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X. Wang, Q. Wang, K. L. Ives and B. M. Evers

Clin. Cancer Res., September 15, 2006; 12 (18): 5346-5355.

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Ethanol sensitizes NF- κ B activation in pancreatic acinar cells through effects on protein kinase C- ϵ

A. Satoh, A. S. Gukovskaya, J. R. Reeve Jr, T. Shimosegawa and S. J. Pandol

Am J Physiol Gastrointest Liver Physiol, September 1, 2006; 291 (3): G432-G438.

[Abstract] [Full Text] [PDF]

Protective roles of redox-active protein thioredoxin-1 for severe acute pancreatitis

S. Ohashi, A. Nishio, H. Nakamura, M. Kido, S. Ueno, N. Uza, S. Inoue, H. Kitamura, K. Kiriya, M. Asada, H. Tamaki, M. Matsuura, K. Kawasaki, T. Fukui, N. Watanabe, H. Nakase, J. Yodoi, K. Okazaki and T. Chiba

Am J Physiol Gastrointest Liver Physiol, April 1, 2006; 290 (4): G772-G781.

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Curcumin ameliorates ethanol and nonethanol experimental pancreatitis

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Gukovsky, Ilya, Christopher N. Reyes, Eva C. Vaquero, Anna S. Gukovskaya, and Stephen J. Pandol. Curcumin ameliorates ethanol and nonethanol experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 284: G85–G95, 2003; 10.1152/ajpgi.00138.2002.—Treatments for pancreatitis are limited. Activation of transcription factor NF- κ B, a key regulator of inflammatory molecule expression, is an early event in experimental pancreatitis and correlates with the inflammatory response. We report here that curcumin, a natural phytochemical known to inhibit NF- κ B and activator protein (AP)-1, another important proinflammatory transcription factor, ameliorates pancreatitis in two rat models. In both cerulein pancreatitis and pancreatitis induced by a combination of ethanol diet and low-dose CCK, curcumin improved the severity of the disease as measured by a number of parameters (histology, serum amylase, pancreatic trypsin, and neutrophil infiltration). Curcumin markedly inhibited NF- κ B and AP-1 activation, assessed by DNA binding and degradation of inhibitory I κ B proteins, and the induction of mRNAs for cytokines IL-6 and TNF- α , the chemokine KC, and inducible nitric oxide synthase in pancreas. Curcumin also blocked CCK-induced NF- κ B and AP-1 activation in isolated pancreatic acini. Our findings indicate that blocking key signals of the inflammatory response ameliorates pancreatitis in both ethanol and nonethanol models. They suggest that curcumin, which is currently in clinical trials for cancer prevention, may be useful for treatment of pancreatitis.

nuclear factor- κ B; activator protein-1; cerulein; cholecystokinin; chemokines; *N*-acetylcysteine

ALTHOUGH THE COMPLETE MOLECULAR mechanism of pancreatitis has not been established, there is a substantial body of evidence suggesting a critical role for the inflammatory response in this disease (4, 31, 40). Recent results from our group and others (13, 14, 17, 20, 22, 25, 31, 47) indicate that the inflammatory response is initiated by injured pancreatic acinar cells that produce inflammatory mediators, such as cytokines (e.g., TNF- α) and adhesion molecules (e.g., ICAM-1), ultimately leading to systemic complications. Blocking the activation of these mediators by using various ap-

proaches ameliorated pancreatitis in different experimental models (4, 9, 11, 13, 14, 17, 20, 25, 31, 47).

Further evidence suggests that the inflammatory response of pancreatitis mediates, at least in part, the parenchymal injury. That is, inhibition of cytokines and chemokines, removal of neutrophils from the experimental animal, or blockade of neutrophil infiltration into the pancreas by genetic deletion of ICAM-1 all lead to attenuation of the increases in serum amylase and lipase, microscopic evidence of pancreatic parenchymal damage, and intrapancreatic activation of trypsin (9, 11, 13, 14, 17, 19, 20, 25, 31, 37, 47).

Considering the central importance of the inflammatory response in pancreatitis, therapeutic strategies should be aimed at the key steps leading to this response. Because of the multitude of cytokines, chemokines, and other inflammatory molecules involved in this disease process, one can argue that the most effective strategy should target upstream “master regulators” of these molecules. Recent data (8, 11, 20, 22, 23, 25, 34, 38, 41, 42, 47) suggest that one such key regulator is NF- κ B.

NF- κ B comprises a family of transcription factors regulating the inflammatory, immune, and cell death responses (1, 12, 36, 45, 46). NF- κ B is composed of hetero- or homodimers of the Rel family proteins. In unstimulated cells, NF- κ B is kept inactive in the cytoplasm by association with the inhibitory (I κ B) proteins. On activation, I κ Bs are phosphorylated by specific IKK kinases and are rapidly degraded via proteasome-involving pathways. NF- κ B then translocates into the nucleus and activates the expression of a multitude of genes that have κ B-binding sites in their promoters/enhancers.

NF- κ B activation, at least in part, regulates the expression of cytokines (TNF- α , IL-6), chemokines (IL-8, KC, Mob-1), and other inflammatory molecules such as ICAM-1 and the inducible nitric oxide synthase (iNOS), all of which are upregulated in pancreatitis (4, 9, 11, 13, 14, 17, 20, 25, 31, 34, 42, 47). Recent data link the inflammatory response to NF- κ B in several exper-

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imental models of pancreatitis (8, 11, 20, 23, 25, 34, 38, 41, 42, 47). In particular, NF- κ B activation in acinar cells is one of the very early events in pancreatitis induced by high-dose cerulein, an analog of CCK, a widely used and well-characterized model (20, 23, 41). CCK and cerulein also activate NF- κ B in isolated pancreatic acinar cells (16, 20–22, 47).

Furthermore, we recently found upregulation of NF- κ B and inflammatory molecule expression in a model of pancreatitis in which an ethanol diet sensitizes rats to pancreatitis induced by low-dose CCK (EtOH + CCK model) (34). The importance of these findings is that they provide data on the mechanism of ethanol pancreatitis, which have been unavailable because of the lack of animal models of alcohol-mediated pancreatitis. Whether the inflammatory response plays a causative role in this model of acute alcoholic pancreatitis, similar to what has been established in nonethanol models, remains to be elucidated.

An attractiveness of NF- κ B as a therapeutic target in pancreatitis is that it is “silent” in normal pancreas (5, 20, 23, 41, 47). In addition, inhibiting NF- κ B activation should alleviate inflammation not only in the pancreas but also in distant organs affected during pancreatitis (25, 38).

Currently (12, 46), there are no specific small-molecule inhibitors of NF- κ B, the type of inhibitor that is most suitable for use in acute pancreatitis. Most of the studies that attempted to ameliorate the pancreatitis inflammatory response by inhibiting NF- κ B activation showed an attenuation of the disease (8, 11, 20, 23, 38, 42), with one notable exception (41). The contradictory results may have been caused by several factors, including differences in the inhibitors used and methods of their administration. Furthermore, an NF- κ B inhibitor that works in vitro may not be effective in vivo, as we found for the proteasomal inhibitor MG-132 (this study, see DISCUSSION; also see Refs. 5 and 47).

Recent studies indicate that the bioflavonoid curcumin inhibits NF- κ B activation in a number of cell types (6, 7, 24, 26, 32, 39). Curcumin is the pigment in turmeric (*Curcuma longa*) that gives the yellow color to curry dishes (2). It has long been known for its anti-inflammatory and cancer chemopreventive activities and is used both in vitro and in vivo (2, 6, 24, 26, 32, 39). The molecular mechanism of NF- κ B inhibition by curcumin involves blockade of IKK activation and subsequent I κ B degradation (26, 32, 39). Of importance, curcumin is not toxic and can be administered in large quantities (2, 24).

Another advantage of curcumin is that it also inhibits activator protein (AP)-1, a transcription factor that often acts in concert with NF- κ B to regulate the expression of chemokines (e.g., IL-8) and other inflammatory molecules (3, 28, 43). We showed that AP-1 is activated in taurocholate-induced pancreatitis and in CCK-stimulated pancreatic acinar cells (21, 42). AP-1 activation in the cerulein and EtOH + CCK models of pancreatitis has not been studied. The mechanism of AP-1 activation involves phosphorylation of c-Jun, a

component of the AP-1 complex, by the JNK kinase (28, 43). Curcumin inhibits JNK (7).

There is a growing interest in natural compounds for the treatment of various inflammatory disorders. In this study, we tested whether curcumin is effective for in vivo application in ethanol and nonethanol pancreatitis. We found that curcumin inhibited the inflammatory response and significantly ameliorated pancreatitis in both models.

MATERIALS AND METHODS

Cerulein-induced pancreatitis. Care and handling of the animals were approved by the Animal Research Committees of the VA Greater Los Angeles Healthcare System and University of Southern California (USC) in accordance with the National Institutes of Health guidelines. Cerulein (5 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) or vehicle control was administered to male Sprague-Dawley rats (275–350 g; Harlan, Madison, WI) by continuous 6-h intravenous infusion as previously described (17, 19, 20, 47). Animals were euthanized by CO₂-induced asphyxiation, and the blood and pancreas were harvested for measurements.

Ethanol and CCK-induced pancreatitis. Sprague-Dawley rats (400–500 g) received intragastric infusion of ethanol-containing or control diet for 6 wk followed by a 6-h infusion of a low dose (3,000 pmol $\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of CCK-8. Animals were euthanized by CO₂-induced asphyxiation, and the blood and pancreas were harvested for measurements. This EtOH + CCK model of pancreatitis has been described in detail (34). Because we previously characterized the effect of ethanol feeding alone in this model, the present study focused on the effect of curcumin on measures of pancreatitis induced by the combined EtOH + CCK treatment.

Preparation of dispersed pancreatic acini. Acini were prepared from rat pancreas using a collagenase digestion method (33) and then incubated at 37°C in 199 medium as previously described (5, 15, 17, 20, 47).

Curcumin administration. Curcumin was dissolved in DMSO and administered to the animal by intravenous infusion together with cerulein or CCK-8 at a dose of 35 mg $\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. Thus, during the 6-h treatment, rats received a total of 200 mg/kg (~0.5 mmol/kg) curcumin. In separate experiments, we tested that administration of the vehicle or curcumin alone to control animals did not have a significant effect on any parameter measured. In experiments on isolated acini, curcumin was added into the cell suspension to a final concentration of 100 μM .

Measures of pancreatitis. For histological evaluation, pancreatic tissue was fixed in 10% buffered formaldehyde, then embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy as described previously (17–19, 34). Serum amylase and lipase were measured by using a Hitachi 707 analyzer (Antech Diagnostics, Irvine, CA). Active trypsin was measured in pancreatic tissue homogenates as described previously (19, 20, 42) by using a specific substrate, Boc-Gln-Ala-Arg-AMC (29). Cleavage of this substrate by trypsin releases 7-amino-4-methylcoumarin (AMC), which emits fluorescence at 440 nm with excitation at 380 nm. Trypsin activity in each sample was determined using a standard curve for purified trypsin.

For measurement of neutrophil infiltration, pancreatic tissue was immunostained for neutrophils as described previously (17, 19, 34, 37, 42). In brief, 8- μm -thick serial cryostat sections were cut and mounted on glass slides. The tissue sections were then stained with FITC-labeled rabbit anti-rat

polymorphonuclear leukocyte antibody (Accurate, Westbury, NY). The number of infiltrating neutrophils was obtained by counting the neutrophils at 40 \times magnification in an average of 50 fields covering at least 1,000 acinar cells. For each animal, neutrophil numbers were expressed as a percentage of acinar cells.

Measurement of pancreatic secretion in vivo. Pancreatic secretion in rats was measured as previously described (19). Briefly, biliopancreatic secretion was allowed to drain freely for 30 min before initiation of the experiment. Then, secretion was collected in tubes in 10-min fractions to determine the basal amylase output, after which 0.25 μ g/kg cerulein was given as an intravenous bolus and collection continued for 40 min in a similar manner. Collection volumes were measured by weight, and the amylase output was calculated by determining amylase concentration in the collected fractions. The mean amylase output obtained from two consecutive 10-min fractions after the stabilization period was taken as the basal secretion and considered as 100%. The results were expressed as percent increase over basal amylase output 40 min after cerulein or vehicle infusion.

Amylase secretion by isolated acinar cells. Secretion was measured spectrophotometrically using Phadebas amylase kit (Pharmacia Diagnostics) as previously described (15). Values for amylase secretion were expressed as ratios between the amount of amylase released into the extracellular medium and the total cellular amylase determined by permeabilizing cells with 0.1% SDS in 10 mM phosphate buffer (pH 7.8).

Measurement of LDH release. Acinar cell necrosis was determined by the release of lactate dehydrogenase (LDH) into the incubation medium, as described previously (15–17). LDH activity in the extracellular medium was measured using LDH detection ELISA kit (Roche Diagnostics, Indianapolis, IN).

Preparation of nuclear and cytosolic extracts. Nuclear protein extracts were prepared as described previously (5, 17, 20, 34, 42, 47). Briefly, samples of pancreatic tissue or isolated acini were rinsed in ice-cold PBS and homogenized in the hypotonic buffer A (20) supplemented with 1 mM PMSF, 1 mM DTT, and 5 μ g/ml each of protease inhibitors pepstatin, leupeptin, chymostatin, antipain, and apronin. The non-ionic detergent Igepal CA-630 was added to a final concentration of 0.3–0.4% (vol/vol) after incubation on ice for 20–25 min, followed by an additional incubation on ice for 1–2 min. The crude nuclear pellet was collected by microcentrifugation for 30 s. The supernatant (cytosolic protein) was removed, and the nuclear pellet was resuspended in the high-salt buffer C (20) containing 1 mM DTT, 1 mM PMSF, and the protease inhibitor cocktail described above. After nuclear membranes were rotated at 4 $^{\circ}$ C for up to 1 h, they were pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at –80 $^{\circ}$ C. Protein concentration in the nuclear extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

EMSA. EMSA was performed as described previously (5, 17, 20, 34, 42, 47). Aliquots of nuclear extracts with equal amounts of protein (2–10 mg) were mixed in 20- μ l reactions with a buffer containing (in mM) 10 HEPES (pH 7.8), 50 KCl, 0.1 EDTA, and 1 DTT with 10% (vol/vol) glycerol and 3 μ g poly(dI-dC). Binding reactions were started by the addition of ~60,000 counts/min of 32 P-labeled DNA probe and were allowed to proceed for 20–25 min at room temperature. The oligonucleotide probe for NF- κ B, 5'-GCAGAGGGGACTTTC-CGAGA, containing a consensus κ B binding site (underlined), or the AP-1 oligo probe, 5'-GGCTTGATGAGTCAGC-

CGGAA, containing phorbol ester-responsive element (TRE; underlined), was annealed with the complementary strands and end-labeled using Klenow DNA polymerase I (Stratagene, La Jolla, CA). Samples were electrophoresed at room temperature in 0.5 \times TBE buffer (1 \times TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) on a nondenaturing 4.5% polyacrylamide gel at 200 V. Gels were dried and directly analyzed in the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Measurement of I κ B α and I κ B β levels. I κ B α and I κ B β levels were measured in cytosolic protein extracts from pancreatic tissue as described previously (20) by Western blot analysis using polyclonal antibodies sc-371 and sc-945, respectively, from Santa Cruz Biotechnology (Santa Cruz, CA).

Cytokine mRNA detection by RT-PCR. The procedures were as we described previously (5, 17, 20, 34, 42, 47). Briefly, total RNA was obtained from pancreatic tissue with TRI reagent (Molecular Research Center, Cincinnati, OH), and its quality was verified by ethidium bromide staining of rRNA bands on a denaturing agarose gel. RNA was reverse transcribed with the SuperScript II preamplification kit (GIBCO-BRL, Rockville, MD) and subjected to PCR with rat gene-specific, intron-spanning primers described previously (5, 17, 20, 34, 42, 47). Target sequences were amplified at 56 $^{\circ}$ C using the same amount of cDNA for all primer sets. The RT-PCR products were all of expected size, and their identity was confirmed by direct sequencing. Negative controls were performed by omitting the RT step or cDNA template from the PCR amplification. The cycle number was adjusted between 22 (for the housekeeping ARP gene) and 36 cycles (for iNOS) to yield visible products within the linear amplification range. Resulting RT-PCR products were run on agarose gel and visualized by staining with ethidium bromide.

Measurement of caspase-3 activation. Caspase activity was measured as previously described (15). Briefly, samples of pancreatic tissue were rinsed with ice-cold PBS and homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Igepal CA-630, and 0.5 mM EDTA. Lysates were centrifuged for 10 min at 16,000 g, and the supernatants were collected. Caspase activity was determined at 37 $^{\circ}$ C in a buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mM DTT, and 20 μ M Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), a specific fluorogenic substrate for measurement of caspase-3-like activity. Cleavage of this substrate releases AMC that emits a fluorescent signal with excitation at 380 nm and emission at 440 nm. Fluorescence was calibrated by using a standard curve for AMC. The data were expressed as moles of AMC per minute per milligram of protein.

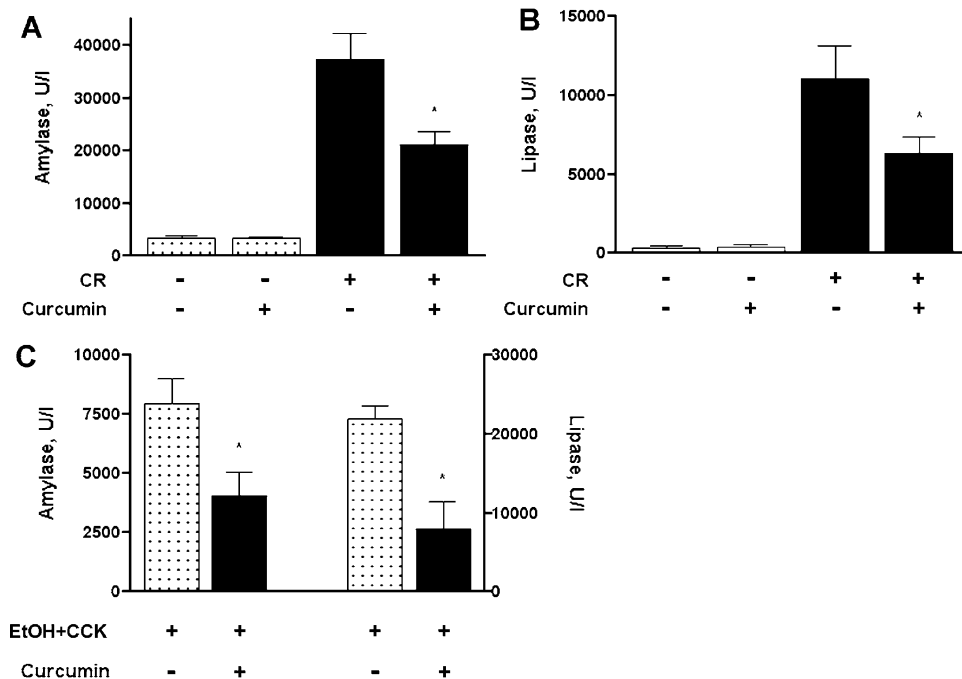
Statistical analysis of the data. Data analysis was done by using two-tailed Student's *t*-test. A *P* value <0.05 was considered statistically significant.

Materials. Cerulein was obtained from American Peptide (Sunnyvale, CA); CCK-8 was from Peninsula Laboratories (Belmont, CA); Ac-DEVD-AMC was from AnaSpec (San Jose, CA); Boc-Gln-Ala-Arg-AMC was from Bachem (Torrance, CA); [α - 32 P]dCTP (3,000 Ci/mmol) was from ICN Pharmaceuticals (Costa Mesa, CA); poly[d(I-C)] was from Boehringer Mannheim (Indianapolis, IN); and curcumin and all other chemicals were from Sigma Chemical (St. Louis, MO).

RESULTS

Effects of curcumin on measures of pancreatitis. The results in Figs. 1–4 show the effects of curcumin on

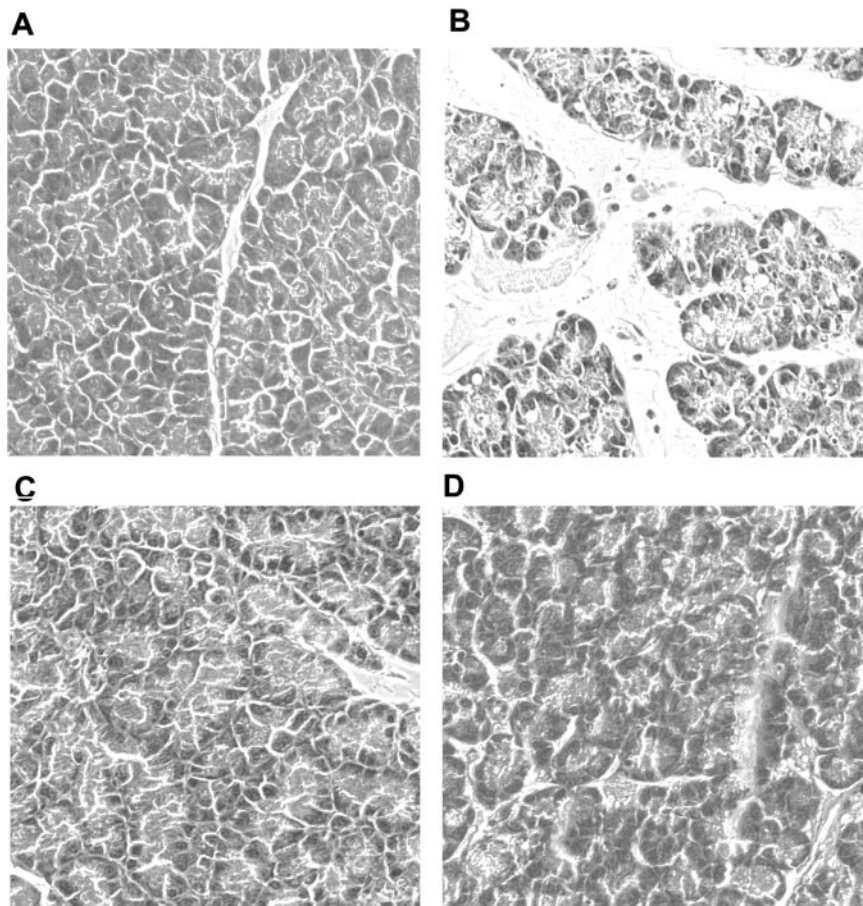
Fig. 1. Curcumin inhibits hyperamylasemia and hyperlipasemia in 2 models of pancreatitis. *A* and *B*: serum amylase and lipase levels in control rats and rats with pancreatitis induced by 6-h infusion of $5 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ cerulein (CR), without and with administration of curcumin. Curcumin was applied as described in MATERIALS AND METHODS. *C*: serum amylase and lipase levels in rats with pancreatitis induced by 6 wk intragastric ethanol feeding followed by a 6-h infusion of $3,000 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ CCK-8 (EtOH + CCK model), without and with administration of curcumin. In this model, the basal level of serum amylase in ethanol-fed rats was $2,140 \pm 320 \text{ U/l}$ ($n = 3$) and was unaffected by curcumin. Values are means \pm SE from at least 4 animals for each group. Values for animals with pancreatitis receiving curcumin were significantly lower ($*P < 0.05$) than for those without curcumin.



measures of pancreatitis in the two experimental models. Both in ethanol and nonethanol pancreatitis, curcumin significantly inhibited the increases in serum amylase and lipase (Fig. 1). Note that, as described

previously (34), the increase in serum amylase in pancreatitis animals is less in the EtOH + CCK model than in the cerulein model, whereas the reverse is true for serum lipase levels.

Fig. 2. Curcumin improves pancreatic histology in rat CR pancreatitis. Representative hematoxylin and eosin staining of pancreatic tissue sections from rats subjected to 6-h infusion of vehicle (*A*), CR (*B*), vehicle + curcumin (*C*), or CR + curcumin (*D*).



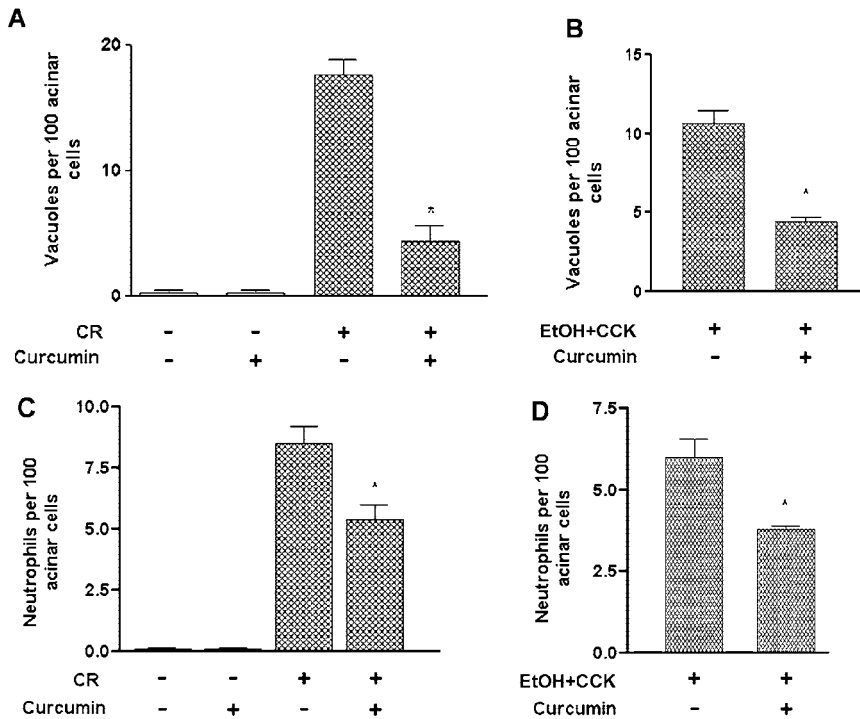


Fig. 3. Curcumin inhibits acinar cell vacuolization and intrapancreatic neutrophil infiltration in 2 models of pancreatitis. *A* and *B*: percentage of acinar cells with vacuoles in pancreatic tissue sections from control rats and rats with CR pancreatitis (*A*) and rats with EtOH + CCK pancreatitis (*B*), both treated without and with curcumin. *C* and *D*: neutrophil infiltration was measured by immunohistochemical detection of neutrophils on sections of pancreatic tissue from control rats and rats with CR pancreatitis (*C*) and rats with EtOH + CCK pancreatitis (*D*), both treated without and with curcumin. Values are means \pm SE from at least 4 animals for each group. Values for animals with pancreatitis receiving curcumin were significantly lower ($*P < 0.05$) than for those without curcumin.

Curcumin treatment improved pancreatic histology (Fig. 2) and decreased acinar cell vacuolization and neutrophil infiltration in the pancreas (Fig. 3). It also inhibited the pathological, intrapancreatic trypsin activation in both models (Fig. 4), which was particularly pronounced in the cerulein model (>70% inhibition).

To rule out the possibility that curcumin's effects were through inhibition of the action of cerulein or CCK-8 on CCK-receptor activation, we performed several control experiments. First, we tested the effect of curcumin on pancreatic secretion in vivo. We infused a bolus of 0.25 μ g/kg cerulein and measured the pancreatic secretion of amylase as described in Ref. 19. The cerulein-induced amylase secretion in rats that did not receive curcumin was $980 \pm 30\%$ ($n = 3$) of the basal amylase output (before administration of cerulein). In rats treated with curcumin, the cerulein-induced amylase secretion was $1,000 \pm 37\%$ ($n = 4$) of the basal, the

two values being statistically not different. Thus curcumin administration did not impair amylase secretion in vivo.

We next tested the effect of curcumin on amylase secretion in isolated acinar cells. Amylase release elicited by both maximal (1 nM) and supramaximal (100 nM) doses of CCK-8 was the same in the absence and presence of 100 μ M curcumin. In particular, 1 nM CCK-8 increased amylase secretion 6 ± 1 -fold ($n = 3$) over basal. Finally, we measured that 100 μ M curcumin did not cause additional LDH release in either control acinar cells or cells stimulated with 100 nM CCK-8, indicating that, under the conditions used, curcumin was not toxic for pancreatic acinar cells. These data imply that, both in vivo and in vitro, curcumin administration did not impair cerulein (or CCK) interaction with its receptor on acinar cells as well as the immediate signaling (i.e., G protein coupling) from the receptor.

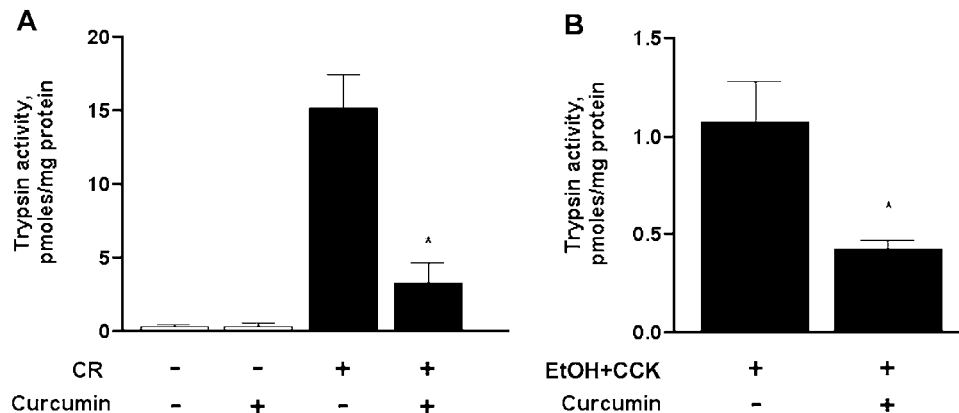
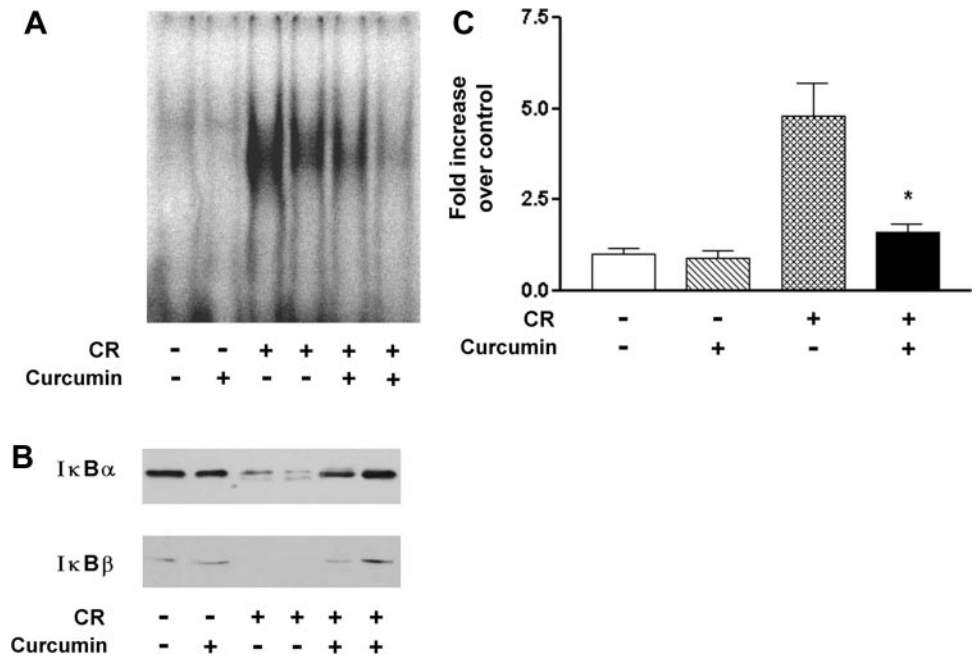


Fig. 4. Curcumin inhibits intrapancreatic trypsin activation in 2 models of pancreatitis. Active trypsin was measured with a fluorogenic assay in pancreatic tissue of control rats and rats with CR pancreatitis (*A*) and rats with EtOH + CCK pancreatitis (*B*), both treated without and with curcumin. In the EtOH + CCK model, the basal level of active pancreatic trypsin in ethanol-fed rats was 0.1 pmol/mg protein ($n = 3$) and was unaffected by curcumin. Values are means \pm SE from at least 4 animals for each group. Values for animals with pancreatitis receiving curcumin were significantly lower ($*P < 0.05$) than for those without curcumin.

Fig. 5. Curcumin inhibits pancreatic activation of NF- κ B and I κ B degradation in rats with CR pancreatitis. **A**: EMSAs for NF- κ B were performed on nuclear extracts from pancreatic tissue of control rats and rats with CR pancreatitis treated without and with curcumin. Each lane represents data for an individual animal. **B**: cytosolic extracts from pancreatic tissue of these rats were analyzed by Western blotting using antibodies against I κ B α and I κ B β . **C**: levels of NF- κ B activation were assessed by quantifying the intensities of NF- κ B bands in the PhosphorImager and shown for each group as means \pm SE relative to control ($n = 4-6$ animals/group). Values for animals with pancreatitis receiving curcumin were significantly lower ($*P < 0.05$) than for those without curcumin.



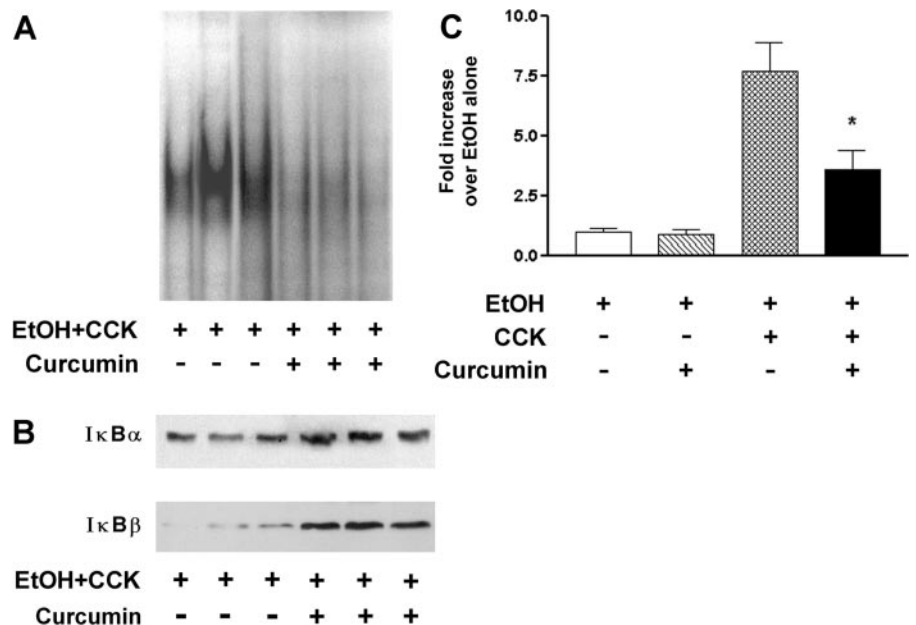
Effects of curcumin on transcription factor activation and inflammatory molecules' expression. Previously, our laboratory (20) demonstrated and characterized in detail NF- κ B activation in rat cerulein pancreatitis. The results in Fig. 5 show the effects of curcumin on NF- κ B DNA binding activity in the pancreas in this model. Virtually no NF- κ B activity was detected in control (vehicle-infused) rats treated either without or with curcumin (Fig. 5A). Cerulein caused severalfold NF- κ B activation, which was greatly attenuated by the curcumin treatment (Fig. 5, A and C).

NF- κ B activation results when the I κ B proteins (I κ Bs) are released from the NF- κ B complex by degra-

ation in the proteasome. As shown by us and others (20, 23, 41), cerulein hyperstimulation caused I κ B degradation in the pancreas. Curcumin prevented I κ B degradation in cerulein pancreatitis, whereas it did not change I κ B α and I κ B β levels in control animals (Fig. 5B).

We showed previously that NF- κ B was activated in the pancreas by the combined EtOH + CCK treatment, whereas neither ethanol diet alone nor the low-dose CCK applied in this model induced NF- κ B activation (34). Curcumin inhibited both the increase in NF- κ B binding activity and I κ B degradation in the EtOH + CCK model of pancreatitis as well (Fig. 6).

Fig. 6. Curcumin inhibits pancreatic activation of NF- κ B and I κ B degradation in rats with EtOH + CCK pancreatitis. **A**: EMSAs for NF- κ B were performed on nuclear extracts from pancreatic tissue of rats with EtOH + CCK pancreatitis treated without and with curcumin. Each lane represents data for an individual animal. **B**: cytosolic extracts from pancreatic tissue of these rats were analyzed by Western blotting using antibodies against I κ B α and I κ B β . **C**: levels of NF- κ B activation were assessed by quantifying the intensities of NF- κ B bands in the PhosphorImager and shown for each group as means \pm SE relative to control ($n = 3-4$ animals/group). Values for animals with pancreatitis receiving curcumin were significantly lower ($*P < 0.05$) than for those without curcumin.



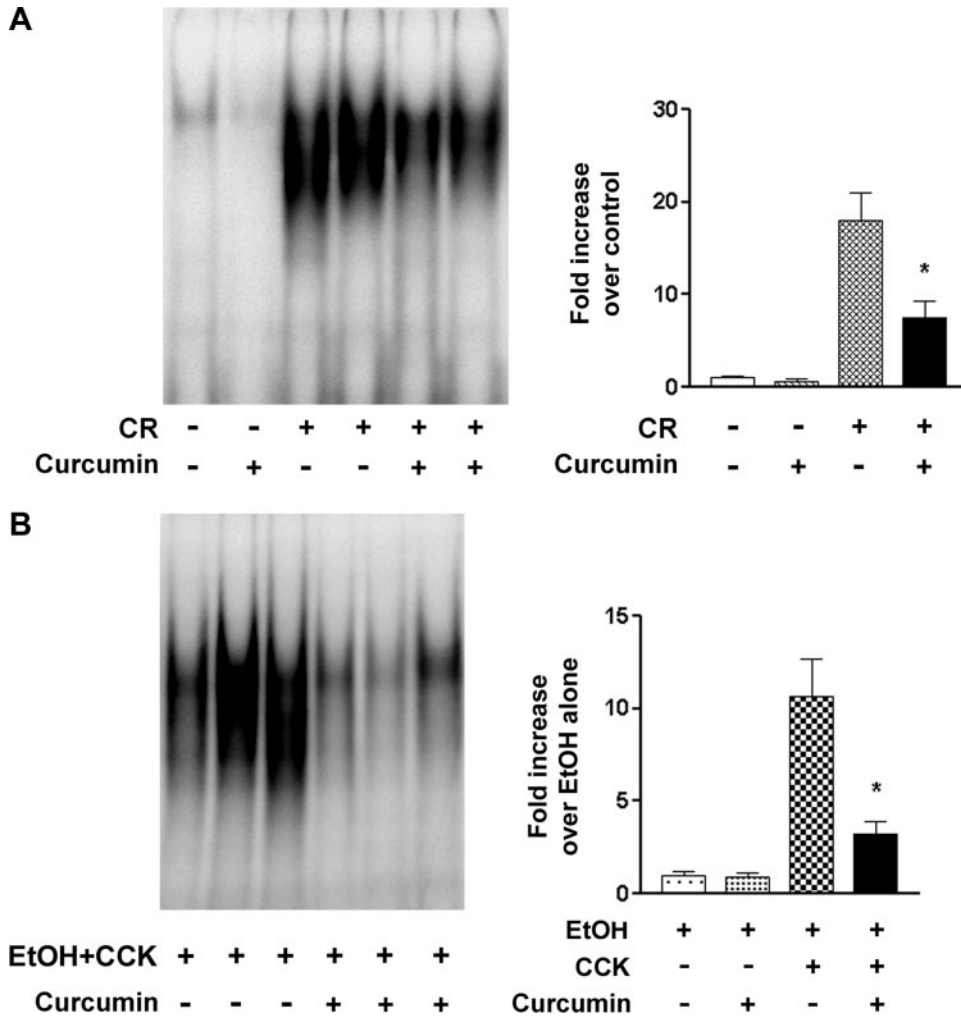


Fig. 7. Curcumin inhibits pancreatic activation of activator protein (AP)-1 in 2 models of pancreatitis. EMSAs for AP-1 were performed on nuclear extracts from pancreatic tissue of control rats and rats with CR pancreatitis (A) and rats with EtOH + CCK pancreatitis (B), both treated without and with curcumin. Each lane represents data for an individual animal. Right shows levels of AP-1 activation assessed by quantifying the intensities of AP-1 bands in the PhosphorImager and given for each group as means ± SE relative to control (n = 3–6 animals/group). Values for animals with pancreatitis receiving curcumin were significantly lower (*P < 0.05) than for those without curcumin.

In previous studies (20, 34), we showed the specificity of our NF-κB binding assay and characterized the subunit composition of activated NF-κB complexes in the pancreas. In particular, with the use of the “super-shift” analysis, we showed that in both models the activated NF-κB complexes are comprised of p65 (RelA) and p50 proteins.

The combined results in Figs. 5 and 6 indicate that in both ethanol and nonethanol pancreatitis, curcumin attenuates pancreatic activation of NF-κB by preventing the degradation of IκBs.

Figure 7 demonstrates that AP-1 is activated in the pancreas in both cerulein and EtOH + CCK models of pancreatitis. Treatment with curcumin attenuated AP-1 activation by >50% in cerulein pancreatitis and by >70% in the EtOH + CCK model.

To determine whether the effects of curcumin on NF-κB and AP-1 activation translated into changes in the expression of inflammatory molecules, we measured the effect of curcumin on pancreatic mRNA expression of several cytokines and other inflammatory molecules. As shown in Fig. 8, curcumin markedly inhibited pancreatic expression of IL-6, TNF-α, the chemokine KC (rodent analog of IL-8/GROα), and iNOS in both models. We and others (4, 14, 20, 31, 34,

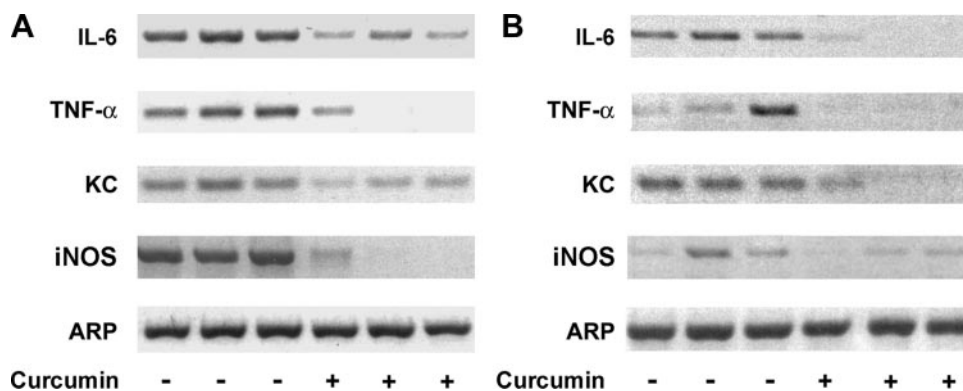
42) previously reported that these molecules’ pancreatic mRNA expression is very low or undetectable in control animals and increases dramatically (up to 100-fold) in experimental pancreatitis.

Effects of curcumin on caspase-3 activity in pancreas. In addition to its role in inflammation, NF-κB activation is generally known to inhibit apoptosis (1, 45, 46). NF-κB inhibits apoptosis by inhibiting activation of caspases, the key mediators of apoptosis (1, 10, 44). We and others (18, 27) showed that the death of parenchymal cells in pancreatitis occurs via both necrosis and apoptosis and, furthermore, that the severity of pancreatitis was less in models with increased apoptosis.

To determine a potential effect of curcumin on apoptosis in the experimental models under study, we measured caspase-3-like (DEVDase) activity in the pancreas. Activation of caspase-3 is a final step in the caspase cascade (44). Our laboratory (15) recently found that caspase-3 mediates CCK-induced apoptosis in isolated pancreatic acinar cells. Our laboratory (16) also showed that NF-κB negatively regulates caspase activation in CCK-stimulated acinar cells.

As shown in Fig. 9, curcumin augmented greater than twofold the increase in DEVDase activity in both models, suggesting that the activated state of NF-κB

Fig. 8. Curcumin inhibits pancreatic expression of inflammatory molecules in 2 models of pancreatitis. Representative RT-PCR for the expression of cytokines IL-6 and TNF- α , chemokine KC, inducible nitric oxide synthase (iNOS), and the housekeeping gene for acidic ribosomal phosphoprotein (ARP) in pancreatic tissue of rats with CR pancreatitis (A) and rats with EtOH + CCK pancreatitis (B), both treated without and with curcumin. Each lane represents data for an individual animal.



attenuates caspase activity (and possibly apoptosis) in pancreatitis.

Effects of curcumin on CCK-induced NF- κ B and AP-1 activation in isolated pancreatic acinar cells. To confirm that the effects of curcumin occur in the pancreatic acinar cell, we measured the effects of curcumin on the activation of NF- κ B and AP-1 in vitro in CCK-stimulated pancreatic acini. Both NF- κ B and AP-1 were activated by 0.1 μ M CCK-8 in the acinar cells, and this activation was abolished by curcumin (Fig. 10). Of note, curcumin inhibited not only the CCK-induced activation of these transcription factors but also their "basal" binding activity in isolated acinar cells, which was more pronounced for AP-1 (Fig. 10). Our laboratory (5) previously reported that such transcription factor activation occurs in pancreatic acinar cells in the process of their isolation from tissue.

DISCUSSION

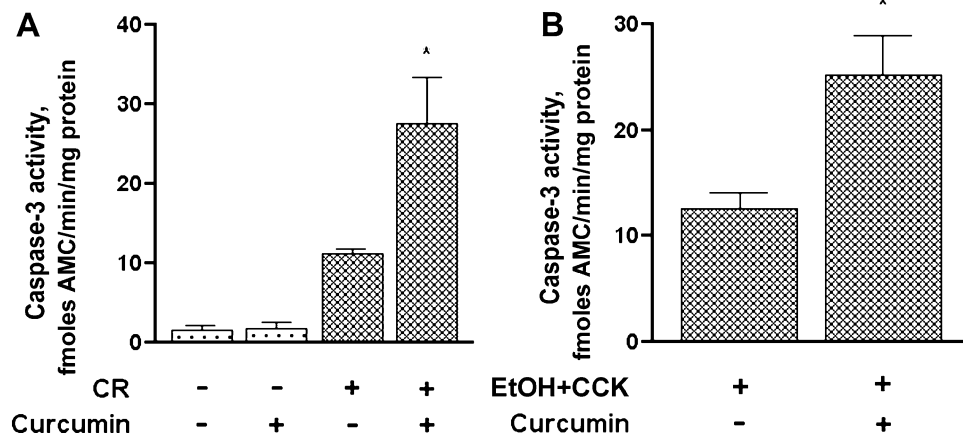
On the basis of the idea that the activation of proinflammatory transcription factors, such as NF- κ B, in pancreas plays a key role in the inflammatory response of pancreatitis, in this study we sought to determine whether an inhibitor of this activation could ameliorate pancreatitis across different experimental models, including alcohol-mediated pancreatitis. We found that curcumin improved the severity of pancreatitis in both ethanol and nonethanol models. The results suggest that the beneficial effects of curcumin are due to its

ability to inhibit activation of NF- κ B and AP-1 and the resulting inflammatory response. In particular, the effects of curcumin on NF- κ B and AP-1 led to inhibition of pancreatic expression of cytokines/chemokines IL-6, TNF- α , and KC and the proinflammatory enzyme iNOS in both models.

The molecular mechanism of curcumin action has not been completely elucidated (2, 6, 7, 24, 26, 30, 32, 35, 39). However, inhibition of NF- κ B and AP-1 is the only common upstream mechanism by which curcumin inhibits gene expression of the above-mentioned and other inflammatory molecules such as ICAM-1 (26) and cyclooxygenase-2 (24). Of note, curcumin does not inhibit other transcription factors, e.g., Sp1 (39). The evidence on cell lines suggests that curcumin inhibits NF- κ B and AP-1 activation upstream of IKK and JNK kinases, probably at the level of MEKK-1 kinase (7, 26, 32, 39). Likewise, our finding that curcumin prevented I κ B degradation in models of pancreatitis suggests that in the pancreas it also acts at one or more steps upstream of I κ B degradation.

As demonstrated by recent studies in experimental models, attenuation of the inflammatory response in pancreatitis either by removing inflammatory cells, preventing neutrophil access to the pancreatic parenchyma, or blocking cytokine action leads to an improvement in the severity of parenchymal injury in pancreatitis (4, 9, 13, 14, 17, 19, 20, 25, 31, 37, 47). Thus the effects of curcumin to inhibit NF- κ B and AP-1 activa-

Fig. 9. Curcumin stimulates pancreatic activation of caspase-3 in 2 models of pancreatitis. Caspase-3-like (DEVDase) activity was measured with a fluorogenic assay in pancreatic tissue lysates from rats with CR pancreatitis (A) and rats with EtOH + CCK pancreatitis (B), both treated without and with curcumin. Values are means \pm SE from at least 4 animals in each group. Values for rats with pancreatitis receiving curcumin were significantly higher (* P < 0.05) than for those without curcumin.



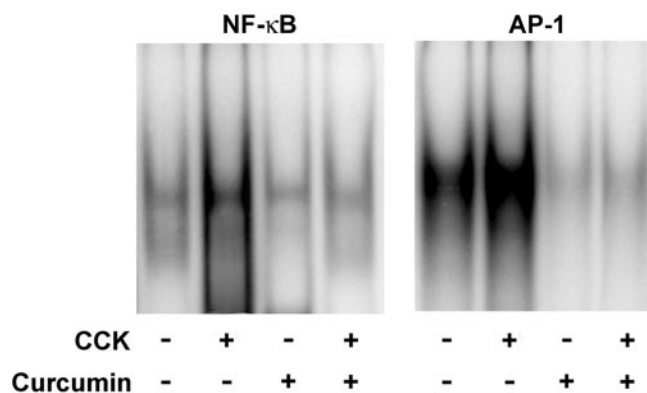


Fig. 10. Curcumin inhibits CCK-induced activation of NF- κ B and AP-1 in isolated pancreatic acini. Rat pancreatic acini were incubated without and with 100 μ M curcumin for 1 h, followed by incubation with 0.1 μ M CCK-8 or vehicle for 30 min. Nuclear extracts were prepared and subjected to EMSAs for NF- κ B and AP-1. Figure is representative of 2 experiments on separate preparations of acini.

tion resulted in the improvement in the measures of parenchymal injury (e.g., histological changes, increases in serum amylase and lipase, and acinar cell vacuolization) as well as the inflammatory response. Of note, in both models, curcumin significantly attenuated the pathological intrapancreatic trypsin activation, which, as was recently shown (19), could result from the decrease in neutrophil infiltration.

The effect of curcumin occurs, at least in part, in the acinar cell, as demonstrated by the experiments on pancreatic acini *in vitro*. Previous work from our and other groups (5, 14, 17, 20–22, 31, 42, 47) indicates that, at the initiation of pancreatitis, the acinar cell is a source of increased inflammatory molecule expression through the activation of NF- κ B and AP-1.

Of importance, the amelioration of both cerulein and EtOH + CCK models of pancreatitis by curcumin suggests that the same mechanisms mediate the development of the disease in both models. Thus the inflammatory response in the ethanol model involves similar mechanisms and plays a similarly critical role to that in nonethanol pancreatitis.

There are several advantages of using curcumin to ameliorate pancreatitis. Curcumin is not toxic and can be given in large quantities (2, 24). Although most of the studies with curcumin were on cell lines, it has been applied *in vivo* (2, 6, 24). Furthermore, recent data (24, 32) indicate that the effects of curcumin administration are produced by curcumin itself and not by hydrogenated products of its metabolism.

Curcumin is one of the few pharmacological NF- κ B inhibitors available for *in vivo* applications (12, 46). The two other NF- κ B inhibitors we tried appeared less promising. Although the proteasomal inhibitor MG-132 greatly inhibited NF- κ B activation *in vitro* in isolated acinar cells (5, 47), it worsened pancreatitis in the two models under study and even increased NF- κ B activation in the pancreas (data not shown). This may

be due to the fact that proteasomes have diverse functions in the organism.

N-acetylcysteine improved cerulein pancreatitis in rats and mice (8, 20) and also pancreatitis induced in rats by taurocholate infusion (42). However, we found that the same *N*-acetylcysteine treatment did not ameliorate the EtOH + CCK pancreatitis (data not shown). Because *N*-acetylcysteine is an antioxidant, this finding indicates that the amelioration of pancreatitis with curcumin that we observed cannot be explained solely by curcumin's antioxidant properties.

Another reason why curcumin was more effective could be that it inhibited both NF- κ B and AP-1, whereas *N*-acetylcysteine inhibited NF- κ B but not AP-1 activation in pancreas (data not shown). AP-1 activation may be necessary for upregulation of some cytokines (e.g., IL-8 and related CXC chemokines; Refs. 3, 43) that are believed to play an important role in the inflammatory response of both human and experimental pancreatitis (4, 20, 31). The results of the present study demonstrate a marked activation of pancreatic AP-1 in the cerulein and EtOH + CCK pancreatitis. On the basis of DNA binding activity measurements, the extent of AP-1 activation in both models was even greater than that of NF- κ B. We also reported AP-1 activation in taurocholate-induced pancreatitis (42).

The observed caspase-3 stimulation by curcumin suggests that curcumin treatment may lead to an increase in apoptosis. This may be a result of NF- κ B inhibition by curcumin, because in many situations NF- κ B plays an antiapoptotic role (1, 46). Caspase-3 is a key "executionary" caspase, activation of which leads to apoptosis (44). On the basis of the results from our group and others, it has been speculated that an increase in apoptosis alleviates the severity of experimental pancreatitis (18, 27). We recently reported (15) that CCK-8 stimulates caspase-3 activation with concomitant apoptosis in isolated pancreatic acinar cells.

There is an increasing interest in the use of natural products to modulate inflammatory disorders. Our data show that curcumin ameliorates pancreatitis in two experimental models by inhibiting the inflammatory response. The results suggest a potential therapeutic role for curcumin, which is currently in clinical trials for cancer prevention, for the treatment of pancreatitis.

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