Transcriptional regulation of CXC-ELR chemokines KC and MIP-2 in mouse pancreatic acini

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Orlichenko LS, Behari J, Yeh T, Liu S, Stolz DB, Saluja AK, Singh VP. Transcriptional regulation of CXC-ELR chemokines KC and MIP-2 in mouse pancreatic acini. Am J Physiol Gastrointest Liver Physiol 299: G867–G876, 2010. First published July 29, 2010; doi:10.1152/ajpgi.00177.2010.—Neutrophils and their chemoattractants, the CXC-ELR chemokines keratinocyte cytokine (KC) and macrophage inflammatory protein-2 (MIP-2), play a critical role in pancreatitis. While acute pancreatitis is initiated in acinar cells, it is unclear if these are a source of CXC-ELR chemokines. KC and MIP-2 have NF-kB, activator protein-1 (AP-1) sites in their promoter regions. However, previous studies have shown increased basal and reduced caerulein-induced AP-1 activation in harvested pancreatic tissue in vitro, which limits interpreting the caerulein-induced response. Moreover, recent studies suggest that NF-kB silencing in acinar cells alone may not be sufficient to reduce inflammation in acute pancreatitis. Thus the aim of this study was to determine whether acinar cells are a source of KC and MIP-2 and to understand their transcriptional regulation. Primary overnight-cultured murine pancreatic acini were used after confirming their ability to replicate physiological and pathological acinar cell responses. Upstream signaling resulting in KC, MIP-2 upregulation was studied along with activation of the transcription factors NF-kB and AP-1. Cultured acini replicated critical responses to physiological and pathological caerulein concentrations. KC and MIP-2 mRNA levels increased in response to supramaximal but not to physiological caerulein doses. This upregulation was calcium and protein kinase C (PKC), but not cAMP, dependent. NF-kB inhibition completely prevented upregulation of KC but not MIP-2. Complete suppression of MIP-2 upregulation required dual inhibition of NF-kB and AP-1. Acinar cells are a likely source of KC and MIP-2 upregulation during pancreatitis. This upregulation is dependent on calcium and PKC. MIP-2 upregulation requires both NF-kB and AP-1 in these cells. Thus dual inhibition of NF-kB and AP-1 may be a more successful strategy to reduce inflammation in pancreatitis than targeting NF-kB alone.

keratinocyte cytokine; macrophage inflammatory protein-2; activator protein-1; nuclear factor-kB; acinar

ACUTE INFLAMMATION IS A HALLMARK of acute pancreatitis (2, 15, 49, 54, 55, 57, 85). Neutrophil recruitment into the pancreas as a part of the inflammatory response worsens pancreatic injury (63). Serum levels of potent neutrophil chemoattractants, the CXC-ELR chemokines, are increased in severe human pancreatitis (68) and rodent pancreatitis (24, 27, 53, 77, 88). In addition, neutralizing antibodies to the CXC-ELR chemokines, cytokine-induced neutrophil chemoattractant (CINC), the rat homologue of keratinocyte cytokine (KC) (CXCL1) or IL-8 (5, 9), and macrophage inflammatory protein-2 (MIP-2) (CXCL2) (56), or their receptor CXCR2 (10), ameliorate the inflammatory response, resulting in decreased local (10, 56) and systemic injury (9, 10, 56) during pancreatitis.

The dependence of the CXC chemokine mob-1 (25, 30, 31) and CC chemokine monocyte chemotactic protein-1 (MCP-1) (8, 25) on NF-kB in acinar cells has been demonstrated. Although NF-kB has been proposed as a therapeutic target in pancreatitis (15) and its activation in acinar cells triggers pancreatitis (6), caerulein-induced pancreatitis in mice with selective inducible deletion of Rela/p65 in the exocrine pancreas was associated with more severe pancreatic neutrophil infiltration, necrosis, and systemic inflammation (2) than in wild-type mice. Therefore, it is important to explore potential NF-kB-independent regulation of neutrophil chemoattractants. Interestingly, CXC-ELR chemokines have both NF-kB and activator protein-1 (AP-1) binding sites in their promoter regions (60, 67, 81). Additionally, MIP-2 regulation depends on cyclic adenosine 5′-monophosphate (cAMP) in other systems (40). These observations, along with the fact that the relative contribution of different transcription factors in regulating these important players in pancreatitis (9, 10, 27, 53, 56, 77, 88) is cell specific (22, 51), persuaded us to study the transcriptional regulation of these chemokines in pancreatic acinar cells.

Whereas acute pancreatitis is thought to be initiated in acinar cells (76, 80), which express tumor necrosis factor-α (26), mob-1, and MCP-1 (8, 25), other cells shown to upregulate CXC-ELR chemokines include periacinar myofibroblasts (3), their precursor stellate cells (79), and later in pancreatitis, inflammatory cells (75). Factors limiting analysis of AP-1 signaling in fresh acinar cells include basal activation of upstream signaling and AP-1 itself (11, 74). This has been thought to be due to the harvesting protocol (11). Consequent basal upregulation of chemokine mRNAs results in decreased responsiveness to further stimulation (11, 25). Therefore, our first aim was to develop a system to study the signaling involved in CXC-ELR expression in acinar cells in vitro. Our second aim was to determine the differential role of NF-kB and AP-1 in the regulation of CXC-ELR chemokines. To explore this, we used acini from TRE-luc mice [these mice have the luciferase gene under the control of the 12-O-tetradecanoylphorbol-3-acetate response element (TRE)] to study AP-1 (16, 38, 59, 89) activation. We preferred this as a measure of transcriptional activation, since several proteins that differ considerably in their ability to activate transcription of target genes can form complexes that bind to AP-1 sites (33, 44) and may be present in nuclear protein extracts analyzed by enzyme-linked immunosorbent assay or electrophoretic mobility shift assays (EMSAs). Moreover, phosphorylation of specific sites...
on proteins enhances their AP-1 transactivating potential without affecting their DNA-binding activities (19, 33). Interestingly, we show in this study that NF-κB inhibition completely prevented KC upregulation, but MIP-2 upregulation was much less affected. On the other hand, dual inhibition of AP-1 and NF-κB with 15-deoxy-D12,14-prostaglandin J2 (PGJ2) prevented the upregulation of both KC and MIP-2. Therefore, dual inhibition of NF-κB and AP-1 may be a more successful strategy than inhibiting NF-κB alone in reducing the inflammatory response of pancreatitis.

MATERIALS AND METHODS

C57bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA). AP-1 luciferase (TRE-luc) mice were a kind gift from Dr. Mercedes Rincon, University of Vermont. Mice were housed with a 12:12-h light-dark cycle at temperatures from 21 to 25°C, were fed standard laboratory chow, and allowed to drink ad libitum. Caerulein was purchased from Research Plus (Bayonne, NJ). RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). Universal 18S Internal Standards containing primers/Competimers were purchased from Applied Biosystems (Foster City, CA). These primers yield a 315-bp band.

Primer sequences used for semiqunatitative PCR

The primer sequences are shown from the 5’–3’ end, along with the base pair size expected from the mRNA sequence and the GenBank accession no. KC, keratinocyte cytokine; MIP-2, macrophage inflammatory protein-2.

Table 1. Primer sequences used for semiquanitative PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size, bases</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC</td>
<td>5’-GACGAGACCAAGGATAAACACGGTT</td>
<td>5’-AACGGAGAAGAGAGACAGCTGCT</td>
<td>533</td>
<td>J04596</td>
</tr>
<tr>
<td>MIP-2</td>
<td>5’-TGGTCGAGATGACTGAATGCC</td>
<td>5’-AGTTCGCTGGACCGGTCGG</td>
<td>466</td>
<td>X53798</td>
</tr>
</tbody>
</table>
Results were calculated as relative light units per microgram protein. Preliminary results revealed a 2.5-fold upregulation at 8 h and 14.3-fold upregulation at 24 h with 100 nM caerulein. We chose the 24-h time point for further studies.

**SDS-PAGE and Western Blotting**

These assays were performed as previously described (52). Protein levels of inhibitory κB (IκB) and chymotrypsin were assessed using polyclonal anti-IκB-α antibody from Santa Cruz (Santa Cruz, CA) and monoclonal anti-chymotrypsin antibody from Millipore (Temecula, CA). Rabbit polyclonal and mouse monoclonal horseradish peroxidase-conjugated secondary antibodies were from Millipore. Secondary antibodies were detected using ECL reagents from Calbiochem (Darmstadt, Germany). All procedures were carried out according to the manufacturers’ instructions.

**Immunofluorescence Studies**

Acini were fixed with 2% paraformaldehyde, permeabilized, blocked with 5% normal goat serum, and exposed to NF-κB p65 polyclonal antibody (Santa Cruz Biotechnology) (1:50) overnight at 4°C. After three washes, goat anti-rabbit Alexa 488 (Invitrogen) and DRAQ5 (1:1,000) for nuclear staining were added for 1 h. After being washed, slides were mounted (fluormount; Sigma) and imaged on a Zeiss Meta (LSM510) confocal microscope using a ×63 lens using 1-μm-thick sections. Images were processed as described previously (71).

**Analysis of Data**

The results represent means ± SE from three or more separate experiments. In Figs. 1–6, vertical bars denote SE values. Statistical evaluation of data was accomplished by using a Student’s t-test to compare two samples or ANOVA for more than two samples, and P values <0.05 were considered significant. All EMSA and RT-PCR gels shown are representative of at least three such gels prepared from independent experiments.

**RESULTS**

**Validation of the Experimental Setup**

Before studying the signaling involved in KC and MIP-2 upregulation, we optimized the experimental setup to replicate in vivo acinar responses. Although fresh acini do replicate this in vivo injury and inflammatory response with caerulein (61), they have basal activation of transcription factors AP-1 and NF-κB along with upstream signaling events (11, 74), with consequent upregulation of chemokine mRNAs and decreased amylase secretion (58). Cultured acini are less responsive to calcium ionophores in the presence of serum or after overnightculture (59). Therefore, we investigated the regulation of KC and MIP-2 expression in cultured acini under basal conditions and after stimulation with 100 nM caerulein. Time-course analyses were performed for both amylase secretion and LDH leakage. The data shown are representative of three experiments, with the mean ± SE for each time point. As shown in Fig. 1A, the amylase secretion is dose-dependent and reaches its maximal value at 100 nM caerulein. In contrast, Fig. 1B shows no significant increase in LDH leakage compared with basal conditions (59). This lack of response is consistent with the reduced amount of basal NF-κB activation as compared with in vivo responses (11, 74).

**Fig. 1.** Acinar cells after overnight culture display dose-dependent physiological and pathological responses to caerulein. Amylase secretion (A) measured over 30 min in response to logarithmic doses of caerulein (shown on the x-axis), and expressed as a percentage of total amylase, peaks at 100 pM caerulein. Cultured acinar cells treated with 100 nM caerulein leak lactate dehydrogenase (LDH) (B) in the medium compared with control acini [basal (BAS)]. LDH leakage in the medium was measured in aliquots taken every hour over 4 h, expressed as a percentage of the total LDH content, and plotted against time (x-axis).

C: comparison of 100 nM caerulein (CER)-mediated NF-κB activation with basal levels as measured by EMSA on nuclear protein extracts of cultured acinar cells. D–I: confocal immunofluorescence images of cultured acinar cells under basal conditions (D–F) and 45 min after stimulation with 100 nM caerulein (G–I), showing lack of nuclear enrichment (D and E, arrows) of p65 under basal conditions (F). Caerulein causes p65 (H) to enrich in the nucleus (arrows in G and I) with a decrease in cytoplasmic staining (dashed oval).
responsiveness to further stimulation (11, 25). A 3-h pacification of these was ineffective in our studies [Supplemental Fig. 3 (Supplemental data for this article may be found on the American Journal of Physiology: Gastrointestinal and Liver Physiology website.)]. Optimization involved culturing the acini overnight. We therefore confirmed viability, secretory responsiveness, and injury response to supraphysiological caerulein. These cells displayed a typical biphasic pattern of amylase secretion (Fig. 1A) that peaked at 0.1 nM as in fresh acini (86). They also displayed an injury response to supramaximal caerulein with blebbing (data not shown) and LDH leakage (Fig. 1B). Trypsinogen activation at 30 min in response to 100 nM caerulein was significantly (P < 0.05) less in cultured acini (1.2-fold basal vs. 2.3-fold in fresh acini). However, this was not a concern since strong evidence supports the independence of transcription factor activation from trypsinogen activation (29, 34, 41). Thus they showed several of the physiological and injury responses of fresh acinar cells and pancreatic exocrine tissue in vivo. The viability of the cultured acini typically exceeded 95%.

To verify that transcription factor activation was indeed in acinar cells and not in contaminating cells (e.g., stellate or duct cells), we studied p65 translocation to the nucleus by immuno-staining. As shown in Fig. 1, E and F, p65 is uniformly distributed all over the acinar cell, with some enrichment in the apical area compared with the basal nuclear area. Stimulation with 100 nM caerulein results in p65 translocating to the nuclear area (Fig. 1, H and I), with depletion in the cytoplasmic area (Fig. 1, H and I). This was verified on EMSA (Fig. 1C), which showed increased NF-kB activation with caerulein.

We also validated the semiquantitative RT-PCR, comparing it with real-time PCR. The semiquantitative method yielded single bands of the appropriate size (Supplemental Fig. 1). Comparison of real-time and semiquantitative PCR methods showed a similar 8.4-fold increase (Supplemental Fig. 2) in MIP-2 mRNA after 90 min of stimulation with 100 nM caerulein compared with basal amounts at the same time in unstimulated cells. We thus chose the semiquantitative method for further studies and verified critical conclusions with real-time PCR.

**Supraphysiological Amounts of Caerulein Increase mRNAs of KC and MIP-2 in Acinar Cells**

To study the regulation of CXC-ELR chemokines in pancreatic acinar cells, we first determined the time course of changes in their mRNA levels in response to supramaximal caerulein (100 nM). The increase was rapid and detectable within 45 min. The mRNA levels peaked at 90 min (KC: 6.6 ± 1.4-fold basal, MIP-2: 4.8 ± 1.5-fold basal), followed by a slower decay until 4.5 h (Fig. 2, A and B). This nature of upregulation for KC/CXCL1 and MIP-2/CXCL2 has been seen in rat β-cells in response to extracellular matrix (58).

![Figure 2](http://example.com/fig2.png)

**Fig. 2.** Supraphysiological but not physiological caerulein increases keratinocyte cytokine (KC) and macrophage inflammatory protein-2 (MIP-2) mRNA levels. A: representative images, and corresponding quantification (B), of changes in levels of MIP-2 (upper band, top) and KC (upper band, bottom) compared with 18S (lower bands) in response to 100 nM caerulein over 4.5 h in cultured acini. There is no change in basal levels (A and B). C and D: supraphysiological (0.1 μM), but not physiological, doses of caerulein (0.1 nM) or the high-affinity receptor agonist JMV (1 nM) cause an increase in KC. *P value <0.01 over basal. Representative images are below the graphs showing the results from 3 experiments.

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**G870 ACINAR CELL CHEMOKINE TRANSCRIPTIONAL REGULATION MODEL**

*AJP-Gastrointest Liver Physiol • VOL 299 • OCTOBER 2010 • www.ajpgi.org*
To study whether the upregulation was in response to a physiological or pathological stimulus, we stimulated the acinar cells with different concentrations of caerulein. No upregulation of either chemokine could be detected in response to physiological doses (up to 0.1 nM caerulein, Fig. 2, C and D). As cholecystokinin (CCK) is known to have two states of the CCKA receptors on the acinar cells, i.e., the low-affinity and the high-affinity state, we stimulated the cells with JMV-180 (73), a well-known selective ligand for the high-affinity state and antagonist for the low-affinity state (64). However, there was no upregulation of KC or MIP-2 mRNA in response to JMV (Fig. 2, C and D). Only supraphysiological doses (0.1 μM caerulein) known to be equivalent to those causing pancreatitis (37, 61) increased the levels of the chemokines (KC: 7.1 ± 0.6-fold basal, P < 0.01 and MIP-2: 9.3 ± 0.9-fold basal, P < 0.01).

KC and MIP-2 mRNA Increase is Transcriptionally Mediated and is Dependent on Calcium and PKC, but not cAMP

We used two approaches to study the intracellular signaling involved in the upregulation of KC and MIP-2 in response to supramaximal caerulein. In the first approach, we stimulated acinar cells after pretreating them with various inhibitors, at concentrations commonly used in acinar cells (30, 36). BAPTA-AM, a calcium chelator, and GF-109203X, a PKC inhibitor, prevented caerulein-induced KC (1.5 ± 0.1- and 1.4 ± 0.1-fold basal, respectively, P < 0.01) and MIP-2 (1.3 ± 0.2- and 1.3 ± 0.4-fold basal, respectively, P < 0.01) upregulation. On the other hand, H-89, a protein kinase A inhibitor, had no effect (Fig. 3, A and B) on these. Therefore, the upregulation of KC, MIP-2 mRNA requires calcium and protein kinase C (PKC), with no involvement of cAMP.

The second approach involved using non-receptor-mediated activation of secondary mediators. We stimulated the acini with the PKC activator 12-O-tetradecanoylphorbol-3-acetate (TPA) and the calcium ionophore ionomycin, which causes extracellular calcium entry. TPA caused upregulation of both chemokine genes (KC: 7.3 ± 0.7-fold and MIP-2: 7.4 ± 0.6-fold, P < 0.01, Fig. 3, C and D). However, ionomycin did not affect their expression (Fig. 3, C and D). Thus calcium is essential but not sufficient for the upregulation of either KC or MIP-2. Actinomycin D at a commonly used concentration (47) prevented the upregulation of both RNAs (Fig. 3, A and B). Thus the upregulation of KC and MIP-2 is transcriptionally mediated and is not due to increased RNA stability.

KC mRNA Upregulation is Dependent on NF-κB While MIP-2 is Transcriptionally Regulated by Both AP-1 and NF-κB

Pretreatment of the acinar cells with the proteasomal inhibitor MG-132 reduced the 100 nM caerulein-mediated increase in NF-κB activation at 45 min from 2.61 ± 0.13-fold over basal.

Fig. 3. Caerulein-induced KC and MIP-2 mRNA increase is dependent on transcription, calcium, and protein kinase C (PKC); H-89 (30 μM) (CER + H-89), 1 μM GF-109203X (CER + GFX), 20 μM BAPTA-AM (CER + BAPTA), or 5 μM actinomycin D (CER + Act.D) were added to the cultured acinar suspension 90 min before stimulation with 0.1 μM caerulein. These were then stimulated for 90 min, and the mRNA levels of KC (A) or MIP-2 (B) were measured by semiquantitative RT-PCR, with 18S as an internal standard. All but H-89 completely prevented the increase in chemokine mRNAs. C and D: cultured acini were stimulated with 0.1 μM caerulein, 1 μM 12-O-tetradecanoylphorbol-3-acetate (TPA), or 1 μM ionomycin (IONO) for 90 min, and the mRNA levels of KC (C) or MIP-2 (D) were measured by semiquantitative RT-PCR, with 18S as an internal standard. Representative images are below the graphs showing the results from 3 experiments. *P < 0.01 compared with basal.
basal to $1.32 \pm 0.04$-fold, ($P < 0.01$) (Fig. 4, A and A’). Lactacystin (10 μM), another proteasomal inhibitor, had a similar effect (results not shown). PGJ_2, at concentrations known to prevent NF-κB activation in other systems (20 μM) (12), also prevented NF-κB activation (1.04 ± 0.11-fold basal, $P < 0.01$) (Fig. 4, A and A’). Reciprocal changes were noted when we studied IκB-α degradation (Fig. 4, B and B’), with caerulein decreasing IκB-α levels to 31.0 ± 5.0% of controls. This was effectively prevented by MG-132 and PGJ_2 (88.1 ± 7.7 and 90.1 ± 15.7%, respectively). Chymotrypsin, used as a loading control, was unaffected by the treatments.

We then studied the effect of these agents on AP-1 activation. Acini were pretreated with MG-132, lactacystin, SP-600125 (25 μM), and PGJ_2, and the luciferase activity after stimulation with 100 nM caerulein under these conditions was compared with basal and 100 nM caerulein-stimulated levels. Caerulein at 100 nM increased luciferase activity 14.3 ± 3.2-fold ($P < 0.01$) over basal levels. This increase was prevented by treatment with SP-600125 and PGJ_2 (12.8 ± 4.6 and 7.8 ± 4.2% of 100 nM caerulein, respectively, $P < 0.05$), but not by MG-132 or lactacystin (249.4 ± 66.2 and 569 ± 59% of 100 nM caerulein, $P < 0.05$), showing that SP-600125 and PGJ_2 inhibited AP-1 activity under the TRE promoter (Fig. 5A). PGJ_2 did not affect the viability of the acini as shown by the unaltered amylase secretion curve (Fig. 5B) and trypan blue uptake (results not shown).

Having determined the effect of MG-132, lactacystin, SP-600125, and PGJ_2 on transcription factor activation, we studied the transcriptional regulation of KC and MIP-2 by stimulating acinar cells with 0.1 μM caerulein after pretreating them with MG-132, lactacystin, or 100 nM caerulein alone, Fig. 6A). AP-1 (PGJ_2) prevented caerulein-induced NF-κB activation, inhibitory κB (IκB) degradation in the cultured acini: The cultured acini were preincubated with or without 20 μM MG-132 (CER + MG-132), or 20 μM PGJ_2 (CER + PGJ_2) for 90 min and then stimulated with 0.1 μM caerulein for 45 min. Electrophoretic mobility shift assay (EMSA) for NF-κB was run on the nuclear protein. A: bands from a representative gel; A’: densitometry; B: IκB-α degradation induced by 100 nM caerulein on Western blotting using chymotrypsin (Chymo) as a loading control, and its prevention by MG-132 and PGJ_2. Quantization of this can be seen in B’. *$P < 0.05$ compared with other values.

DISCUSSION

In this study, we developed a system to study the regulation of CXC-ELR chemokine synthesis in pancreatic acinar cells and show that both KC and MIP-2 are upregulated by supra-physiological doses (sufficient to induce pancreatitis in vivo) but not by physiological doses of caerulein or JMV-180 (62). This upregulation of chemokine genes was dependent on calcium and PKC, but not cAMP. Interestingly, while KC is transcriptionally regulated by NF-κB, MIP-2 requires both AP-1 and NF-κB.

There are several potential endogenous sources of chemokines apart from acinar cells in the normal pancreas. These sources include duct cells, islet cells, stellate cells, periacinar myofibroblasts, resident macrophages, and endothelial cells, which could be activated in a paracrine manner [e.g., trypsin released from acinar cells cleaving protease-activated receptors on these (50), unpublished observations] during pancreatitis. The multiple potential sources of pancreatic chemokines, in addition to infiltration of inflammatory cells during pancre-
atitis, limit our ability to study the signaling involved in transcriptional regulation in a clean manner in vivo. Our study involved generating an in vitro system in which well-characterized in vivo responses of acinar cells are replicated.

Elevated mRNA levels of KC and MIP-2 have been previously shown in pancreatitis (24, 27, 53, 77, 88). KC, a rapidly upregulated gene (13, 14), is a murine equivalent of human Gro-α/H9251 and rat CINC (83). It is a potent neutrophil chemoattractant and upregulates Mac-1 on the surface of neutrophils (13). MIP-2, while being a chemoattractant, elicits significantly greater release of elastase (14). KC and MIP-2 have been shown to be upregulated in murine models of peritonitis (14), meningitis (20), glomerulonephritis (23), and endotoxia-induced lung injury (66). These act synergistically to induce increased leukocyte rolling, adhesion, and tissue extravasation dependent on P-selectin (90, 91). Their principal receptor CXCR2 is expressed on neutrophils (39, 42) and mast cells (46). Neutralizing antibodies to CINC, MIP-2, and CXCR2 reduced local and systemic injury during pancreatitis (9, 10, 56).

MIP-2 (67, 81) and IL-8 (60) both have AP-1 and NF-κB binding sites in their promoter region. MIP-2 is solely dependent on AP-1 in the mouse liver (22) and dually dependent on NF-κB and AP-1 activation, like in our studies in a model of myocardial ischemia and reperfusion (51). These along with cAMP response element-binding protein (CREB) regulate

Fig. 5. PGJ2 inhibits activator protein-1 (AP-1) activity but does not affect stimulation secretion coupling in acini. A: cultured acini were either left unstimulated (BASEL) or were stimulated with 0.1 μM caerulein after a 90-min pretreatment with 20 μM MG-132 (CER + MG-132), 10 μM lactacystin (CER + Lacta), 25 μM SP-600125, or 20 μM PGJ2 (CER + PGJ2) for a total duration of 24 h. Luciferase activity (arbitrary units/mg protein) was measured in each sample, and results were plotted as a percentage of caerulein. The luciferase activity in freshly ground pancreatic tissue was also measured (PANC. TISSUE). †Significant (P < 0.05) increase compared with 100 nM caerulein. *Significant (P < 0.05) decrease compared with caerulein. Cultured acini were washed and suspended in HEPES buffer to measure amylase release (B). Some of these were then preincubated with 20 μM PGJ2 for 15 min (filled bars), and the amylase release was measured over 30 min from acini that were either unstimulated (BASEL) or in response to 0.1 nM and 0.1 μM caerulein. Open bars: amylase from acini untreated with PGJ2. Secretion was expressed as a percentage of total amylase content of the acini.

Fig. 6. KC upregulation is NF-κB dependent, but that of MIP-2 requires both NF-κB and AP-1. MG-132 (20 μM) (CER + MG-132), 10 μM lactacystin (CER + Lacta), 25 μM SP-600125, or 20 μM PGJ2 (CER + PGJ2) were added to the acinar culture before stimulating these with 0.1 μM caerulein for 90 min. mRNA levels of KC (A) or MIP-2 (B) were measured by real-time PCR. The graphs show the means ± SE from 3 separate experiments. *P < 0.01 compared with CER.
MIP-2 in murine macrophages (40). However, CREB seems to be an unlikely mediator in our system since the protein kinase A inhibitor H-89 had no effect on the caerulein-stimulated increase in either chemokine.

Dual requirement of AP-1 and NF-κB in the transcriptional regulation of MIP-2 suggests that, while NF-κB does have a prominent role in pancreatitis (1, 27, 74, 84), its silencing may not be sufficient to prevent neutrophil infiltration. MG-132, lactacystin, and BAY11–7082, which inhibit NF-κB, did not significantly inhibit the caerulein-induced MIP-2 increase. This is in agreement with the study by Algul et al. (2) in which selective conditional deletion of Rela/p65 in the exocrine pancreas resulted in worse neutrophil infiltration and local, systemic injury during pancreatitis. The finding that dual inhibition of NF-κB and AP-1 with curcumin (28) resulted in reduced neutrophil infiltration in two different models of pancreatitis resonates well with our finding, since inhibiting AP-1 alone did not reduce caerulein-mediated upregulation of KC or MIP-2. Constitutive expression of peroxisome proliferator-activated receptor (PPAR) γ has previously been shown to decrease AP-1 and NF-κB activation (82). PGJ_2, which is a PPARγ ligand [PPARγ forms heterodimers with the retinoid X receptor (65)], has previously been shown to prevent both NF-κB and AP-1 activation (12). PGJ_2’s mechanisms may involve covalent adduct formation with thiol residues on specific proteins altering redox-sensitive cell pathways and inhibiting IkB kinase (43, 65). Our findings are in agreement with its dual role in AP-1, NF-κB inhibition. Hashimoto et al. (32) have shown PGJ_2 to reduce intercellular adhesion molecule-1 expression and decrease NF-κB activation, inflammation, and local injury during pancreatitis (32).

TPA activates NF-κB in acini (30). It also results in AP-1 binding to the TPA response element (TRE) (4). TRE corresponds to the human collagenase promoter and is the consensus site for the binding of the Fos/Jun and Jun/Jun dimers that comprise AP-1 (4, 33, 59). Their activity is regulated by transcription, protein amount, nuclear translocation, and phosphorylation (48). AP-1 EMSAs, which we plan to do in future studies, would help to determine which of these is activated by specific stimuli. AP-1 also transcriptionally regulates proinflammatory genes like MCP-1 (69), IL-2 (17), IL-6 (18), iNOS (45), and cyclooxygenase-2 (78).

The proteasome inhibitors MG-132 and lactacystin did not inhibit, but rather increased, AP-1 luciferase activity in response to caerulein. This is seen with proteasome inhibitors in other systems (35, 87) and probably results from decreased proteasomal degradation of luciferase protein. Whether this holds true for inflammatory proteins is unknown.

In summary, we have shown that inhibiting NF-κB alone does not completely prevent the upregulation of MIP-2 in response to caerulein. Although KC is regulated by NF-κB, dual inhibition of NF-κB and AP-1 is essential for preventing the upregulation of MIP-2. Therefore, dual AP-1, NF-κB inhibition may be a more successful strategy in decreasing pancreatic inflammation during pancreatitis than silencing NF-κB alone (2). Future experiments will be directed toward determining whether deletion of both AP-1 and p65 can prevent the worsening of neutrophil infiltration and injury caused by deleting Rela/p65 alone during pancreatitis.

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DISCLOSURES
No conflicts of interest exist.

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