Early NF-κB activation is associated with hormone-induced pancreatitis

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Gukovsky, Ilya, Anna S. Gukovskaya, Thane A. Blinman, Vjekoslav Zaninovic, and Stephen J. Pandol. Early NF-κB activation is associated with hormone-induced pancreatitis. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1402–G1414, 1998.—Inflammation and cell death are critical to pathogenesis of acute pancreatitis. Here we show that transcription factor nuclear factor-κB (NF-κB), which regulates these processes, is activated and plays a role in rat cerulein pancreatitis. NF-κB was strongly activated in the pancreas within 30 min of cerulein infusion; a second phase of NF-κB activation was prominent at 3–6 h. This biphasic kinetics could result from observed transient degradation of the inhibitory protein IκBα and slower but sustained degradation of IκBβ. The hormone also caused NF-κB translocation and IκB degradation in vitro in dispersed pancreatic acini. Both p65/p50 and p50/p50, but not c-Rel, NF-κB complexes were manifest in pancreatitis and in isolated acini. Confusion of CCK JMV-180, which abolishes pancreatitis, prevented cerulein-induced NF-κB activation. The second but not early phase of NF-κB activation was inhibited by a neutralizing tumor necrosis factor-α antibody. Antioxidant N-acetylcysteine (NAC) blocked NF-κB activation and significantly improved parameters of pancreatitis. In particular, NAC inhibited intrapancreatic trypsin activation and mRNA expression of cytokines interleukin-6 and KC, which were dramatically induced by cerulein. The results suggest that NF-κB activation is an important early event that may contribute to inflammatory and cell death responses in acute pancreatitis.

Despite considerable progress in understanding pathophysiology of pancreatitis, the mechanisms of the development of this disease remain obscure (52). A number of animal models of experimental pancreatitis have been developed and show biochemical, morphological, and pathophysiological similarities to various aspects of human pancreatitis (14, 31, 51, 54). One of the best-characterized and widely studied experimental models involves administration of high doses of cerulein, a CCK analog (14). Doses of CCK or cerulein beyond those that cause maximal pancreatic secretion of amylase and lipase (26, 46) result in pancreatitis characterized by dysregulation of production and secretion of digestive enzymes, in particular, inhibition of pancreatic secretion and elevation of their serum levels, cytoplasmic vacuolization and death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas (14, 31, 44, 51, 54).

Over the past several years, evidence has been accumulating on the involvement of inflammatory mediators, such as cytokines-chemokines interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α), and platelet-activating factor (PAF), in the development of pancreatitis (8, 12, 15–17, 19, 25, 36–39, 45, 57). Systemic levels of IL-6 and IL-8 increase in patients with acute pancreatitis and correlate with the severity of the disease (12, 16, 17, 25, 36). In experimental pancreatitis, the levels of PAF, IL-1α, and TNF-α are elevated not only in blood but also in the pancreas, and their blockade attenuates the disease (8, 15, 19, 36–39, 45, 57). Furthermore, we found recently that the pancreatic acinar cell itself is capable of producing and releasing TNF-α (19). Although cytokines have been implicated in the development and clinical course of pancreatitis, their source, mechanism of regulation, and role at the early stages of the disease remain obscure.

A key regulator of cytokine induction is the pleiotropic transcription factor nuclear factor-κB (NF-κB). NF-κB represents a family of proteins sharing the Rel homology domain, which bind to DNA as homo- or heterodimers, and activate a multitude of cellular stress-related and early response genes such as the genes for cytokines, growth factors, adhesion molecules, and acute phase proteins (reviews in 3, 32, 50, 55). Unlike most transcription factors, NF-κB is kept silent in the cytoplasm via interaction with inhibitory proteins of IκB family (IκBα, IκBβ, Bcl-3, etc.) that masks the nuclear localization signal. NF-κB is activated by a variety of agents ranging from cell-damaging physical factors and viruses to mitogens and cytokines. On activation IκB proteins become hyper-phosphorylated and proteolytically degraded, thus allowing NF-κB dimers to rapidly translocate into the nucleus where they bind to DNA sites containing κB motifs. The selectivity of binding is controlled, at least partially, by distinct protein subunits of NF-κB, such as p65 (RelA), p50, and c-Rel (32, 50).

The two most abundant members of IκB family, IκBα and IκBβ, are differentially regulated by NF-κB itself (55). The gene encoding IκBα contains functional NF-κB sites and can be transcriptionally activated by NF-κB, which leads to rapid resynthesis of IκBα and blockade of NF-κB nuclear translocation (1, 24, 53). In contrast to IκBα, no κB motifs have been found in the gene for IκBβ, and NF-κB does not regulate IκBβ synthesis (24, 57).
Apartment from phosphorylation, the oxidative state of the cell has been shown to play a role in NF-κB activation (11, 42, 47, 48, 50, 55). Several antioxidants, such as N-acetylcysteine (NAC), block activation of NF-κB by a number of inducers, indicating the involvement of reactive oxygen intermediates (11, 42, 47, 55).

Little is known about NF-κB in the pancreas. NF-κB activation plays a central role in regulating the expression of genes involved in inflammation, cell injury, and cell death. These processes mediate the development of acute pancreatitis; however, apart from limited observations (10, 20–22, 35), the role of NF-κB in pancreatitis has not been studied.

The purpose of the present study was to determine if NF-κB is activated and plays a role in hormone-induced experimental pancreatitis. We found that 1) in rat cerulein pancreatitis NF-κB was rapidly and strongly activated through degradation of both IκBα and IκBβ; 2) cerulein-induced NF-κB activation was prevented by co-incubation of the CCK analog J MV-180, a treatment that abolishes pancreatitis; 3) cerulein directly activated NF-κB through degradation of IκB in isolated pancreatic acini; 4) blockade of NF-κB activation by NAC improved the parameters of the disease; and 5) the expression of NF-κB-regulated genes for cytokines IL-6 and KC (murine analog of chemokines IL-8/ GRO-α) was dramatically induced in cerulein pancreatitis and was inhibited by NF-κB blockade. Thus NF-κB activation is an early event that may have an important part in the development of hormone-induced pancreatitis.

**METHODS**

Experimental model of pancreatitis. We randomized male Sprague-Dawley rats (Harlan, Madison, WI), weighing 270–320 g, into several groups in two categories (A and B). The rats in category A received continuous intravenous infusion of 5 µg·kg⁻¹·h⁻¹ cerulein (in physiological saline solution plus 30 U/l heparin) for a period of up to 6 h at a rate of 0.6 ml/h. Rats in the main group were infused with cerulein only; rats in other groups received, in addition to cerulein, the CCK analog J MV-180, the antioxidant NAC, or a neutralizing TNF-α antibody. CCK J MV-180 was infused at 5 mg·kg⁻¹·h⁻¹ in combination with cerulein or alone. NAC was given in various regimens; most of the data were obtained on rats that were given an intravenous bolus of 200 mg/kg NAC 2 h before cerulein treatment followed by continuous infusion of 25 µg·kg⁻¹·h⁻¹ NAC for the duration of the experiment. Thus a 6-h cerulein-infused rat received a total of 400 mg/kg (2.4 mmol/kg) NAC. Polyclonal rabbit anti-mouse neutralizing TNF-α antibody (Genzyme, Cambridge, MA) was given at 1:100 dilution as an intravenous bolus (0.1 µl diluted in 990 µl sterile PBS). Category B consisted of rats used as controls in each group, which instead of cerulein received an infusion of physiological saline solution plus 30 U/l heparin at the same rate.

After treatment the rats were killed, blood was collected for serum amylase and lipase determinations, the pancreas was removed, and a 50- to 100-µg piece of the gland close to the spleen was cut out, rinsed in ice-cold PBS, and snap frozen in liquid nitrogen for subsequent RNA isolation. Portions of the pancreas were used for nuclear protein extraction and measurement of active trypsin, and the rest of the tissue was saved for morphological examination.

Isolation of dispersed pancreatic acini. Dispersed pancreatic acini from rats were prepared using a previously published collagenase digestion method (41). To culture acinar cells, dispersed pancreatic acini were washed and resuspended in medium 199 supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 0.5% BSA. Cells were plated at a concentration of 5 × 10⁶/ml in 25-cm tissue culture flasks and incubated with or without 0.7 µM cerulein for indicated times in a 5% CO₂-humidified atmosphere at 37°C.

Preparation of nuclear extracts. Nuclear protein extracts were prepared essentially as described by Dignam et al. (9). A 150- to 200-µg portion from the splenic area of the gland or a sample of isolated pancreatic acinar cells was rinsed in ice-cold PBS and lysed on ice in the hypotonic buffer A (9) by 20 or 5 strokes, respectively, in a glass Dounce homogenizer. Just before use, buffer A was supplemented with phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) to the final concentration of 1 mM each and with the protease inhibitor cocktail containing 5 µg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin. The homogenate was left on ice for 15–20 min, and then Nonidet P-40 was added to the final concentration of 0.3–0.4% (vol/vol), and the samples were briefly vortexed and incubated on ice for an additional 1–2 min. Crude nuclear pellet was collected by centrifugation of the lysed tissue or cell samples for 30 s in a Microfuge. The supernatant (cytosolic protein) was saved for Western blot analysis of IκB, and the nuclear pellet was resuspended in the high-salt buffer C (9) containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM β-glycerophosphate, 10 mM Na₂MoO₄, 50 µM Na₃VO₄, 1 mM DTT, 1 mM PMSF, and the protease inhibitors cocktail described previously. After being rotated at 4°C for up to 1 h, the nuclear membranes were pelleted by microcentrifugation for 10 min and the clear supernatant (nuclear extract) was aliquoted and stored at −80°C. Protein concentration in the nuclear extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assay. Aliquots of nuclear extracts with equal amount of protein (2–10 µg) were mixed in 20 µl reactions with a buffer containing 10 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% (vol/vol) glycerol, and 3 µg poly(dI·dC) (9). After aliquots were equilibrated on ice for 5 min, binding reactions were started by the addition of 20–60,000 counts/min (20 fmol) of 32P-labeled DNA probe and allowed to proceed for 25–30 min at room temperature or up to 1 h on ice. The oligonucleotide probe 5’-GCAGAGGGGACCTTTCCGAGA containing a κB binding motif (underlined) was annealed to the complementary oligonucleotide with a 5’-G overhang and end-labeled using Klenow DNA polymerase I. In mutated oligonucleotide, the κB motif was changed (lowercase) to GGCCACTaaCC. For cold competition, a 60–300× molar excess of nonlabeled wild-type or mutated κB oligonucleotide was added to the reaction together with the probe. For supershift experiments, 1 µg of specific antibodies against NF-κB proteins p65 (RelA), p50, or c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction 15 min before the addition of the probe. Samples were electrophoresed at room temperature in 0.5 × TBE buffer (1× TBE 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) on nondenaturing 4.5% polyacrylamide gel at 200 V. Gels were dried and directly analyzed in the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or exposed at −80°C to Fuji RX film with intensifying screens. In this case the intensity of bands on gel fluorograms was quantified using the image analysis system AMBIS (Scanalytics, San Diego, CA).
Western blot analysis of IxB. Proteins in the cytosolic extract (see Preparation of nuclear extracts) were separated by SDS-PAGE at 120 V using precast Tris-glycine gels and Mini-Cell gel apparatus (Novex, San Diego, CA). Separated proteins were electrophoretically transferred to polyvinylidene difluoride membrane for 2 h at 30 V, using a Novex blot module. Nonspecific binding was blocked by overnight incubation of the membrane in 5% (wt/vol) non-fat dry milk in Tris-buffered saline (TBS, pH 7.5). Blots were then incubated for 2 h at room temperature with primary antibodies in antibody buffer containing 1% (wt/vol) nonfat dry milk in TBS (0.05% vol/vol Tween 20 in TBS), washed three times with TBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer.

Immunoblotting of IxB and IxB was performed using monoclonal sc-1643 and polyclonal sc-945 antibodies, respectively, from Santa Cruz Biotechnology. Blots were developed for visualization using enhanced chemiluminescence detection kit (Pierce Chemical, Rockford, IL).

Quantification of mRNA levels for IL-6 and KC by RT-PCR. Total RNA was extracted from pancreatic tissue or isolated acinar cells using the method of Han et al. (23) or TRizol reagent (GIBCO BRL). RNA quality was verified by better than 2:1 ethidium bromide staining of rRNA bands on denaturing agarose gel. Total RNA (5 µg) was reverse-transcribed according to the manufacturer’s protocol (SuperScript II Pre amplification System, GIBCO BRL) using oligo(dT) as a primer. cDNA prepared from 0.5 to 1 µg total RNA was subjected to PCR using gene-specific primers described below. Negative controls were performed by omitting the RT step from PCR amplification.

To quantitate changes in mRNA expression, we applied quantitative competitive (QC) PCR. For this analysis, a homologous external standard DNA (mimic) was created and coamplified with each of the cDNAs of interest, based on a modified procedure similar to that used previously (7, 29). Briefly, after the sense and antisense primers for the specific cDNA (target) were chosen, a “composite” primer was designed consisting of the sense primer spliced to a sequence 50–100 bp downstream in the cDNA of interest. Amplification of the target cDNA with the composite and antisense primers generates the same size amplicon as amplification of mRNA of interest plus a difference in their efficiencies of their amplification (that is expected to be minimal).

The amplified PCR products were all of expected size. They were separated on agarose gel stained with ethidium bromide and the intensity of the bands was quantified by densitometry in GelBlot UVP (Synoptics, Upland, CA). The ratio of specific mRNA of interest was determined from the competition curve derived by plotting the ratio of the densities of the target to mimic PCR products coamplified in the same tube vs. the concentration of the mimic (see Fig. 11A). The equivalence point, where this ratio equals 1.0, was estimated, and a correction was made for the difference in the target and mimic sizes. The reproducibility of our QC-PCR was tested by performing parallel series of PCR reactions on one and the same cDNA sample. For all of the PCR reactions the competition curve was well described by a straight line. In most cases the slope of this line was close to the theoretical value of −1.0 (43), indicating similar amplification efficiencies for the target and mimic (see Fig. 11B). To allow comparison between different RNA samples and experimental conditions, the amount of specific mRNA in a sample was further normalized to the expression level of a housekeeping mRNA in the same sample that was determined in a similar manner.

For the following murine gene-specific, intron-spanning primers were used for: IL-6, sense 5′-GAAATCTCCTGGTC-TCTCGG and antisense 5′-TTTCTGACACGCTGAGGA-ATG primers (nucleotide positions 397–419 and 732–754 in rat IL-6 cDNA, GenBank accession number M26744); for the chemokine KC, sense 5′-CAATGGACTGCTGTCATG and antisense 5′-CTTGGGACACCTTTGACATC primers (nucleotide positions 135–155 and 318–339 in rat KC cDNA, GenBank M86536); for the housekeeping acidic ribosomal phosphoprotein P0 gene (ARP), sense 5′-GGTGAACATCTTCCCCCTTTCTC and antisense 5′-ATGCCCTATGGATGCTCC primers (nucleotide positions 560–580 and 941–961 in rat ARP cDNA, GenBank Z29530). The amplification was performed for 32 cycles at the annealing temperature of 54°C.

Serum amylase and lipase measurements. Serum amylase and lipase levels were determined by using a Hitachi 707 analyzer (Antech Diagnostics, Irvine, CA).

Acinar cell vacuolization. Pancreatic tissue was fixed immediately in 10% buffered formaldehyde. The tissue embedded in paraffin was sectioned and stained with hematoxylin and eosin. Values were obtained by counting the number of vacuoles per 100 acinar cells in an average of 50 fields at magnification ×40 (at least 1,000 acinar cells).

Determination of apoptotic acinar cells. Apoptotic acinar cells were determined as described previously (19, 45) on sections of pancreatic tissue stained with 8 mg/ml of Hoechst 33258. The stained sections were examined by fluorescence microscopy at excitation 380 nm and emission 510 nm. Scoring and classification of nuclei were performed based on the condensation state of chromatin. Acinar cells were considered apoptotic if their nuclei contained condensed and/or fragmented chromatin. A total of 5,000 to 10,000 cells were counted per slide.

Myeloperoxidase activity measurements. Myeloperoxidase (MPO) activity, a measure of neutrophil infiltration, was determined using the method of Bradley et al. (5). Briefly, pancreatic tissue was homogenized in a solution containing 50 mM EDTA plus the cocktail of protease inhibitors (5 µg/ml each) pepstatin, aprotinin, leupeptin, chymostatin, and antipain, and 1 mM PMSF. The homogenate was centrifuged at 40,000 g for 15 min, the supernatant was discarded, and the pellet containing membrane-bound MPO was saved. MPO was solubilized by incubation with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0), 5 mM EDTA. Subsequently, the suspension was rehomogenized, sonicated, and centrifuged at 40,000 g for 15 min. The supernatant was collected, heated at 60°C for 2 h, and centrifuged at 10,000 g for 5 min. The supernatant containing MPO activity was saved. An aliquot of the supernatant was mixed with o-dianisidine and 0.005% hydrogen peroxide in 50 mM phosphate buffer (pH 6.0), and changes in absorbance at 460 nm were measured.

Measurements of active trypsin. Trypsin activity in pancreatic tissue homogenates was measured by a fluorometric assay according to the method of Kawabata et al. (28). Briefly, a 10-µl aliquot of the tissue homogenate was added to 2 ml of the assay buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM CaCl2, and 0.1 mg/ml BSA, in a stirred cuvette at 37°C. The reaction was started by adding the Boc-Glu-Ala-Arg-AMC substrate (28) and was followed for 6–10 min. The increase in fluorescence (excitation 380 nm, emission 440 nm)
NF-κB is rapidly activated in rat cerulein pancreatitis. In the pancreas of normal and saline-infused rats, DNA binding activity of NF-κB was virtually undetectable. Infusion of a supramaximal dose (5 μg·kg⁻¹·h⁻¹) of cerulein caused a strong pancreatic NF-κB activation (Fig. 1). The specificity of NF-κB DNA binding activity detected by our electrophoretic mobility shift assay (EMSA) in the pancreas was demonstrated by cold competition experiments using wild-type and mutated κB oligonucleotides (Fig. 1) and by supershift experiments described below.

NF-κB activation during rat cerulein pancreatitis displayed a biphasic time course (Fig. 2). Infusion of the supramaximal dose of the hormone rapidly activated NF-κB. The DNA binding activity increased several-fold within 15 min of cerulein hyperstimulation; it peaked at around 30 min and then decreased substantially by 1.5 h. A second wave of NF-κB activation was prominent at 3 h cerulein infusion, and it remained elevated at 6 h at a level two to three times above the control (Fig. 2, A and C).

Figure 2B shows serum amylase and lipase (common markers of acute pancreatitis) for the animals subjected to NF-κB EMSA presented in Fig. 2A. Note that NF-κB is fully activated within 30 min cerulein hyperstimulation, when serum amylase and lipase levels only start to rise. Thus NF-κB becomes activated in the pancreas at the onset of the disease.

CCK JMV-180 does not activate NF-κB in pancreas. Infusion of a low, physiological dose of cerulein for 6 h did not evoke NF-κB activation (Fig. 3A, lane 8). To further prove that NF-κB activation was associated with the development of the hormone-induced pancreatitis we made use of another derivative of CCK, CCK JMV-180. This peptide, which binds to the same CCK-A receptor at concentrations about 1,000 times higher than CCK, is considered to be an agonist for the high-affinity state and antagonist for the low-affinity state of the CCK-A receptor (26, 33, 46). In contrast to CCK or cerulein, at high doses JMV-180 does not have an inhibitory effect on pancreatic enzyme secretion (26, 33, 44, 46) nor does it induce pancreatitis (44). Therefore, activation of a low-affinity state of the CCK-A receptor is thought to mediate pancreatitis (44).

Treatment of rats with 5 mg·kg⁻¹·h⁻¹ JMV-180 did not activate NF-κB (Fig. 3A, lane 6). Furthermore, confusion of J MV-180 with the supramaximal dose of cerulein, which prevents cerulein-induced rat pancreatitis (44), completely abolished NF-κB nuclear translocation at both 30 min and 3 h (Fig. 3A, lanes 3 and 5). The absence of pancreatitis in the animals treated with the low dose of cerulein or with J MV-180 was confirmed by the normal levels of serum amylase and lipase (Fig. 3B) and by morphological evaluations (data not presented). Thus intrapancreatic NF-κB activation in cerulein-infused rats is specifically related to the development of the hormone-induced pancreatitis.

IκBα and IκBβ display different kinetics of degradation in cerulein pancreatitis. Because NF-κB is activated via degradation of the inhibitory IκB proteins we analyzed IκBα and IκBβ in the cytosolic protein extracts from pancreatic tissue by Western blotting. Figure 4 shows that cerulein hyperstimulation caused degradation of both IκBα and IκBβ but with drastically different kinetics. The IκBα was greatly decreased by the hormone at the earliest time point studied (15 min), but it subsequently recovered, and at 3 h cerulein...
infusion IκBα was at its normal level in the control (Fig. 4). No degradation of IκBα was seen at 6 h cerulein infusion. In contrast, IκBβ degradation developed more slowly and was sustained for up to 6 h (Fig. 4). Thus the early phase of cerulein-induced pancreatic NF-κB activation (Fig. 2) is due mainly to IκBα degradation, whereas the second phase is mediated by IκBβ.

As distinct from cerulein, no degradation of either IκBα or IκBβ was observed in pancreas under the action of CCK JMV-180 (Fig. 4). This parallels the absence of NF-κB nuclear translocation in animals treated with JMV-180 (Fig. 3).

Cerulein directly activates NF-κB in pancreatic acinar cells. To investigate whether acinar cells could be one source of cerulein-induced pancreatic NF-κB activation, we asked if the hormone activates NF-κB in vitro. Dispersed acini, obtained from normal pancreas by standard collagenase digestion (41), displayed a certain level of NF-κB DNA binding activity on isolation. However, incubation of isolated acinar cells with cerulein caused a pronounced increase in nuclear NF-κB compared with cells incubated without the hormone (Fig. 5A). Acinar cells comprise >97% in our preparations of dispersed pancreatic acini (19), and they do not contain any neutrophils or macrophages, a potential source of activated NF-κB. Thus the hormone directly activates NF-κB in acinar cells.

The detailed time and concentration dependencies of cerulein-induced NF-κB activation in pancreatic acinar cells are a subject of our separate study. Here we show (Fig. 5B) that in isolated acini the hormone induces degradation of both IκBα and IκBβ with kinetics similar to what we observed for cerulein pancreatitis. A supramaximal concentration of cerulein caused a rapid but transient IκBα degradation: there was essentially no IκBα in the cytosol after 15-min incubation with cerulein, but at 3 h IκBα returned to the same level as in control cells. By contrast, IκBβ was intact at 15 min but remained degraded after 3- and 6-h incubation of the cells with cerulein (Fig. 5B).

Subunit composition of pancreatic NF-κB complexes. We used antibodies against different NF-κB proteins to characterize subunit composition of pancreatic NF-κB complexes activated by cerulein (Fig. 6). Both in tissue and in isolated acinar cells stimulated with cerulein, two NF-κB-DNA bands could be distinguished by EMSA, the lower (faster migrating) one being usually more diffuse. Antibodies to NF-κB proteins p65 (RelA) and
p50, but not to c-Rel, either elicited a “supershifted” complex or inhibited the DNA binding. The p50 antibody supershifted the lower band and attenuated the upper one, whereas the antibody to p65 supershifted the upper band without changing the lower one, which is seen more clearly on dispersed pancreatic acini EMSA (Fig. 6B). These data indicate that both the classic p65/p50 heterodimer (slower migrating complex) and p50/p50 homodimer (the faster migrating complex) are formed on cerulein hyperstimulation. The same types of NF-κB complexes are activated by the hormone in vivo and in vitro. No complexes involving c-Rel could be detected in pancreatic tissue (Fig. 6A) or dispersed acini (data not shown).

TNF-α involvement in NF-κB activation in cerulein pancreatitis. To provide an insight into the mechanism of pancreatic NF-κB activation, we examined whether TNF-α had a role in this process. TNF-α is a potent activator of NF-κB (50, 55). Recently we showed that pancreatic acinar cells are able to produce and respond to TNF-α and that neutralization of this cytokine with an antibody improved the parameters of rat cerulein pancreatitis (19). Therefore, we tested if the neutralizing anti-TNF-α antibody could prevent or inhibit NF-κB activation induced by cerulein hyperstimulation. Immediately before cerulein infusion, rats were given an intravenous bolus of the antibody that was shown (19) to produce an improvement in pancreatitis (see METHODS). This appeared to have no effect on the early NF-κB activation caused by 30-min cerulein infusion (Fig. 7A). Moreover, the early phase of cerulein-induced NF-κB translocation was not inhibited by pretreating the rat with one more such bolus of the TNF-α antibody 1 h before hormone infusion (Fig. 7A, lane 3).

The neutralizing TNF-α antibody, however, caused 45–63% inhibition (n = 5) of the second phase of NF-κB activation at 6 h cerulein infusion (Fig. 7B). Similar inhibition was observed for 3 h of cerulein infusion (not illustrated). These data suggest that TNF-α does not mediate the initial NF-κB activation in rat cerulein pancreatitis, but it is involved on later stages. In control experiments TNF-α neutralization with the antibody did not cause any NF-κB binding activity in saline-infused rats.
Blocking of NF-κB activation improves parameters of rat cerulein pancreatitis. To study the role of NF-κB activation in the hormone-induced pancreatitis, we used the antioxidant NAC to inhibit NF-κB. This compound is commonly used to block NF-κB activation (50, 55) and is believed to act both via its direct reducing action and as glutathione precursor (6, 11, 42, 47, 48). We applied NAC in various regimens, but most of the data were obtained on rats that were given an intravenous bolus of 200 mg/kg NAC 2 h before cerulein treatment followed by continuous infusion of 25 mg·kg⁻¹·h⁻¹ NAC for the period of the experiment. That is, a 6-h cerulein-infused rat received a total of 400 mg/kg (2.4 mmol/kg) NAC during 8 h. This treatment almost completely abolished NF-κB activation caused by cerulein hyperstimulation at both 30 min and 6 h (Fig. 8).

For instance, NF-κB DNA binding activity at the early 30-min peak decreased in NAC-treated animals as much as 10-fold (n = 3) compared with rats infused with cerulein only (Fig. 8A). In control (saline-infused) animals NAC treatment did not produce any changes in the measured parameters, e.g., serum amylase and lipase levels.

Another NF-κB inhibitor, pyrrolidinedithiocarbamate (PDTC; Refs. 6, 11, 48), also blocked cerulein-induced NF-κB DNA binding activity to about the same degree as NAC (not shown). However, at the doses necessary for this inhibition (several hundred milligram per kilogram) PDTC appeared toxic for the rats. This could be due to other actions of PDTC, e.g., its known effects as a potent metal chelator (6, 11, 42, 47, 48). Therefore, we did not use PDTC to study the effects.

Fig. 6. Both p65/p50 and p50/p50 dimers but not NF-κB complexes involving c-Rel are activated by cerulein. A: to characterize NF-κB complexes activated in cerulein pancreatitis, nuclear protein from pancreatic tissue of a rat infused with 5 µg·kg⁻¹·h⁻¹ cerulein for 30 min was subjected to EMSA either alone (lane 1) or in the presence of antibodies (Ab) to different NF-κB proteins (lanes 2-4). Positions of supershifted bands are shown by arrows. B: similar supershift analysis was performed on dispersed pancreatic acini incubated with 0.7 µM cerulein for 30 min.

Fig. 7. Tumor necrosis factor-α (TNF-α) does not mediate initial NF-κB activation but is involved in second phase of cerulein-induced pancreatic NF-κB activation. Treatment with neutralizing TNF-α antibody, which ameliorates rat cerulein pancreatitis (see METHODS), did not prevent NF-κB activation caused by 30-min cerulein hyperstimulation (A), but it inhibited by 45–63% (n = 5) the second phase of cerulein-induced NF-κB activation at 6 h (B). Lane 1, pancreatic tissue from rats infused with 5 µg·kg⁻¹·h⁻¹ cerulein for 30 min (A) or 6 h (B); lane 2, immediately before cerulein infusion rats were given intravenous bolus of 1:100 neutralizing TNF-α antibody; lane 3, rats were given 1 more such bolus of neutralizing TNF-α antibody 1 h before cerulein infusion.
Activation caused by 30-min infusion of 5 µg·kg⁻¹·h⁻¹ cerulein. Lane 1, cerulein treatment alone; lane 2, 2 h before cerulein infusion rat was given intravenous bolus of 0.2 g/kg NAC followed by continuous infusion of 25 mg·kg⁻¹·h⁻¹ NAC. B: effect of NAC on pancreatic NF-κB activation caused by 6-h infusion of 5 µg·kg⁻¹·h⁻¹ cerulein. Lane 1, cerulein treatment alone; lane 2, 1 h before cerulein infusion rat was given intravenous bolus of 0.2 g/kg NAC followed by continuous infusion of 25 mg·kg⁻¹·h⁻¹ NAC; lanes 3 and 4, 2 h before cerulein (lane 3) or saline (lane 4) infusion rats were given intravenous bolus of 0.2 g/kg NAC followed by continuous infusion of 25 mg·kg⁻¹·h⁻¹ (total NAC, 2.4 mmol/kg).

Blocking of NF-κB with the NAC treatment improved parameters of rat cerulein pancreatitis: serum levels of amylase and lipase, the percentage of apoptotic acinar cells was not affected by NAC (Fig. 9). The results obtained in this study show that NF-κB inhibition was achieved in rat cerulein pancreatitis is and inhibited by NAC. To look into another functional aspect of NF-κB activation in hormone-induced pancreatitis, we investigated the expression of NF-κB-regulated genes for cytokines IL-6 and KC in rat cerulein pancreatitis and is inhibited by NAC. To look into another functional aspect of NF-κB activation in rat cerulein pancreatitis, we investigated the expression of NF-κB-regulated genes for the acute phase cytokine IL-6 and the chemokine KC (a murine analog of IL-8 and GRO-α). It has been shown that patients with acute pancreatitis have elevated serum levels of IL-6 and IL-8 that correlate with the severity of the disease (12, 16, 17, 25, 36). KC expression in experimental pancreatitis has not yet been characterized, and the induction of IL-6 has not been quantified (13, 36, 37).

Total RNA was isolated from pancreata of control, cerulein-infused, and cerulein plus NAC-treated rats, and RT-PCR was performed using intron-spanning primers specific for rat KC and for the housekeeping gene ARP (see Methods). The number of cycles was chosen well below saturation to enable visualization of the PCR products on the agarose gel. Figure 10A demonstrates that the expression of KC mRNA in the pancreas was greatly upregulated in cerulein-infused animals compared with the control group. The effect of cerulein hyperstimulation was inhibited by NAC. To obtain an estimation of these changes, the intensities of KC bands were quantified by densitometry and normalized to ARP for differences in the amount of cDNA between samples. The results of this semiquantitative RT-PCR (Fig. 10B) indicate that relative to the control group cerulein hyperstimulation increased the level of KC mRNA expression more than 40-fold, which was inhibited about three times by NAC.

Similar estimations of the relative changes in mRNA expression were performed for IL-6 (Fig. 10, A and C). The levels of pancreatic IL-6 mRNA were barely detectable in the control group, and therefore their upregulation by cerulein appeared even greater than for KC. With semiquantitative RT-PCR, NAC inhibited cerulein-induced upregulation of IL-6 mRNA about 10 times (Fig. 10C).

For a quantitative analysis of IL-6 mRNA expression, we used QC-PCR (7, 29). Exogenous homologous standards (mimics) were created for both IL-6 and ARP, based on the published rat cDNA sequences (see Methods). Serial dilutions of a known concentration of the mimic were coamplified with a constant amount of reverse-transcribed RNA, using gene-specific primers, and a competition curve was derived from densitometric analysis of the PCR products (Figs. 11, A and B). The amount of IL-6 mRNA in a sample was estimated from the equivalence point at which the ratio of the densities of the target to mimic PCR products was equal to 1.0 (Fig. 11B), after correcting for the difference in the target and mimic sizes. This analysis was done for each RNA sample. To allow comparison between different samples, the calculated amount of IL-6 mRNA was further normalized to the level of the housekeeping ARP mRNA in the same sample, which was determined in a similar manner.

Cerulein hyperstimulation for 6 h caused a 100-fold increase in the steady-state level of intrapancreatic IL-6 mRNA (up to about 40 amol/mg total RNA compared with a value of <0.3 amol/mg total RNA in the control group). This upregulation was inhibited more than six times in rats in which NF-κB activation was blocked by NAC (Fig. 11C). The expression levels for ARP varied within one order of magnitude (mean 14.6 ± 2.2 amol/mg total RNA, n = 13) in the cDNA samples studied. They were randomly scattered among the control, cerulein-infused, and NAC-treated groups of rats (data not presented), validating the use of ARP as a housekeeping gene. A comparison of the data in Figs. 10C and 11C shows that there is a good correlation between the results of the semiquantitative and QC-RT-PCR for IL-6 mRNA expression.

**DISCUSSION**

The results obtained in this study show that NF-κB is rapidly and strongly activated in rat cerulein pancreatitis. Within 15 min of infusion of a supramaximal dose of the hormone, NF-κB DNA binding activity in the pancreas increases several-fold, and it remains above...
Fig. 9. Blocking of NF-κB activation with NAC improves parameters of rat cerulein pancreatitis. a, 6-h saline infusion (n = 3–5 for different parameters); b, 6-h infusion of 5 μg·kg⁻¹·h⁻¹ cerulein (n = 3–8); c, 6-h cerulein infusion together with 2-h NAC pretreatment described in legend to Fig. 8 (n = 3–6). MPO, myeloperoxidase. Data represent means ± SE; *P < 0.03 relative to cerulein alone (b).

the basal level at 6 h cerulein hyperstimulation. Rapid kinetics is typical for NF-κB activation. However, most of the data have been obtained for cultured cells, and apart from lung and liver, there are few measurements of NF-κB in normal and injured tissues in the development of a disease (3, 55). Except for abstract communications (20–22, 35) and a recent observation of NF-κB activation in biliary pancreatitis (10), this is the first detailed study of NF-κB activation in the pancreas, in particular, of its role in hormone-induced pancreatitis.

We observed a two-phase time course of the pancreatic NF-κB activation: an initial increase in DNA binding activity which peaked at around 30 min and subsided by 1.5 h, and the second phase which was most prominent at 3 h cerulein infusion. This kinetics can be explained by the involvement of different species of inhibitory IκB proteins, IκBα and IκBβ, in the first and second phases of hormone-induced NF-κB activation. We found that IκBα was rapidly but transiently degraded in pancreatitis, and it completely returned to normal levels at 3 and 6 h cerulein infusion. In contrast, degradation of IκBβ developed more slowly and was sustained up to 6 h. Thus, the early phase of pancreatic NF-κB activation is due mainly to IκBα degradation, whereas the second phase is mediated by IκBβ.

The different behavior of IκBα and IκBβ is explained by differences in transcriptional regulation of their genes by NF-κB. The gene encoding IκBα contains functional NF-κB binding sites and is transcriptionally activated by NF-κB, which leads to rapid resynthesis of IκBα and blockade of NF-κB nuclear translocation (1, 24, 27, 53, 55). Moreover, the resynthesized IκBα can diffuse into the nucleus, bind there to NF-κB dimers, and disassociate them from DNA sites (1, 56). In contrast to IκBα, no κB motifs have been found in the gene for IκBβ and its expression is not regulated by NF-κB (1, 24, 27, 53, 55).

A biphasic NF-κB nuclear translocation with a very similar time course was reported recently in TNF-α-
stimulated human hepatoblastoma cells (24). It was shown that the transient early (15 min to 1 h) phase of NF-κB activation in these cells was due to a rapid IκBα resynthesis and that persistent IκBβ proteolysis induced by TNF-α was necessary for the second (>2 h) phase.

Another factor contributing to the second wave of NF-κB activation could be inflammatory cell infiltration into the pancreas, which in the rat model becomes pronounced after 2–3 h of cerulein hyperstimulation (14, 49). Inflammatory cells produce cytokines, such as IL-1 and TNF-α, known to activate NF-κB. As shown by us and others (13, 19, 36–38), TNF-α is upregulated in the pancreas during cerulein hyperstimulation. We showed that TNF-α induces NF-κB activation in isolated pancreatic acinar cells; we also found that the acinar cell itself produced and released TNF-α (19). The results obtained in the present study on the effects of TNF-α neutralization on NF-κB (Fig. 7) suggest the involvement of this cytokine in NF-κB activation at 3 and 6 h of cerulein hyperstimulation but not at the early phase. The source of TNF-α could be both infiltrating inflammatory cells and acinar cells.

No NF-κB binding activity was detected in response to a low, physiological dose of cerulein or infusion of the CCK analog JMV-180 that does not induce pancreatitis. Furthermore, cerulein-induced NF-κB activation was abolished by coinfusion of J MV-180, a treatment that is known (44) to prevent pancreatitis. These results show that pancreatic NF-κB activation caused by cerulein hyperstimulation is associated with the development of the disease.

Several indirect lines of evidence in the present study implicate acinar cells as one source of pancreatic NF-κB activation in rat cerulein pancreatitis: 1) the first, main peak of NF-κB activation develops within 30 min of hormone hyperstimulation, much faster than the infiltration of inflammatory cells; 2) cerulein directly activates NF-κB in isolated acinar cells through degradation of IκB; and 3) the same types of NF-κB complexes

![Fig. 10. NF-κB-regulated mRNA expression for cytokines KC and interleukin-6 (IL-6) is greatly activated in rat cerulein pancreatitis and is inhibited by NAC. A: representative RT-PCR for pancreatic expression of cytokines KC and IL-6 and housekeeping gene acidic ribosomal phosphoprotein (ARP) in saline control, cerulein-infused, and cerulein plus NAC-treated rats. cDNA from 3 animals in each group was reverse-transcribed from 0.5 µg of total RNA and amplified as described in METHODS. PCR products were resolved on agarose gel stained with ethidium bromide, and band intensity was quantified by densitometry. B and C: densities of KC and IL-6 RT-PCR products were normalized to those of housekeeping gene ARP in same cDNA sample. For each group of rats, KC and IL-6 expression levels are given as means ± SE relative to control. *P < 0.001 (n = 5) compared with the saline (S) group. **P < 0.05 (n = 3) compared with cerulein only (CR) group.

![Fig. 11. Quantitation of pancreatic IL-6 mRNA expression by competitive RT-PCR. A: representative gel showing detection of target IL-6 cDNA (T) and its mimic (M). Constant amount of cDNA derived from 0.5 µg total RNA was coamplified with sequential 1:2 dilutions of IL-6 mimic. PCR products were resolved on agarose gel stained with ethidium bromide and band intensity was quantified by densitometry. B: ratio of densitometric values for IL-6 target and mimic PCR products shown in A was plotted against amount of mimic in reaction tube, yielding straight line with slope of approximately –1.0. Absolute amount of IL-6 cDNA in sample was calculated from equivalence point where intensities of target and mimic bands were equal, after correcting for difference in target and mimic size. C: for each animal, level of IL-6 mRNA expression was quantified as shown in A and B and then normalized to expression of ARP determined in similar manner. In most control rats, amount of IL-6 message was less than the smallest amount of mimic detectable with ethidium bromide, 0.3 amol/mg total RNA. Data represent means ± SE. *P < 0.001 (n = 5) compared with saline. **P < 0.05 (n = 3) compared with cerulein only.]
are activated by the hormone in pancreas and in isolated acinar cells.

The isolation procedure caused a certain level of NF-κB binding activity in acinar cells, probably triggered by the disruption of pancreatic extracellular matrix (18). However, incubation of acinar cells with cerulein resulted in a marked NF-κB activation. Moreover, we found that supramaximal concentrations of the hormone-induced degradation of both IκBα and IκBβ in isolated acinar cells, with kinetics similar to that observed in pancreatitis.

The results of supershift experiments indicate that at least two types of NF-κB complexes are activated in the pancreas of rats with cerulein pancreatitis: p65/p50 and p50/p50 dimers. We have not detected NF-κB complexes involving c-Rel. One may speculate that different types of NF-κB complexes play distinct functional roles in hormone-induced pancreatitis by selectively activating NF-κB target genes with different binding sites (32, 50). On the other hand, p50 homodimers often behave as transcriptional inhibitors (32, 50, 55) and in this way they may, for example, counteract an “excessive” transactivation by p65/p50.

We used the antioxidant NAC to inhibit NF-κB activation evoked by cerulein. This compound is well tolerated and is commonly applied to inhibit NF-κB in vivo (11, 47, 55). Both the early and late phases of hormone-induced NF-κB activation were blocked by NAC. This resulted in an improvement of all measures of rat cerulein pancreatitis, suggesting that NF-κB activation is involved in the development of the disease. Of importance, NAC significantly reduced pancreatic trypsin activation, a key indicator of acinar cell injury. The only parameter unchanged by NAC was the percentage of apoptotic acinar cells, which can be affected by a number of factors involved in pancreatitis (19, 45).

Besides NAC, with two more treatments we found a similar correlation between inhibition of pancreatic NF-κB and amelioration of rat cerulein pancreatitis. One is TNF-α neutralization with an antibody that both inhibited NF-κB activation (this study) and improved cerulein-induced pancreatitis (19); another example is neutralization of PAF by an antagonist, which inhibited both the development of cerulein pancreatitis (45) and NF-κB activation (20).

NF-κB plays a central role in regulating cytokine gene expression. Using RT-PCR for quantitation of mRNA levels, we found that cerulein hyperstimulation greatly induced mRNA expression of cytokine IL-6 and chemokine KC. The latter is a murine analog of IL-8 and GRO-α, major mediators of neutrophil activation and recruitment (2, 4). NF-κB binding sites were found in the promoters of IL-8, KC, and IL-6, and the expression of these genes in a number of cell lines was shown to be regulated by NF-κB (4, 30, 40, 50, 55). Our results show that, in rat cerulein pancreatitis, intrapancreatic mRNA levels for both IL-6 and KC increase up to 100-fold. This induction is inhibited by NAC, indicating the involvement of NF-κB in cytokine activation by the hormone.

Our quantitative analysis of IL-6 expression correlates well with the data obtained by Northern blot and conventional RT-PCR (13, 36, 37). mRNA expression of KC (or IL-8) in experimental pancreatitis has not been characterized yet. In addition to IL-6 and KC, NF-κB may also be involved in the induction of other cytokines, in particular, “first-line” cytokines IL-1 and TNF-α, which are upregulated in cerulein-induced and other models of pancreatitis (36).

The observed upregulation of KC may play a role in activation and recruitment of neutrophils into the pancreas. The neutrophils not only mediate the inflammatory response but they also regulate acinar cell death in the disease, as we recently found for rat cerulein pancreatitis (45). The infiltrating inflammatory cells may contribute to NF-κB activation at later stages of pancreatitis.

Based on our results, several lines of indirect evidence suggest a functional role for NF-κB activation in cerulein pancreatitis: 1) hormone hyperstimulation causes a rapid and strong NF-κB activation in the pancreas; 2) NF-κB activation is associated with the development of the disease, as shown by experiments with JMV-180; 3) cerulein directly activates NF-κB in isolated acinar cells; both the kinetics of IκB degradation and the subunit composition of activated NF-κB complexes in vitro are similar to those observed in pancreatitis; 4) treatments with NAC, neutralizing TNF-α antibody or PAF antagonist all blunt pancreatitis and inhibit NF-κB activation; and 5) NF-κB-regulated genes for IL-6, KC, IL-1, and TNF-α are all induced in the pancreas by hormone hyperstimulation. In this study we showed that the expression of the first two is inhibited by NAC and the last two have been shown to mediate cerulein pancreatitis (19, 36–39).

This evidence is of a correlating character, it only shows association between NF-κB inhibition and improvement of pancreatitis. More specific, precise ways of inhibiting NF-κB, e.g., by using “decoy” NF-κB-binding oligonucleotides (34) or knockout animals (3, 55), are needed to prove the causative role of NF-κB activation in the pathogenesis of pancreatitis.

NF-κB activation was reported recently in a model of biliary pancreatitis induced by duct ligation (10). Its inhibition by amobarbital improved serum amylase levels, one of the parameters of pancreatitis. Interestingly, from that study one can also infer a correlation between NF-κB activation and the development of the disease: biliary pancreatitis develops more slowly, and the NF-κB activation is also delayed (Fig. 4 in Ref. 10) compared with cerulein pancreatitis.

In conclusion, based on the data obtained, we can hypothesize that supramaximal doses of cerulein activate NF-κB in acinar cells resulting in upregulation of certain cytokines, like TNF-α, IL-6, and KC, which mediate both acinar cell death (19) and inflammation (12, 36). The results suggest that NF-κB activation is an important early event that may link the initial...
NF-κB IN CERULEIN PANCREATITIS

Acinar cell injury to the inflammatory and cell death responses, the hallmarks of acute pancreatitis.

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