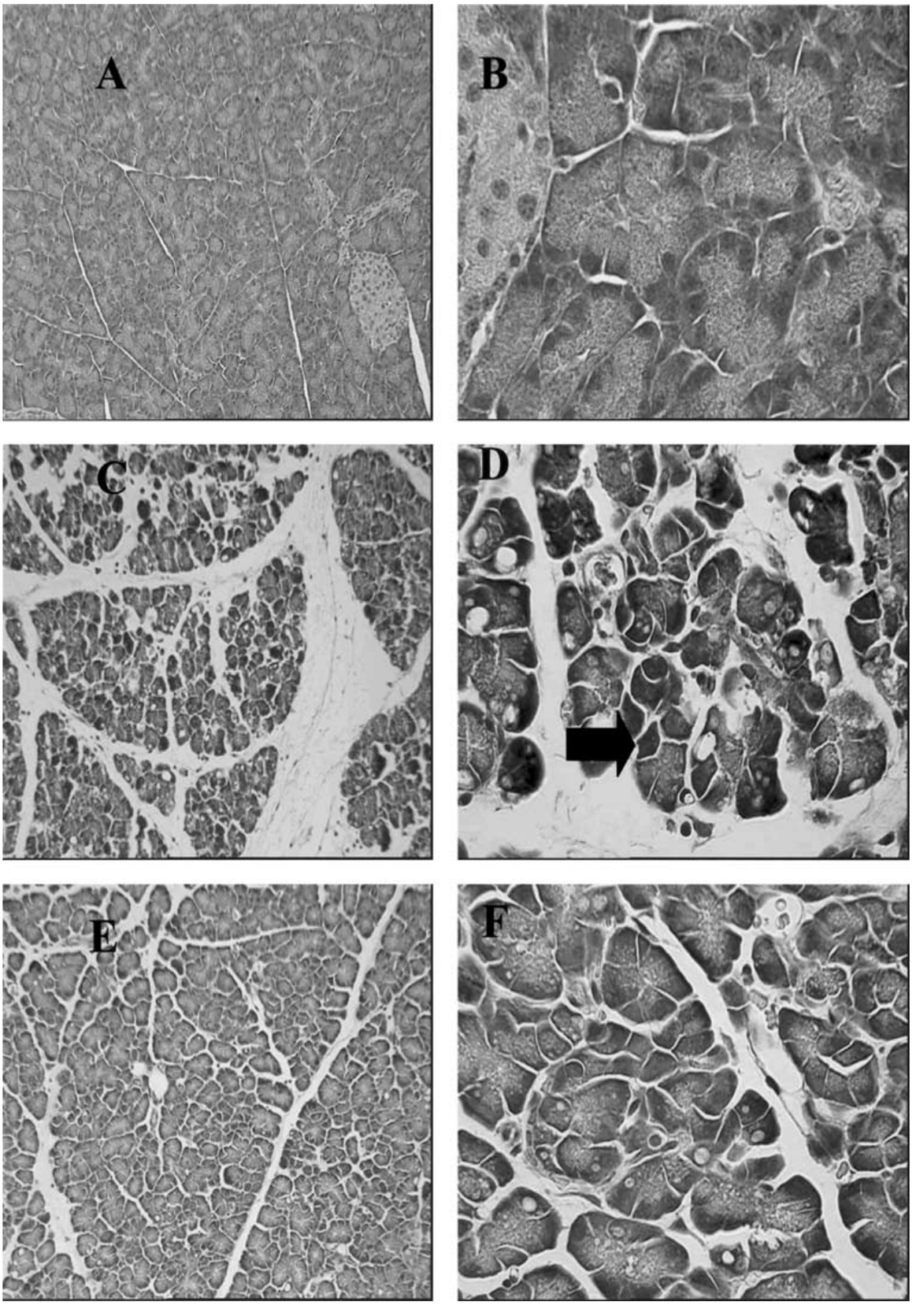

TABLE I Histology of pancreas harvested from control (CTRL) and cerulein (CER) injected rats treated with either vehicle or raxofelast

Groups	Grade of interstitial edema	Grade of inflammatory cell infiltrate	Grade of vacuolization
CTRL+vehicle	0	0	0
CTRL+raxofelast	0	0	0
CER+vehicle	3	3	3
CER+raxofelast	1	1	1

Histologic grading of edema and infiltrate of inflammatory cells were based on the following scale: 0, absent; 1, mild; 2, moderate; 3, severe. Grading of vacuolization was based on the approximate fraction of cells involved; 0, 0–25%; 1, 25–50%; 2, 50–75%; 3, 75–100%. The statistical analysis of the histological grading was performed using the method of the "mode" which identifies the predominant value in each group ($n = 7$).

this mechanism, it may cause additional injury and inflammation.[27]

The administration of raxofelast resulted in a blunted leukocyte sequestration and accumulation in the pancreas: in fact the drug significantly decreased the levels of pancreatic MPO activity, studied as a means to measure the deleterious phenomenon of leukocyte sequestration.

A central regulator of cytokine induction is the pleiotropic transcription factor NF-κB.

NF-κB is an early transcription factor which modulates gene expression in various situations that require rapid and sensitive immune and inflammatory response. The prototypic inducible form of NF-κB is a heterodimer composed of NF-κB1 and Rel A, which both belong to the NF-κB/Rel family of proteins. Inactive NF-κB is present in the cytoplasm complexed with the inhibitory proteins of IκB (IκBα, IκBβ, Bcl-3, etc).

NF-κB is activated by a number of incoming signals from the cell surface. On activation IκB proteins become hyper-phosphorylated and proteolytically degraded. Released from IκBα inhibition, NF-κB translocates into the nucleus and binds to κB motif of the target gene, in turn, causing activation of several factors (cell adhesion molecules; cytokines) involved in the inflammatory response. The selectivity of binding is controlled, at least in part, by distinct protein subunits of NF-κB, such as p65 (RelA), p50 and c-Rel.

It has been demonstrated that cerulein markedly stimulates NF-κB binding activity in experimental pancreatitis and in isolated pancreatic acini.[28,29] Supershift EMSA studies indicated that both p65/p50 and p50/p50 dimers, but not c-Rel or p52 complexes, were involved in cerulein-induced pancreatitis and in isolated acinar cells.[29]

In agreement with previous experimental evidence, our data suggest that cerulein administration in rats evoked a sustained activation of the cytoplasm transcription factor NF-κB. This event was accompanied by a concomitant depletion of the inhibitory protein IκBα. Thus, NF-κB activation has central role in controlling the inflammatory cascade reaction that occurs in experimental pancreatitis induced by cerulein. Apart from phosphorylation, the oxidative state of the cells has been shown to play a key role in NF-κB activation.[30,31] Several antioxidants such as *N*-acetylcysteine (NAC) inhibit NF-κB activation induced by several inducers, again reinforcing the notion that reactive oxygen intermediates are deeply involved in this triggering. Moreover it has been shown that NAC administration in cerulein induced pancreatitis blocks the activation of the transcription factor and improves the parameters of the disease.[29] However, at present, there is no conclusive information as to whether lipid peroxidation in acute pancreatitis is a cause or consequence of NF-κB activation.

Raxofelast is a synthetic analog of vitamin E with a hydrophilic character and powerful antioxidant properties. This compound has been shown to be a potent inhibitor of lipid peroxidation.[17]

Our data clearly indicate that administration of raxofelast, in addition to inhibit MAL and to dramatically ameliorate the severity of cerulein induced pancreatitis, succeeded in blunting NF-κB activation and in preventing the depletion of the inhibitory protein IκBα. In contrast, raxofelast did not suppress other cerulein-induced effects such as the neuroleptic-like-induced behavior and the hypothermic activity. These findings rule out the possibility that in acute pancreatitis the drug may work by antagonizing cerulein action or accelerating its metabolism.

Indeed our experimental evidence lead us to hypothesize that, at least in cerulein-induced pancreatitis, lipid peroxidation has a fundamental role in NF-κB activation. Therefore, the halting of the inflammatory cascade triggered by transcription

FIGURE 7 (A) Normal histology of a representative control pancreas. Original magnification 25×. (B) Normal histology of a representative control pancreas. Lobules and acini of the pancreas are intact. Note uniform size of acinar cells and the peripheral localization of acinar nuclei. Original magnification 100×. (C) Representative cerulein-injected pancreas. Edema, inflammatory cell infiltrates of both interlobular and intralobular interstitium are present. Original magnification 25×. (D) Representative cerulein-injected pancreas. Presence of vacuoles appearing as round dark bodies within the acinar rosettes (arrows). Original magnification 100×. (E) Representative cerulein-injected pancreas treated with raxofelast. Significant reduction in the edema and in the inflammatory cell infiltrates. Original magnification 25×. (F) Representative cerulein-injected pancreas treated with raxofelast. The edema is minimal and the presence of vacuoles is rare. Original magnification 100×.

factor (i.e. increased cytokines production, enhanced leukocyte sequestration and accumulation in the pancreas) represents an important component of the protective effects of vitamin E-like drugs in acute pancreatitis. Theoretically, vitamin E could also represent a good candidate to inhibit NF-κB activation. However, its poor pharmacokinetic profile precludes the use in pathological situations that require an acute administration and a prompt bio-availability. This justifies the search for alternative analogs that could overcome this problem.

In conclusion, our data suggest that lipid peroxidation plays a pivotal role in triggering NF-κB activation and the inflammatory response in cerulein-induced pancreatitis and that raxofelast might be considered a potential therapeutic agent to prevent the severe damage in acute pancreatitis.

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