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Induction of IκB-Kinase by Cholecystokinin Is Mediated by Trypsinogen Activation in Rat Pancreatic Lobules

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Key Words
NF-κB · IκB-kinase · Trypsinogen · Trypsin activation · Acute pancreatitis · Concentrations of cholecystokinin · Serine protease inhibitor

Abstract
Background and Aims: Supramaximal concentrations of cholecystokinin (CCK) or cerulein induce the intracellular activation of trypsinogen and the transcription factor NF-κB, a key regulator of inflammatory gene expression. Both events occur early in the development of an acute pancreatitis. The aim of this study was to examine the relationship between intracellular trypsinogen and NF-κB activation. ***Methods:*** We detected NF-κB-binding activity in electromobility shift assays, IκB proteolysis in Western analysis and endogenous IκB-kinase (IKKα and β) activation using immune complex kinase assays following treatment with CCK in rat pancreatic lobules. To block intrapancreatic trypsinogen activation, a potent and cell-permeable serine-protease inhibitor, Pefabloc, was used. ***Results:*** CCK-induced IκBα degradation and subsequent NF-κB activation correlated closely with the catalytic activity of IKKs to phosphorylate IκBα in vitro. Activation is dose-dependent and peaked at 30 min. Doses of Pefabloc sufficient to inhibit trypsin activation reduced CCK-induced activation of NF-κB whereas TNF-α-induced NF-κB activation was not blocked but slightly increased. Moreover, treatment with Pefabloc as well as another serine protease inhibitor, FUT175, inhibited CCK-induced IKK activation. ***Conclusion:*** These results suggest that intrapancreatic activation of trypsinogen may contribute to NF-κB signaling via IKK activation in cerulein pancreatitis. This also explains the fact that only doses of CCK which activate trypsinogen induce NF-κB activation in pancreatic acinar cells. Thus, trypsinogen activation is likely to modulate signaling events in acinar cells in the initial phase of acute pancreatitis.

Introduction

Under physiological conditions, pancreatic enzymes are synthesized and secreted as inactive proenzymes and zymogens. In the duodenum the brush-border enzyme enteropeptidase hydrolyzes trypsinogen to trypsin and the N-terminal trypsinogen-activation peptide (TAP). Active trypsin subsequently activates other zymogens. Intrapancreatic activation of digestive enzymes, in particular trypsinogen, leads to injury of the pancreas by autodigestion and is believed to be a key event in acute pancreatitis.

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This is based on the detection of premature and intrapancreatic trypsinogen activation under experimental conditions and the identification of point mutations in the trypsinogen gene in hereditary pancreatitis [1–5].

Stimulation of the pancreas with supramaximal concentrations of cholecystokinin (CCK) or the CCK analog cerulein leads to acute edematous pancreatitis and the activation of trypsinogen within acinar cells [6, 7]. Accumulating evidence suggests that a number of early signaling pathways are induced in addition to trypsinogen activation. This includes activation of mitogen-activated protein kinases (MAPKs) and heat-shock proteins [reviewed in 8]. The induction of these signaling molecules may be involved in mediating the pancreatic response to supramaximal CCK stimulation and have a protective or detrimental effect during acute pancreatitis. Recently, we and others have shown that the transcription factor NF-κB is activated following cerulein pancreatitis and exposure of pancreatic lobules to supramaximal doses of cerulein in vitro [9–11].

NF-κB is localized in the cytoplasm complexed to its endogenous inhibitor proteins, IκBs, which sequester NF-κB in the cytoplasm. Following extracellular stimulation including cytokines, mitogens and microbial pathogens, IκBα is phosphorylated at serine residues 32 and 36, which initiates ubiquitination and rapid degradation of this inhibitor by the nonlysosomal, ATP-dependent 26S proteasome [reviewed in 12]. A 700-kDa protein complex containing two related IκB kinases, IKKα and IKKβ, has been identified which is able to phosphorylate IκBα at the two critical serines [13–16]. Various extracellular signaling pathways which activate NF-κB converge at the level of IKK activation, implicating this complex as the critical regulator of NF-κB activation.

The present study was designed to explore the effect of CCK on IKK activation and to examine the relationship between trypsinogen activation and NF-κB induction. CCK-induced IκBα degradation and subsequent NF-κB activation correlated closely with the catalytic activity of IKKs to phosphorylate IκBα in vitro, both in terms of concentration and in the temporal response. In an attempt to characterize the effect of intrapancreatic trypsinogen activation on CCK-mediated NF-κB induction, we used the serine protease inhibitor Pefabloc to inhibit trypsinogen activation. CCK-induced IκBα degradation and subsequent NF-κB activation was blocked by pretreatment with Pefabloc in contrast to NF-κB activation induced by TNF-α. Moreover, Pefabloc or FUT175, both serine protease inhibitors, inhibited CCK-induced IKK activity. Therefore, these data suggest that in rat pancreatic lobules, trypsinogen activation plays a role in CCK-mediated NF-κB induction upstream of the IKK complex.

Materials and Methods

Reagents

Cholecystokinin octapeptide, sulfated (CCK-8), was purchased from Tocris (Bristol, UK). Pefabloc SC (4-[2-aminoethyl]benzolsulfonylfluoride hydrochloride) was from Merck (Darmstadt, Germany). FUT175 (6-amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate) was from Torii Pharmaceutica Co. (Tokyo, Japan). TNF-α was from Sigma-Aldrich Chemie (Steinheim, Germany), as were all other chemicals which were of the highest purity commercially available.

Male Wistar rats (250–300 g b.w.) were obtained from the breeding colony of Ulm University Animal Facilities. They were housed in nalgene shoebox cages under a 12:12 h light-dark cycle with free access to standard diet and water. All animal experiments were conducted according to the guidelines of the local Animal Use and Care Committees and executed according to the National Animal Welfare Law.

Preparation of Pancreatic Lobules

Pancreatic lobules were prepared as previously described [9, 17]. In brief, after an overnight fast, rats were sacrificed by exsanguination under light ether anesthesia. The pancreas was removed and incubated in Dulbecco's Modified Eagle's Medium (Gibco Life Technologies, Paisley, UK). Equal quantities of lobules were incubated in medium for 15 min at 37°C under continuous oxygenation in a shaking water bath. Following this adaptation period, lobules were incubated with Pefabloc (10, 100 or 1,000 μ*M*) or FUT175 (1,000 μ*M*) as pretreatment or left with medium alone. Thereafter, lobules were stimulated with CCK-8 (0.1, 1, 10 or 100 n*M*) or 150 U/ml of TNF-α. After respective incubation periods, lobules were immediately frozen in liquid nitrogen and stored at –70°C.

Protein Extracts

Nuclear protein extracts were prepared essentially as described [9, 17], with some minor modifications: Pancreatic lobules were homogenized in a sucrose buffer containing protease inhibitors (0.1 *M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 14 μg/ml pepstatin, 10 μg/ml aprotinin, 1 *M* DTT and 10 m*M* DFP). Nuclei were separated by centrifugation (14,000 rpm for 70 min at 4°C) and proteins were eluted using a high salt buffer. Aliquots of the nuclear protein extracts were stored at –70°C. For cytoplasmic protein extracts, pancreatic lobules were homogenized in 150 m*M* NaCl, 50 m*M* Tris-HCl, 50 m*M* $CaCl_2$, 1.0% NP-40, 5 m*M* NaF, 100 m*M* PMSF, 10 μg/ml leupeptin, 14 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 *M* DTT, pH 7.2, and centrifuged at 15,000 rpm for 20 min at 4°C. Aliquots of the supernatant were stored at –70°C. Protein concentrations were determined by the method of Bradford (BioRad Laboratories, Munich, Germany).

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed as previously described [9, 17]. The DNA probes used for EMSAs corresponded to the high affinity κB sequences found in the mouse κ light

chain enhancer and in the HIV-1 promoter region. Two oligonucleotides were annealed to generate a double-stranded probe: sense 5′-AGCTTGGGGACTTTCCACTAGTACG-3′ and antisense 5′-AATTCGTACTAGTGGAAAGTCCCCA-3′ (the binding site is underlined). Labeling was accomplished by treatment with Klenow in the presence of dGTP, dCTP, dTTP and [^{32}P]-dATP. Labeled oligonucleotides were purified on push columns (Stratagene, Heidelberg, Germany). A labeled double-stranded probe (80,000 cpm) was added to 5 μg of nuclear protein in the presence of 5 μg poly(dIdC) as nonspecific competitor (Pharmacia Biotech Enzyme, Freiburg, Germany). Binding reactions were carried out in 10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 4% glycerol, 1 m*M* EDTA, 5 m*M* DTT for 20 min at room temperature. DNA protein complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel in 1 × Tris-glycine-EDTA buffer. Gels were vacuum dried and exposed to Kodak Bio Max MS-1 film at –70°C with intensifying screens.

Western Blotting

Cellular protein extracts were analyzed by immunoblotting. Samples were diluted in SDS-PAGE loading buffer in a ratio of 1:5 and heated at 97.5°C for 5 min. Recombinant IκBα or IκBβ was prepared by transfecting 293 human embryonic kidney cells with the respective eukaryotic expression vector as previously described and loaded as controls (data not shown) [17]. Protein complexes were resolved by electrophoresis on 10% polyacrylamide gels in 1 × Tris-glycine-SDS buffer at room temperature. After SDS-PAGE, separated proteins were transferred to 0.45 μm polyvinylidene difluoride membranes (Sigma-Aldrich Chemie) for 60 min at 40 mA at room temperature. Nonspecific binding was blocked in 5% (w/v) skim milk in Tris-buffered saline (TBS), pH 7.5 at 4°C. Blots were then incubated for 1 h with primary antibodies IκBα, IκBβ (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at a dilution of 1:1,000 in 5% (w/v) skim milk in TBS, washed 3 times with 0.05% (v/v) Tween 20 in TBS (T-TBS), and incubated for 1 h with a secondary antibody, goat anti-rabbit IgG peroxidase (Dianova-Immunotech, Hamburg, Germany) at a dilution of 1:5,000 in 5% (w/v) skim milk in TBS. After that, blots were washed 3 times with T-TBS; they were developed with enhanced chemiluminescence reagents (Amersham Buchler, Braunschweig, Germany) to Kodak Bio Max MR-1 film.

Measurement of Trypsinogen Activation Peptide in Pancreatic Lobules

Trypsinogen activation peptide (TAP) concentrations in pancreatic lobules were detected by a method as previously described [8, 19]. In brief, pancreatic lobules were boiled in 200 m*M* Tris-HCl and 20 m*M* EDTA, pH 7.3, for 15 min at 100°C and homogenized for 30 s. The resulting homogenates were centrifuged at 1,500 rpm for 10 min at 4°C and the supernatants were taken for an enzyme immunoassay using Biotrin TAP EIA kit (Biotrin International, Sinsheim-Reihen, Germany).

Immunoprecipitation IκB-Kinase Assay

IκB-kinase activity was detected by immunoprecipitation of IKK followed by a kinase assay using GST-IκBα(1–54) substrate as previously described with some modifications [20]. Pancreatic lobules were homogenized in lysis buffer containing 150 m*M* NaCl, 25 m*M* Tris-HCl, 2 m*M* EGTA, 2 m*M* EDTA, 1.0% Triton-X 100, 10% glycerin, 50 m*M* NaF, 25 m*M* Na-pyrophosphate, 50 m*M* β-glycerophosphate, pH 8.0, and complete protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany) and centrifuged at 14,000 rpm for 20 min at 4°C. Anti-IKKα antibody (Santa Cruz Biotechnology) and 35 μ*M* of washed protein A agarose (Boehringer Mannheim) was added to the supernatant (3 mg protein) and mixed at 4°C for 2 h. Immunoprecipitated material was washed 3 times with lysate buffer and once with kinase buffer containing 25 m*M* HEPES, 150 m*M* NaCl, 25 m*M* β-glycerophosphate and 10 m*M* $MgCl_2$. Kinase activity was assayed in 40 μl of kinase buffer containing 10 μ*M* [γ-^{32}P]dATP and 3 μg GST-IκBα(1–54) for 20 min at room temperature. The reaction was stopped by the addition of SDS gel sample buffer and analyzed by SDS-PAGE and autoradiography.

Statistical Analysis

Data are expressed as the mean of at least four independent experiments ± SEM. Statistical comparison was performed using unpaired Student's t test. In case of failed normality test, we used the Mann-Whitney rank sum test. Significant changes were defined as those with a p value <0.05.

Results

Time- and Dose-Dependent Activation of IκB-Kinase by CCK

To assess the ability of CCK on NF-κB-binding activity, IκBα proteolysis and IKK activity, rat pancreatic lobules were incubated with CCK-8 at different doses for 30 min. CCK induced a dose-dependent increase in NF-κB-binding activity with a maximal stimulatory effect detected at 10 and 100 n*M* (fig. 1, *top*, lanes 5 and 6). The specificity of NF-κB-binding activity was confirmed by competition assays (data not shown). Cytoplasmic extracts were analyzed by Western blotting using anti-IκBα and anti-IκBβ antibodies. CCK 1–100 n*M* induced a dose-dependent degradation of cytoplasmic IκBα (fig. 1, *middle*, lanes 4–6). IκBβ was not affected by CCK stimulation. To analyze whether degradation of IκBα correlates with IκB-kinase activity, endogenous IKK activity was immunoprecipitated with an anti-IKK mAb. Immunocomplexes were assayed for kinase activity by incubation with GST-IκBα(1–54) in the presence of [γ-^{32}P]ATP. An activation of IKK activity was detected at 1 n*M* CCK with a maximum at 10 and 100 n*M* CCK (fig. 1, *bottom*). The amounts of precipitated IKKα and IKKβ were verified on the same membrane. The activation of IKK correlates with IκBα degradation and the appearance of NF-κB-binding activity in the nucleus.

To investigate the kinetics of IKK activation in response to CCK, pancreatic lobules were incubated with 100 n*M* CCK and assayed for NF-κB-binding activity, IκBα degradation and IKK activity (fig. 2). NF-κB activity was detected after 10 min *(top)* and peaked after 30 min of stimulation *(top)*. The IκBα signal decreased

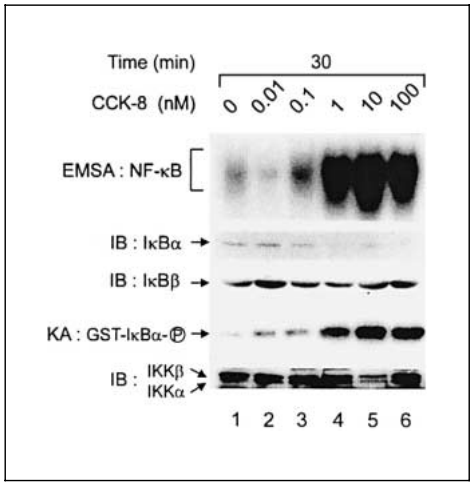

Fig. 1. CCK induces activation of IκB-kinase in a dose-dependent manner. Pancreatic lobules were left untreated (lane 1) or incubated with the indicated concentrations of CCK-8 for 30 min (lanes 2–6). *Top*: Nuclear proteins were extracted and EMSAs performed using a κB-specific probe. Running position of the NF-κB complex is indicated. *Middle*: Cytoplasmic protein extracts were prepared and equal amounts were analyzed by immunoblotting (IB) as indicated in Methods using IκBα- and IκBβ-specific antibodies. *Bottom*: Cytoplasmic protein extracts were immunoprecipitated with an IKKα-specific antibody and incubated with GST-IκBα(1–54) as substrate in the presence of [γ-^{32}P]ATP (KA). The amounts of immunoprecipitated IKKα and IKKβ were verified on the same membrane (IB).

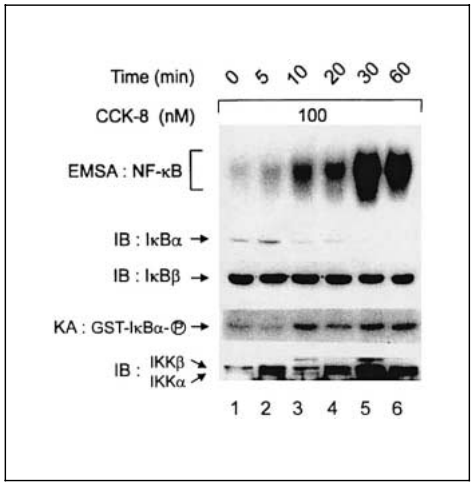

Fig. 2. CCK induces activation of IκB-kinase in a time-dependent manner. Pancreatic lobules were left untreated (lane 1) or incubated with CCK-8 100 n*M* for time periods indicated (lanes 2–6). *Top*: Nuclear proteins were extracted and EMSAs performed using a κB-specific probe. Running position of the NF-κB complex is indicated. *Middle*: Cytoplasmic protein extracts were prepared and equal amounts were analyzed by immunoblotting (IB) as indicated in Methods using IκBα- and IκBβ-specific antibodies. *Bottom*: Cytoplasmic protein extracts were immunoprecipitated with an IKKα-specific antibody and incubated with GST-IκBα(1–54) as substrate in the presence of [γ-^{32}P]ATP (KA). The amounts of immunoprecipitated IKKα and IKKβ was verified an the same membrane (IB).

gradually beginning at 10 min and was completely absent at 30 min *(middle)*. IKK activity was increased after 10 min following stimulation with CCK and lasted up to 60 min (fig. 2, *bottom*). As expected IKK activity correlated with degradation of IκBα and the appearance of NF-κB-binding activity.

Kinetic of TAP Formation and Inhibition by Pefabloc in Isolated Pancreatic Lobules

In an attempt to investigate the effect of blocking intrapancreatic trypsinogen activation on CCK-mediated NF-κB induction, we used the potent and cell-permeable serine-protease inhibitor Pefabloc SC. Pefabloc inhibited CCK-induced intrapancreatic trypsinogen activation as detected by the amount of free TAP. Pefabloc at 1,000 μ*M* suppressed CCK-induced TAP release to less than 30% (fig. 3a). Activation of trypsinogen was detected within 10 min of exposing rat pancreatic lobules to a supramaximally stimulating concentration of CCK-8 in vitro. The increase in TAP concentration was maximal at 30 min following CCK-8 stimulation (fig. 3b).

The Serine Protease Inhibitor, Pefabloc, Abrogates CCK-Induced NF-κB Activation by Blocking IκBα Degradation

Pancreatic lobules were pretreated with the serine protease inhibitor Pefabloc for 15 min at varying doses and stimulated with 100 n*M* CCK for 30 min. CCK-induced NF-κB activation was inhibited by Pefabloc in a dose-dependent manner (fig. 4a). 1,000 μ*M* Pefabloc completely blocked CCK-induced NF-κB activation (lanes 2 and 5). This result does not allow a conclusion regarding the stage at which blockage of NF-κB activation occurs. To test whether degradation of IκBα could take place in the presence of Pefabloc, cytoplasmic extracts were analyzed by Western blotting using anti-IκBα and anti-IκBβ antibodies (fig. 4b). The cytoplasmic IκBα signal completely disappeared after stimulation with CCK-8 (compare lanes 1 and 2). IκBα protein levels were not affected by CCK-8 when pancreatic lobules were preincubated with Pefabloc 1,000 m*M* (lane 5). In contrast, IκBβ protein levels did not change.

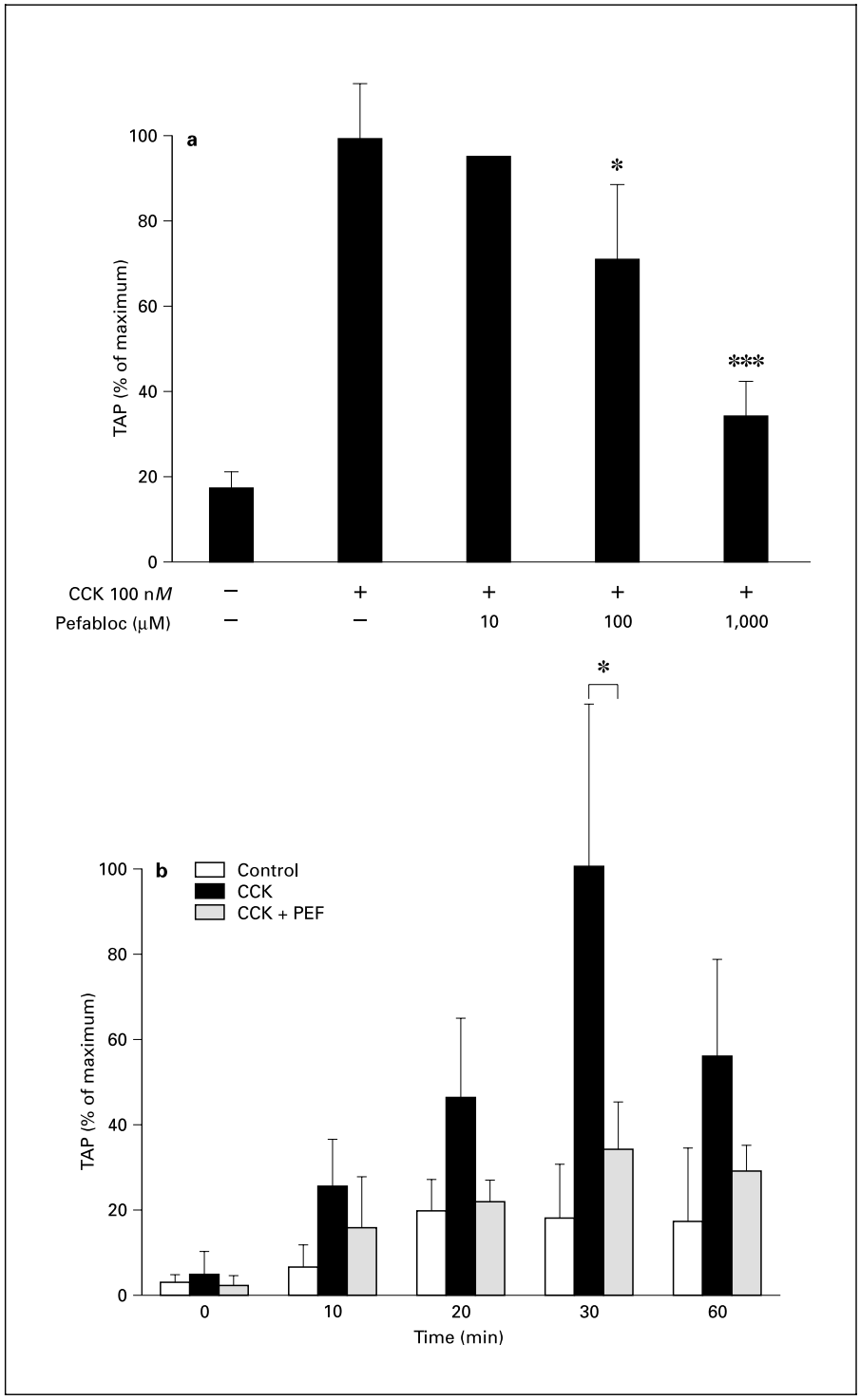

Fig. 3. Pefabloc inhibits TAP release in isolated pancreatic lobules following incubation with supramaximal CCK. a Pancreatic lobules were left untreated or pretreated with Pefabloc at doses indicated for 15 min followed by incubation with CCK-8 100 n*M* for 30 min. b Pancreatic lobules were pretreated with Pefabloc 1,000 μ*M* or medium and incubated with CCK-8 100 n*M* or medium (control) for time periods indicated. Pancreatic lobules were homogenized and supernatants assayed for TAP concentration as described in Methods. * $p < 0.05$, *** $p < 0.005$.

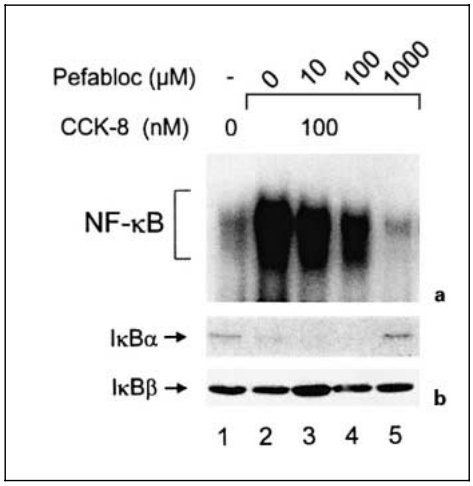

Fig. 4. Inhibition of trypsinogen activation prevents NF-κB induction and IκBα degradation by CCK in rat pancreatic lobules. a Pancreatic lobules were left untreated (lane 1) or pretreated with Pefabloc at doses indicated for 15 min followed by incubation with CCK-8 100 n*M* for 30 min (lanes 2–5). Nuclear proteins were extracted and EMSAs performed using a κB-specific probe. Running position of the NF-κB complex is indicated. b Cytoplasmic protein extracts of same samples were separated in SDS-PAGE and immunoblotted with antibodies specific for IκBα and IκBβ as described in Methods.

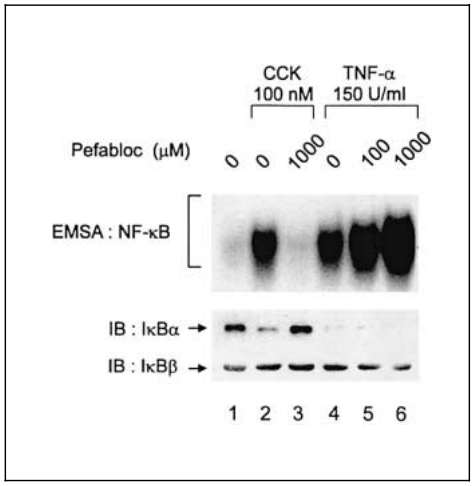

Fig. 5. Inhibition of trypsinogen activation prevents NF-κB induction by CCK but not TNF-α in rat pancreatic lobules. Pancreatic lobules were left untreated (lanes 1, 2 and 4) or pretreated with Pefabloc 100 μ*M* (lane 5) or 1,000 μ*M* (lanes 3 and 6) for 15 min followed by incubation with CCK-8 100 n*M* (lanes 2 and 3) or TNF-α 150 U/ml (lanes 4–6) for 30 min. Nuclear proteins were extracted and EMSAs performed using a κB-specific probe. Running position of the NF-κB complex is indicated. Cytoplasmic protein extracts of same samples were separated in SDS-PAGE and immunoblotted (IB) with antibodies specific for IκBα and IκBβ as described in Methods.

Pefabloc Abrogates NF-κB Activation by CCK But Not by TNF-α

To investigate whether the inhibitory effect of Pefabloc is specific for CCK-induced NF-κB activation, pancreatic lobules were pretreated with Pefabloc for 15 min and subsequently stimulated with CCK-8 or TNF-α. Pancreatic lobules were harvested and nuclear extracts were prepared and incubated with a κB-specific probe (fig. 5). CCK-8 as well as TNF-α induced a strong NF-κB-binding activity (*top*, lanes 2 and 4). Pretreatment with Pefabloc resulted in a complete inhibition of NF-κB activity induced by CCK (*top*, lane 3). In contrast, in pancreatic lobules stimulated with TNF-α, NF-κB-binding activity was slightly increased by Pefabloc (*top*, lanes 5 and 6). These data suggest different mechanisms of NF-κB activation by TNF-α and CCK in pancreatic lobules. To investigate whether NF-κB activation correlates with IκBα degradation, cytoplasmic extracts were subjected to Western analysis. IκBα is completely degraded following stimulation with TNF-α even when pancreatic lobules were pretreated with Pefabloc (*middle*, lanes 4–6). In contrast, Pefabloc completely prevented degradation of IκBα by CCK-8 (*middle*, lane 3). IκBβ was not affected (*bottom*). These data argue against an inhibitory effect of Pefabloc on the proteasome.

Serine Protease Activity Participates in the Activation of IKK by CCK

To test whether Pefabloc pretreatment interferes with endogenous IKK activation, pancreatic lobules were pretreated with different concentrations of Pefabloc followed by incubation with CCK-8. Endogenous IKK activity was immunoprecipitated with an anti-IKK mAb. Immunocomplexes were assayed for kinase activity by incubation with GST-IκBα(1–54) in the presence of [γ-^{32}P]ATP. An activation of IKK activity was detected at 100 n*M* CCK and inhibited in a dose-dependent fashion with the maximal effect at 1,000 μ*M* Pefabloc (fig. 6a). To verify that this effect is not specific for Pefabloc, we used FUT175, another serine protease inhibitor. Preincubation of pancreatic lobules with FUT175 prevented IKK activation induced by CCK similar to Pefabloc (fig. 6b). Taken together, these data suggest that serine protease activity participates in the activation of IKK by CCK.

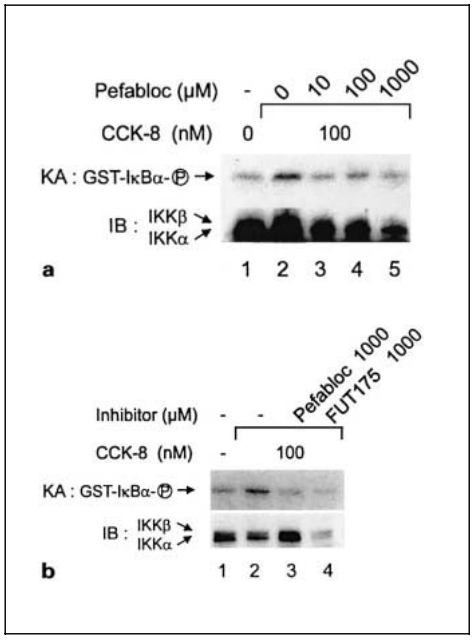

Fig. 6. Inhibition of trypsinogen activation prevents IKK activation by CCK in rat pancreatic lobules. a Pancreatic lobules were left untreated (lane 1) or pretreated with Pefabloc at doses indicated for 15 min followed by incubation with CCK-8 100 n*M* (lanes 2–5) for 30 min. b In a similar experiment pretreatment with Pefabloc 1,000 μ*M* was compared to FUT175 1,000 μ*M*. Endogenous IKK complexes were immunoprecipitated and incubated with GST-IκBα(1–54) as substrate in the presence of [γ^{32}P]ATP (KA). The amount of immunoprecipitated IKKα and IKKβ was verified on the same membrane by immunoblotting (IB).

Discussion

We and others have previously shown that supramaximal concentrations of cerulein which induce pancreatitis lead to the induction of the transcription factor NF-κB. The onset of NF-κB activation correlated with the degradation of IκBα [9–11]. Treatment with various inducers of NF-κB leads to the phosphorylation of two critical N-terminal serine residues at positions 32 and 36 of IκBα. Once phosphorylated, IκBα becomes a target for degradation by the ubiquitin-26S proteasome pathway leading to translocation of NF-κB/Rel dimer into the nucleus. A high-molecular-weight multisubunit kinase complex has been isolated from cytosolic extracts of HeLa cells, which specifically phosphorylates serines 32 and 36 of IκBα. This complex contains two closely related kinases, IKKα and IKKβ [13–16]. IKKα and IKKβ form homo- and heterodimers, but the active form of the protein in vivo may be the heterodimer [14, 21]. Here we show that CCK-induced IκBα degradation and NF-κB activation correlate with the induction of IKKα/IKKβ activity. IKK activity is only induced at supramaximal doses of CCK and detected as early as 10 min following incubation with CCK. How CCK signaling relates to the IKK-complex is unclear.

NF-κB is activated within the pancreas during the very early stages of cerulein pancreatitis and can be detected long before evidence of either morphological or biochemical injury to the gland [9–11]. This NF-κB activation requires both Ca^{2+} and protein kinase C as messengers in vitro [17]. Interestingly, cerulein-induced trypsinogen activation is dependent on Ca^{2+} as well [22]. Despite this evidence it remains unclear whether the activation of NF-κB occurs as an independent step in the pathogenesis of pancreatitis, or whether it is the result of zymogen activation, in particular of trypsinogen. In the present study, the relation of serine protease activity and NF-κB activation was investigated more directly. We used an in vitro model of isolated pancreatic lobules exposed to supramaximal CCK concentrations. As a marker for trypsinogen activation we measured TAP, the N-terminal part of trypsinogen which is cleaved to activate trypsin [18]. Quantification of TAP is a direct measurement of the amount of activated trypsinogen as one TAP molecule is generated for each molecule of trypsinogen cleaved.

Based on the following observations, we hypothesize that trypsinogen activation is involved in the induction of NF-κB: (i) kinetics of NF-κB and trypsinogen activation are very similar, activation is detected at 10 min and peaks at 30 min; (ii) only supramaximal doses of CCK which are known to activate trypsinogen induce NF-κB; (iii) doses of potent and specific serine protease inhibitors which block trypsinogen activation prevent CCK-induced NF-κB activation, and (iv) this inhibition is specific for CCK-induced NF-κB activation while other stimuli such as TNF-α are not affected by trypsin inhibitors.

It has been previously shown that activated trypsinogen and elevated TAP levels can be detected in the pancreas within 15–30 min of supramaximal secretagogue stimulation in vivo [23]. Exposure of isolated pancreatic acini to hyperstimulation by CCK results in the rapid (<15 min) conversion of procarboxypeptidase A_1 to carboxypeptidase A_1 [24] and to enhanced trypsin activity [25, 26]. A similar time course has been observed using direct detection of premature protease activation in living pancreatic acinar cells [27]. We and others have previously shown that NF-κB is activated following cerulein pan-

creatitis within 15 min with a maximum at 30 min [9–11]. In pancreatic lobules, NF-κB binding and IKK activity is detected 10 min after stimulation with supramaximal doses of CCK with a maximal effect after 30 min [17]. Similar results have been shown using isolated pancreatic acini [10, 11]. Recently we were able to provide evidence for different modes of NF-κB/Rel activation in pancreatic lobules [30].

There is the possibility that serine protease inhibitors which block trypsinogen activation also interfere with proteasome-dependent degradation of IκBα. To circumvent this problem we used an NF-κB inducer which is known to be independent of trypsinogen activation. TNF-α-induced NF-κB is not blocked by preincubation of pancreatic lobules with Pefabloc, suggesting that Pefabloc does not interfere with proteasome-mediated degradation of IκBα. Indeed, Singh et al. [33] were able to show that Pefabloc has a specific effect on a still unknown serine protease which seems to be responsible for maintaining the normal apical F-actin distribution. Furthermore, they could not detect any effect of Pefabloc on protein kinase C, calmodulin-dependent protein phosphatase (calcineurin), or calpain activity. Interestingly, Pefabloc further increased TNF-α-stimulated NF-κB activation which in fact indicates that serine proteases act antagonistically on that pathway. In contrast, Pefabloc completely blocked CCK-induced NF-κB activation. The interference of the compound involves signaling upstream of IKK activation since Pefabloc treatment prevented CCK-induced activation of IKKs. To rule out nonspecific effects of this inhibitor, we used another serine protease inhibitor, FUT175, which showed similar results.

The inhibitory effect of Pefabloc on NF-κB activation correlates with the inhibition of CCK-induced TAP levels. This leaves the question how serine proteases contribute to NF-κB activation in the pancreas? A recent study has demonstrated that after cerulein hyperstimulation TAP immunoreactivity appeared in a vesicular supranuclear compartment that did not overlap with zymogen granules. This compartment demonstrated immunoreactivity for a marker for lysosomes and recycling endosomes [28]. Another study has suggested that trypsinogen subsequent to its activation is released into the cytosol [24, 29]. Although the cytosolic compartment of the IKK complexes and their activation is unknown, it is possible that active trypsin or other serine proteases change protein/protein interactions or the conformation of the IKK complexes directly and thereby contribute to their activation. These findings further underline the observation of an additional pathway in which activation of proteases stimulate NF-κB via activation of the IKK complex. The role of trypsin activation in NF-κB signaling has been discussed in several studies providing different results. Our data together with data presented by Hietaranta et al. [32] support a participating role of trypsin or other serine proteases in NF-κB activation in acinar cells. In contrast, others could show that CCK independently activates intracellular trypsinogen and NF-κB in rat pancreatic acinar cells [31]. Using Chinese hamster ovary-CCK_A cells, which do not express trypsinogen, they were able to inhibit CCK-mediated NF-κB activation by using protease inhibitors. All studies agree that pancreatic acinar cells do not require NF-κB for basal or CCK-mediated trypsinogen activation. In light of different modes of NF-κB activation in pancreatic lobules, various signaling pathways and cross-talks between them seem to be responsible for these discrepancies. Furthermore, cytoskeletal compartments are involved in NF-κB and trypsinogen activation. This might be an additional explanation for the discrepancies presented in different studies.

In summary, our results provide evidence that intrapancreatic activation of proteases might contribute to NF-κB induction in response to supramaximal concentrations of CCK. Moreover, this study shows that IKK activation is involved in CCK signaling and is blocked by serine protease inhibitors after CCK stimulation.

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