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Nuclear Factor-κB Increases Intracellular Calcium by Upregulation of Na^+-Ca^{2+} Exchanger 1 in Cerulein-Induced Acute Pancreatitis

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Objectives: The mechanisms underlying pathogenesis of acute pancreatitis (AP) are still not completely understood. An early, critical feature of AP is aberrant calcium (Ca^{2+}) signaling, termed Ca^{2+} overload, within pancreatic acinar cells. This study aimed to develop a model system in rats for AP induction to study the contribution of the Na^+-Ca^{2+} exchanger 1 (NCX1) ion channel in AP pathogenesis.

Methods: To establish a rat model of AP induction, cerulein or L-arginine were intraperitoneally injected and tissue was histologically analyzed by hematoxylin and eosin staining. A cell culture-based model for AP induction was similarly created through cerulein treatment of AR42J cells. Induction of AP was also examined following exposure to the NXC1-targeted inhibitor KB-R7943. The expression of each gene was detected by Western blotting, immunofluorescence, immunohistochemistry, or quantitative reverse transcription polymerase chain reaction. Transcriptional regulation by nuclear factor (NF)-κB was detected using an NCX1 promoter-fusion dual luciferase reporter system. Cytosolic Ca^{2+} was measured using a fluorescent calcium indicator.

Results: We found that cerulein induced NCX1 expression via activation of nuclear factor NF-κB, which potentially binds to the NCX1 promoter to induce its transcription.

Conclusions: Our findings reveal a regulatory pathway through NF-κB/NCX1 governing Ca^{2+} overload in AP development, thus providing potential targets for AP treatment.

Key Words: acute pancreatitis, calcium channel, NCX1, NF-κB, cerulein, transcriptional regulation

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Acute pancreatitis (AP) is an inflammatory disease in which pancreatic enzymes are activated by variety of causes.[1] It is always accompanied by pancreatic tissue digestion, edema, hemorrhage, and necrosis, leading to multiple organ failure.[2] Although treatment of AP has been studied extensively, the mechanism of pathogenesis remains unclear. The etiology of AP is complex, and calcium overload is considered an essential component of mechanisms that are currently understood to contribute to AP.[3,4]

In prior work, Ca^{2+} signaling and ion channels have been examined for their role in pancreatic acinar cells.[5,6] For example, several studies have reported that Ca^{2+} overload is a key contributor to pancreatic acinar cell injury because prolonged and global elevation of the intracellular Ca^{2+} concentration leads to trypsin activation, inflammation, necrosis, and vacuolization.[5,7] Other, more recent, studies have also suggested that calcium overload in acinar cells may be an early event in the pathogenesis of AP.[8–10] Clinical studies show that patients with severe pancreatitis often have associated hypocalcemia with later stages,[11] and blood calcium concentration is an important indicator of a pancreatitis prognosis.[12] Calcium overload in cells is therefore a predominant factor in pancreatitis development. Furthermore, activation of the CARC and TRPV1 channels has been closely associated with alcohol-induced pancreatitis.[13,14] Similarly, calcium overload during biliary pancreatitis is primarily regulated by phospholipase C and TRPC3 calcium channels on the membrane.[15]

The bidirectional transporter Na^+-Ca^{2+} exchanger 1 (NCX1) is necessary for ion transport functions across the cell membrane.[16] The NCX1 is expressed in a variety of tissues such as the pancreas, myocardium and neurons, and it plays critical roles in several human physiological processes and diseases.[17] During the development of many diseases, the function of NCX1 is reversed and the direction of ion exchange is altered.[18] The accumulation of intracellular calcium has been reported in pathological processes, such as cerebral ischemic injury, proliferation of liver cancer, and TGF-β-induced pancreatic cancer invasion and metastasis.[19] In pathological processes, NCX1 functions in pumping Ca^{2+}, subsequently increasing the concentration of intracellular Ca^{2+}, which consequently triggers a series of biological effects, such as mitochondrial dysfunction, increased generation of reactive oxygen species, and dismantling of the cytoskeleton.[20] In addition, NCX1 is involved in the regulation of calcium ions in inflammatory diseases and has been reported, through a study on airway inflammation, to be an important ion channel that mediates an increase in the concentration of intracellular calcium ions.[21] In light of these roles, NCX1 may act as a regulatory target in the response to inflammatory diseases and can potentially provide an effective avenue for development of treatments.[22]

In this study, we established an in vitro and in vivo model in rats to understand the mechanisms of AP using cerulein or L-arginine treatment. We then explored the role of NCX1 and its mediation of calcium signaling during AP. We also elucidated the effect of calcium overload in AP development. This work provides valuable insight into the mechanisms underlying AP, which may further lead to development of novel therapeutics for this disease.

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MATERIALS AND METHODS

Reagents and Cell Cultures

The commercially available reagents cerulein (MCE, Shanghai, China), L-arginine (MCE), NCX1 inhibitor KB-R7943 (Sigma-Aldrich, St. Louis, Mo), nuclear factor-κB (NF-κB) inhibitor Bay 11–7082 (Sigma-Aldrich), and NF-κB activator Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) were all purchased through the indicated manufacturers. Anti-NCX1 antibody was produced by Abcam (Cambridge, UK). Antibodies against P65, pP65 (ser536), GAPDH, β-actin, and goat anti-rabbit/mouse IgG (H + L) secondary antibodies were purchased from Cell Signaling Technologies (Danvers, Mass). The rat pancreatic acinar cell line AR42J and human embryonic kidney cell line HEK-293T were both supplied by the Cell Line Resource Center of the Chinese Academy of Medical Sciences (Beijing, China). All cell lines were stored frozen in liquid nitrogen and after thawing, less than 20 of passages were used over the course of 3 months for the present experiments.

Experimental Model and Groups

Healthy, adult male Sprague-Dawley rats (180–220 g, 4–5 weeks) were obtained from the Laboratory Animal Center of Xinqiao Hospital, Army Medical University (Chongqing, China). Before experiments, rats were housed under controlled day-night cycles and adapted to the experimental environment for 1 week.

For experiments, rats were starved overnight, and the AP groups were injected with cerulein (dose, 50 ng/g, dissolved in 1 mL of saline solution) hourly in the abdominal cavity for 6 hours, following previously described methods.[15] Experimental groups treated with NCX1 inhibitor were injected with KB-R7943 (3 mg/kg) at 30 minutes before cerulein or L-arginine injection. The rodents allocated to the control group received 1 mL of sterile saline intraperitoneally. At 6 hours after the last injection, the rats were killed to excise the pancreas and collect blood. The plasma was used to measure serum amylase and lipase. Half of each pancreatic sample was formalin-fixed and embedded in paraffin, and the other half was immediately frozen and stored at −80°C until further analyses.

The rats allocated to L-arginine–induced AP groups were injected twice with 25% L-arginine (2.5 g/kg) in 1 hour, then separately killed at 3, 6, and 12 hours after the second injection to excise the pancreas. The pancreatic samples were formalin-fixed and embedded in paraffin for further analyses.

Generation of Stable Cell Lines

The lentivirus vectors Lv-shNCX1 (3636, 4082, 4351) and Lv-shNC empty vector were purchased from Genepharma (Shanghai, China). Generation of shNCX1 stable cell lines was performed following the protocols provided by Genepharma.

Enzymatic Activity Assays

Rat serum amylase and lipase activity were measured using an AU 5822 automated chemistry analyzer (Beckman Coulter, Brea, Calif). Amylase and trypsin activities in the supernatant of AR42J cells were evaluated using assay kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Immunofluorescence

AR42J cells grown on coverslips were fixed with ice-cold formaldehyde for 20 minutes, then blocked with phosphate-buffered saline (PBS) (5% bovine serum albumin) for 30 minutes and incubated overnight at 4°C with primary antibodies against NCX1 (1:100 dilution) (Abcam). Cells were incubated with Fluorescein isothiocyanate (FITC)-IgG fluorescent secondary antibody (1:500 dilution, ZSGB-Biotechnology Co., Ltd., Beijing China) at room temperature for 1 hour, and DNA was counterstained with 1× 4′6-diamidino-2-phenylindole diluted in PBS for 1 minute. The 4′6-diamidino-2-phenylindole was removed, and cells were washed three times with PBS, mounted on coverslips, observed, and photographed using confocal endoscopy (Leica TCS SP5; Leica Microsystems, Inc., Buffalo Grove, Ill).

Hematoxylin and Eosin Staining and Immunohistochemistry

Pancreases were removed immediately after bleeding animals and fixed in 4% paraformaldehyde. Fixed pancreases were embedded in paraffin and sectioned at 4 μm with a microtome. Sections were stained with hematoxylin and eosin (H&E). Five fields were observed for each sample.

Slides with rat pancreatic samples were incubated with anti-NCX1 monoclonal antibody (1:100 dilution, Abcam) or anti-P65 (1:100 dilution, Cell Signaling Technology). Primary antibodies were detected with biotinylated goat anti-mouse IgG secondary antibodies (Vector Laboratories, Inc., Burlingame, Calif). Immunoreactivity was detected using horseradish peroxidase (30-diaminobenzidine) kits (BioGenex, Fremont, Calif) followed by counterstaining with hematoxylin, dehydration, and mounting.

Western Blotting

Pellets or cell lysates were resuspended in 2× loading buffer, boiled for 5 minutes, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Resolved proteins were transferred to polyvinylidene difluoride membranes (MilliporeSigma, Burlington, Mass). Membranes were blocked with blocking buffer with 5% nonfat milk powder, followed by incubation with monoclonal antibody against NCX1 (1:1000), Phospho-p65 (Ser536) (1:1000; Cell Signaling Technology), P65 (1:1000; Cell Signaling Technology), or GAPDH (1:5000; Ambion, Inc., Austin, Texas). After washing with tris-buffered Tween 20 solution, secondary antibodies were applied. Signals were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, Mass) with a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, Calif).

Reverse Transcription-PCR

Total RNA from pancreatic samples and AR42J cells were extracted by RNAiso plus reagent (Takara Bio, Mountain View, Calif), then reverse transcribed to cDNA by PrimeScript RT-polymerase (Takara Bio). Polymerase chain reaction (PCR) was performed using cDNA primers specific for rat NCX1 with Taq PCR Master Mix kit (BBI Solutions, Portland, Maine). The rat gapdh gene was used as an internal control. The PCR primers were as follows:

Rat-NCX1-Fwd: 5′-AAGGAGACAGACCAGCTTCC-3′
Rat-NCX1-Rev: 5′-GGCTCTTTTGCTGCTGACTT-3′
Rat-gapdh-Fwd: 5′-AACGACCCCTTCATTGACCT-3′
Rat-gapdh-Rev: 5′-CCCCATTTGATGTTAGCGGG-3′

The PCR conditions were as follows: denaturation at 94°C for 4 minutes, annealing at 55°C for 30 seconds, extension at 72°C for 20 seconds, 35 cycles, and final extension at 70°C for 10 minutes.

Luciferase Assay

For the NCX1 promoter reporter assays, 293T cells in a 24-well plate were transfected with 100 ng of the empty vector or plasmids carrying the NCX1 promoter, then cerulein or PMA

was added to cultures at 48 hours posttransfection. Cell lysates were collected in the indicated time. Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter System (Promega, Madison, Wis) according to the manufacturer's instructions. The firefly luciferase activity in each sample was normalized to that of renilla luciferase.

Measurement of Cytosolic Ca^{2+}

AR42J cells grown on coverslips or inserts were loaded with 5 mmol/L fura-2-acetoxymethyl ester (fura-2) calcium indicator in physiological salt solution at 37°C for 50 minutes, then washed in physiological salt solution for 30 minutes. Cells on coverslips were mounted in a standard perfusion chamber on a Leica DMi8 microscope stage (Leica Microsystems, Inc.). Cells on inserts were mounted in a special dual perfusion chamber so that monolayers could be treated with drugs separately from the apical or basolateral side of the cells. The ratio of fura-2 fluorescence with excitation at 340 or 380 nm (F340/380) was followed over time and captured with an intensified CCD camera (ICCD200) and a Meta Fluor Imaging System 7.10.2.240 (Molecular Devices, LLC, San Jose, Calif).

Statistical Analysis

All data were expressed as means ± standard error of the mean (SEM) for a series of n experiments and analyzed by one-way ANOVA followed by the Student-Newman-Keuls post hoc test or by Student *t* tests for paired or unpaired samples with GraphPad Prism 5.0 (GraphPad Software, San Diego, Calif). $P < 0.05$ was considered statistically significant.

Study Approval

All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Army Medical University, Chongqing, China.

RESULTS

Elevated NCX1 Expression in Cerulein-Induced AP Rat Models

To study the expression and possible function of NCX1 in AP, we established an in vivo cerulein-induced AP rat model. Rats were given cerulein by intraperitoneal injection 6 times (50 $\mu g \cdot kg^{-1} \cdot h^{-1}$) and killed at 6 hours post–final injection. Biochemical assays of amylase and lipase activity showed that the rats in the AP group were characterized by a significant increase in serum amylase activity, compared with the control group (Fig. 1A). Moreover, serum lipase activity was also distinctly elevated in the AP group (Fig. 1B). Staining with H&E revealed that rat pancreases from the AP group were edematous, with obscured structural outlines (Fig. 1C). Immunohistochemical staining showed that expression of NCX1 was increased in the AP group compared with the untreated control rats (Fig. 1D). Western blot and real-time reverse transcription (qRT)-PCR analyses indicated that both protein and mRNA levels of NCX1 were substantially increased in AP group (Figs. 1E, F).

To compare the effects of cerulein on NCX1 expression with the effects of L-arginine in the induction of AP in AR42J cells, we seeded AR42J cells into 6-well plates (1×10^6/well) and exposed them to a range of L-arginine concentrations for 24 hours. We found that cell viability was increasingly impaired, commensurate with L-arginine concentration (Fig. 1G). At 1 and 2.5 mM 90% of

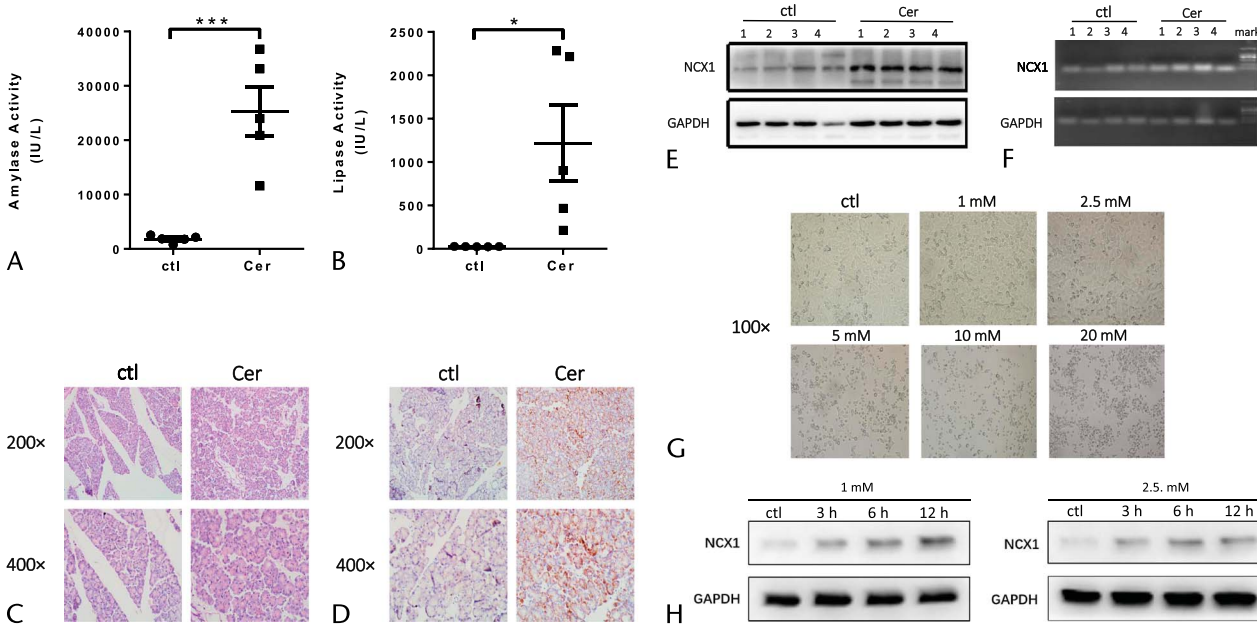

FIGURE 1. NCX1 was upregulated in cerulein-induced rat AP models and pathological changes in cerulein-induced AP rat models. A and B, Concentrations of serum amylase (A) and lipase (B) were measured using a clinical biochemistry analyzer. C, Histological changes were analyzed by H&E staining. NCX1 increased in cerulein-induced AP rat models. D, E, and F, NCX1 protein expression was observed by immunohistochemical staining (D) and Western blot (E); mRNA was measured by qRT-PCR (F). ctl, control group. Cer, intraperitoneal injection of cerulein for 6 hours. GAPDH was the internal control. Data were from three independent experiments and expressed as means ± SEM. $^*P < 0.05$, $^{***}P < 0.001$, n = 5 rats per group in this experiment. G and H, NCHX1 upregulation in AR42J cells with AP induced by a range of L-arginine concentrations. AR42J cells exposed to 0, 1, 2.5, 5, 10, and 20 mM L-arginine for 24 hours, showed impaired viability at 5 mM and higher L-arginine (G). Western blot showing increased NCX1 expression in AR42J cells compared with uninduced controls following treatment with 1 or 2.5 mM L-arginine (H).

cells survived. In contrast, at 5 mM or greater, cell proliferation was significantly inhibited, and at 20 mM of L-arginine, the majority of cells did not survive. In light of this result, we selected 1 mM and 2.5 mM L-arginine to induce AR42J cells. Expression of NCX1 protein increased gradually from 3 to 12 hours of L-arginine induction over its expression in the control group, the trend of which was more distinct in the 1 mM treatment group (Fig. 1H).

Elevated NCX1 Expression in Cerulein-Induced AP Cell Models

We purchased rat exocrine pancreas AR42J cells to establish a cell culture-based model of cerulein-induced AP. The NCX1 protein was expressed at low levels at 1 hour postcerulein treatment; at 3 hours, upregulation was clearly observed, increasing to stable, high levels of expression for at least 12 hours posttreatment compared to the untreated control; expression finally diminished by 24 hours treatment (Fig. 2A). Supporting the Western blot analysis of protein expression, qRT-PCR showed that NCX1 mRNA levels were consistently higher for 12 hours postcerulein treatment (Fig. 2B). Immunofluorescence of AR42J cells showed that NCX1 expression was upregulated by cerulein treatment. NCX1 was localized in the cytoplasm and cell membrane of pancreatic acinar cells (Fig. 2C).

NCX1 Upregulation Leads to Calcium Overload and Exacerbates Pancreatitis

To distinguish if Ca^{2+} levels increased in response to cerulein treatment in pancreatic acinar AR42J cells, cerulein was applied in a Na^{+}-free solution. Application of the cerulein-free control solution did not elicit any change in $[Ca^{2+}]_{cyt}$ levels (Fig. 3A), whereas addition of 100 nM cerulein led to immediate elevation of $[Ca^{2+}]_{cyt}$ (Figs. 3B). In addition, to explore whether NCX1 was involved in this calcium influx, we knocked down NCX1 protein expression by transfecting 3 separate lentiviral constructs carrying shRNAs targeting NCX1 mRNA and an empty vector control. The shRNA constructs were transfected at high efficiency, as observed by immunofluorescent staining of the vector (Fig. 3C), and successfully knocked down levels of NCX1 (Fig. 3D). Measurement of Ca^{2+} showed that cerulein treatment did not elevate $[Ca^{2+}]_{cyt}$ in the NCX1 knockdown cells, even in the presence of extracellular 5 μM $CaCl_2$ in the treatment solution (Figs. 3E), while a significant change in intracellular Ca^{2+} could only be observed in the unsilenced control cells (Fig. 3F).

To further explore the physiological effects of shRNA NCX1 knockdown on AR42J cells, we quantified the activities of amylase and trypsin in cell supernatant. Inhibition of NCX1 expression produced no significant effect on either amylase activity (496 ± 61 U/dL compared with 527 ± 87 U/dL in the control group versus the shNCX1 group, respectively [$P > 0.05$]) or trypsin activity (450 ± 70 U/dL vs 404 ± 115 U/dL in the controls vs shNCX1 cells). After 6 hours of cerulein induction in AR42J cells, inhibition of NCX1 expression in silenced cells led to reduced activities for both amylase (965 ± 116 U/dL vs 634 ± 43 U/dL, controls vs shNCX1, $P < 0.01$) (Fig. 3G) and trypsin (943 ± 196 U/dL vs 715 ± 191 U/dL, controls vs shNCX1, $P < 0.05$) (Fig. 3H) in cell supernatants.

Potential NF-κB-Binding Sites in the NCX1 Promoter and NF-κB Is Increased After Cerulein Treatment

To explore the regulatory mechanism by which cerulein induced NCX1 expression, we searched for potential transcription

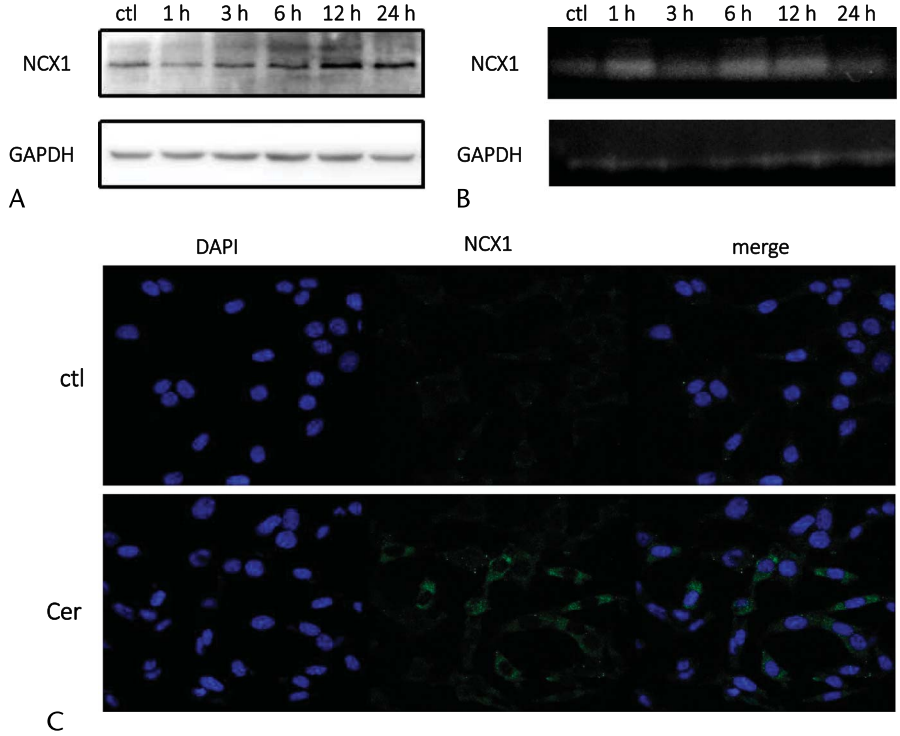

FIGURE 2. NCX1 was upregulated in cerulein-induced cell culture-based AP models. A and B, NCX1 mRNA and protein levels in AR42J cells were analyzed by qRT-PCR and Western blots at the indicated times of sampling during 100 nM cerulein treatment. GAPDH served as the internal control. C, NCX1 (green) expression observed by immunofluorescence in AR42J cells (blue) after 100 nM cerulein treatment for 12 hours. Data were from three independent experiments.

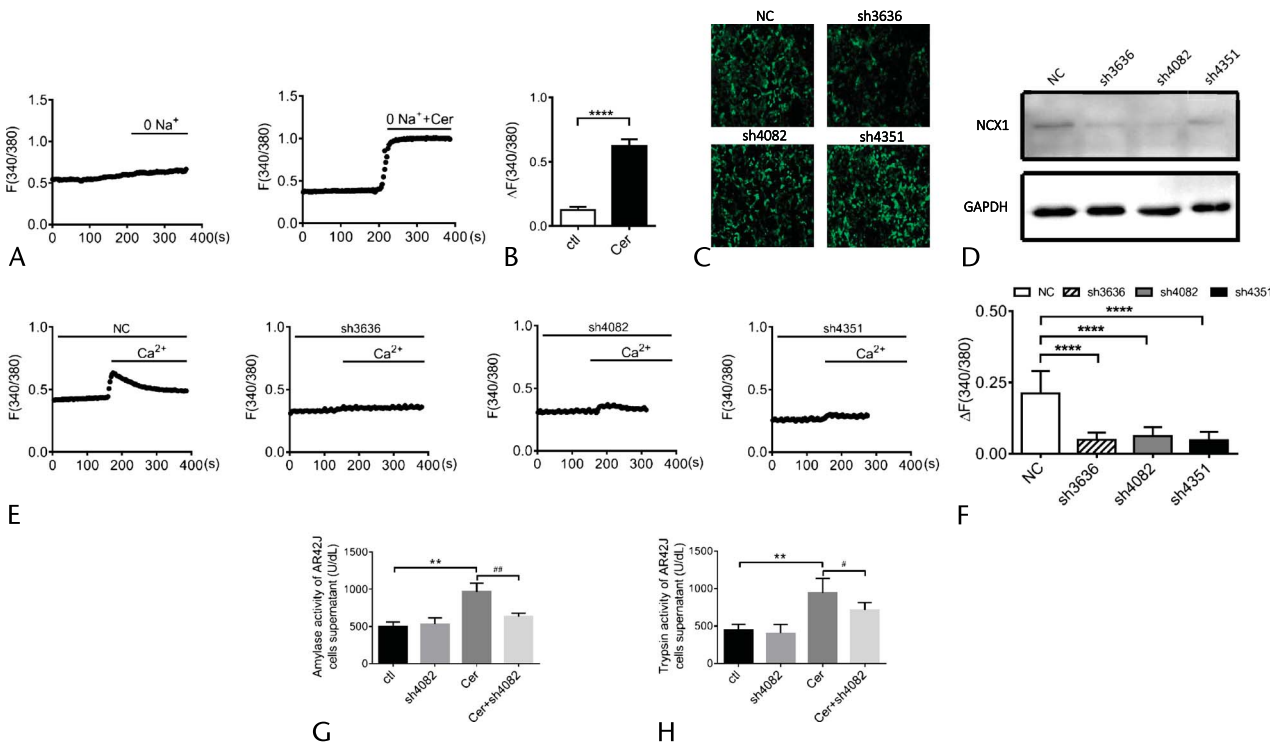

FIGURE 3. Cerulein mediates extracellular calcium influx via NCX1 in AR42J cells. A and B, Cerulein-induced increase in $[Ca^{2+}]_{cyt}$ in AR42J cells (100 nM; n = 20 cells). C and D, Transfection and lentivirus knockdown efficiency in AR42J cells observed by fluorescence microscopy (C) and Western blot (D). GAPDH served as the internal control. E and F, The cerulein-induced increase in $[Ca^{2+}]_{cyt}$ in AR42J cells (E) was attenuated after knockdown of NCX1 (100 nM; n = 20 cells), with equivalent differences in $[Ca^{2+}]_{cyt}$ from control among the three shNCX1 cell lines (F). NC, negative control group. G and H, Effects of NCX1 knockdown on amylase (G) and trypsin (H) in supernatant of AR42J cells treated or not with cerulein. Cer, cerulein. Data were from three independent experiments and expressed as means ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

factor binding sites in the NCX1 promoter region using the University of California Santa Cruz genome browser (https://genome.ucsc.edu/) database. Several NF-κB binding sites were identified in the −400 to −1000 bp region of the NCX1 promoter. This finding comports with several previous studies that implicated NF-κB activation as an early and central event in the progression of AP-associated inflammation. We focused on possible regulatory activity by NF-κB on NCX1 expression (Fig. 4A). Western blots showed a significant increase in pP65(ser536) protein, an active form of an NF-κB pathway component, in AR42J cells at 1 hour after incubation with cerulein. High expression of pP65 was sustained for at least 24 hours postcerulein treatment (Fig. 4B).

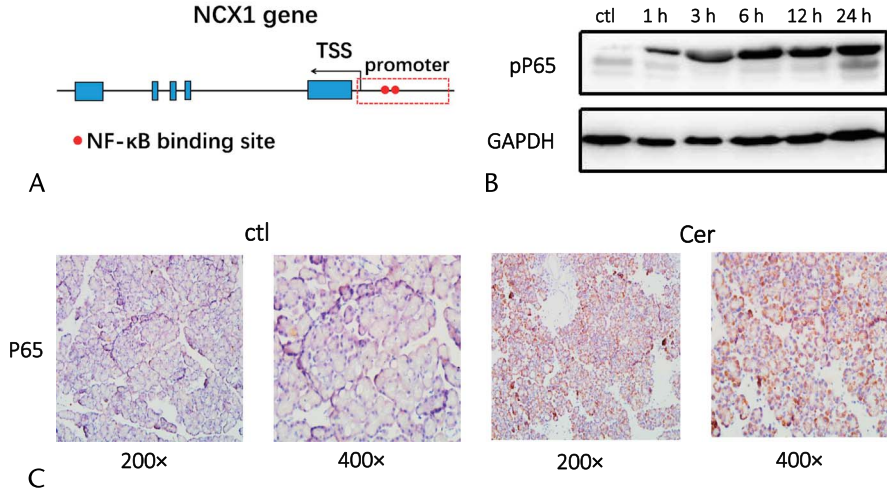

FIGURE 4. Potential NF-κB binding sites in the NCX1 promoter region and cerulein-induced increase in NF-κB expression. A, Structure of NCX1 gene. B, Expression of pP65 protein measured by Western blot after 100 nM cerulein treatment in AR42J cells. GAPDH was used as the internal control. C, Expression of p65 protein (brown) in cerulein-induced AP rat model was measured by immunohistochemical staining. n = 5 rats per group in this experiment.

Immunohistochemical staining confirmed that P65 was highly expressed in cerulein-induced AP rat pancreatic tissue, indicating that NF-κB expression also increased compared with the uninduced control (Fig. 4C).

Cerulein Upregulates NCX1 Through Transcription Factor NF-κB

To examine the effect of transcription factor NF-κB on the regulation of NCX1, an agonist (PMA) and an inhibitor (Bay11-7082) were used to induce or suppress NF-κB, respectively, for subsequent analysis of differences in NCX1 expression. AR42J cells were treated with different concentrations of PMA for 6 or 12 hours, after which Western blot analysis confirmed that PMA treatment led to an accumulation of phosphorylated P65 protein. Significant upregulation of NCX1 protein expression was observed in treatments of 25 to 100 nM PMA compared with cells treated with the DMSO vehicle only (Fig. 5A). After treatment with the NF-κB inhibitor Bay11-7082, expression of NF-κB was slightly upregulated at 6 hours, then decreased significantly at 12 and 24 hours, similar to Western blots showing that NCX1 protein expression was suppressed throughout the treatment (Fig. 5B).

As discussed above, database analysis of the NCX1 promoter sequence identified several conserved NF-κB binding motifs. To determine if NCX1 transcriptional activity was responsive to NF-κB, we cloned the −1 to −1464 bp region of NCX1 promoter into luciferase-fusion reporter constructs (Fig. 5C) which were then transfected into 293 T cells. By 24 hours after transfection, luciferase activity had significantly increased steadily over 5 to 20 minutes in cells treated with cerulein (Fig. 5D), and also increased compared with the control cells after treatment with PMA, though not in a time-dependent manner (Fig. 5E). In contrast, treatment of 293 T cells with the NF-κB inhibitor Bay11-7082 before luciferase reporter transfection resulted in a significant decrease in NCX1 promoter-driven fluorescence in approximately 30% of control cells, regardless of exposure to cerulein (Fig. 5F).

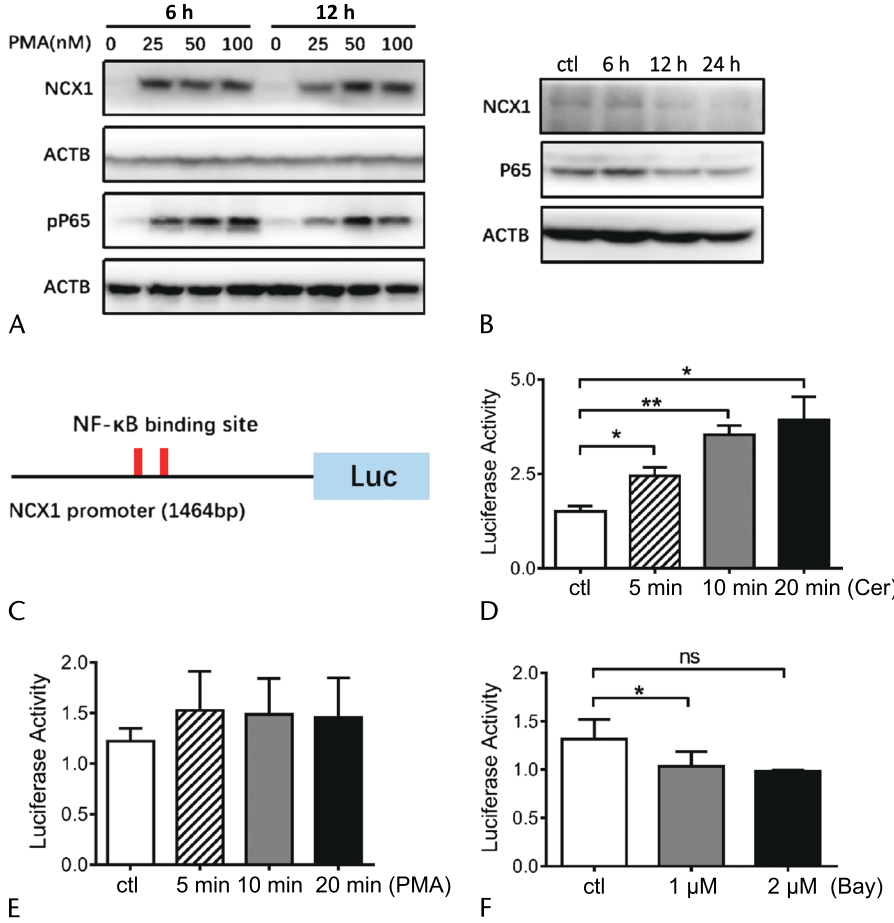

FIGURE 5. Cerulein upregulated NCX1 through transcription factor NF-κB. A, NCX1 expression was upregulated at the indicated times and during exposure to a range of PMA concentrations. PMA, activator of NF-κB; pP65(536), phosphorylation-activated p65. B, NCX1 was downregulated at the indicated times after exposure to 10 μM Bay11-7082. Bay11-7082, NF-κB inhibitor. ACTB served as the internal control. Data were from three independent experiments. C, Schematic diagram of the luciferase reporter constructs with NCX1 promoter. Genomic fragments of the NCX1 promoter region were cloned into psiCHECK2 plasmid. D, E, F, Activity of luciferase reporters in 293 T cells treated with cerulein (D) or PMA (E) for 5, 10, or 20 minutes after transfection with reporter constructs at 24 hours before luciferase activity assays (F). Cell line 293 T was treated with Bay11-7082 (1 μM or 2 μM) for 12 hours before transfection with luciferase-NCX1 promoter fusion constructs at 24 hours before treatment with 100 nM cerulein. Luciferase activity of untreated cells was set to 1. Data were from three independent experiments and expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$, ns, not significant.

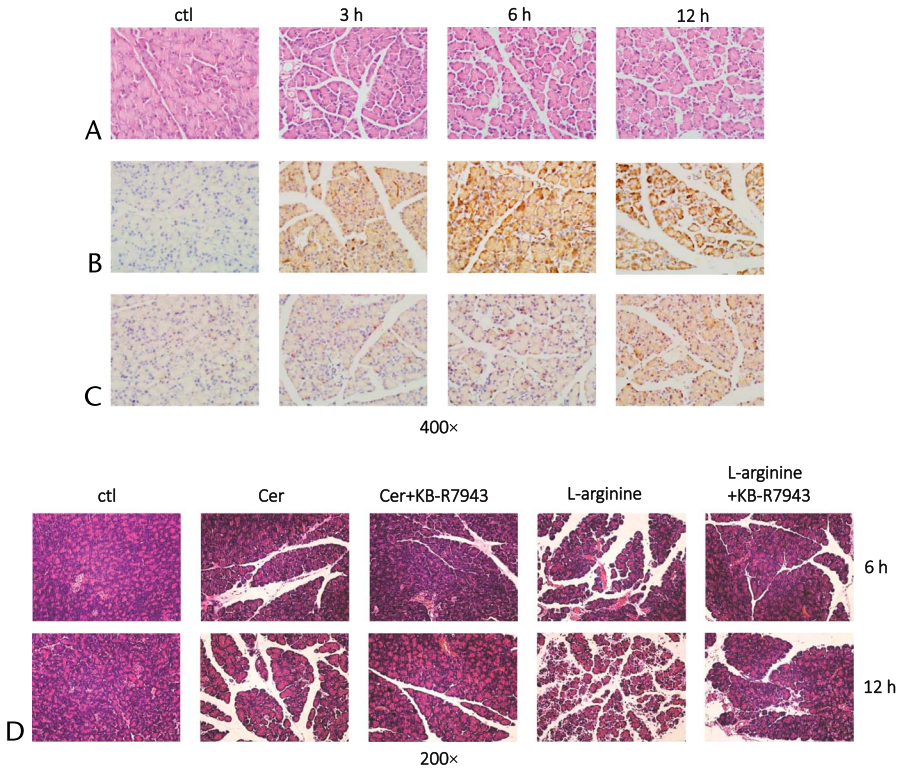

FIGURE 6. NF-κB and NCX1 levels were both increased in L-arginine-induced rat models. A, Histological changes were analyzed by H&E staining. B and C, NF-κB and NCX1 protein was measured by immunohistochemical staining (B) and Western blot (C), Rats were killed at 3, 6, 12 hours, after intraperitoneal injection of L-arginine. n = 6 rats per group in this experiment. D, Decreased symptoms of AP in rat pancreatic tissue following 3 mg/kg KB-R7943 treatment and AP induction with 2.5 g/kg L-arginine or 50 ng/g cerulein, visualized by H&E staining. KB-R7943, NCX1-targeted inhibitor.

Elevated NF-κB and NCX1 Expression in L-Arginine-Induced AP Rat Models

To confirm the possible regulatory activity by NF-κB on NCX1 expression during AP, we established another AP rat model induced by L-arginine. Staining with H&E revealed that rat pancreases were in the early to middle phases of acute inflammation between 3 and 12 hours following L-arginine treatment (Fig. 6A). Furthermore, immunohistochemical staining showed that both NCX1 (Fig. 6B) and NF-κB (Fig. 6C) expression increased gradually during the early to middle phases of L-arginine-induced AP.

To determine the effects of cerulein- or L-arginine-induced AP following chemical suppression of NCX1 in vivo, we treated rats intraperitoneally with the NCX1-targeted inhibitor KB-R7943 (3 mg/kg), followed by injection of cerulein (50 ng/g) once per hour for 6 hours, or 25% L-arginine (2.5 g/kg) once per hour for 2 hours. Staining with H&E of rat pancreatic tissue showed edema, exudation, necrosis, and inflammatory cell infiltration in cells treated with cerulein or L-arginine only, whereas AP was alleviated in cells exposed to KB-R7943 (Fig. 6D).

DISCUSSION

In this study, we found that NCX1 was expressed in rat pancreatic acinar cells and treatment with cerulein increased levels of both NXC1 mRNA and protein. Immunohistochemical staining of NCX1 and H&E staining showed that NCX1 expression was commensurate with the severity of pancreatitis. Together, these results strongly suggested that NCX1 played a critical role in pancreatitis development. Further experiments revealed that expression of the transcription factor NF-κB was also increased during AP, and that NF-κB inhibition led to downregulation of NCX1, indicating that NF-κB may primarily serve as a positive regulator of NCX1.

Although the majority of cases of AP are mild and patients tend to recover quickly, severe cases of AP can potentially result in death, with mortality rates of roughly 5% to 10%.[23] The pathogenesis of pancreatitis is still not well understood. Cytosolic calcium ($[Ca^{2+}]_{cyt}$) is a versatile signal molecule in the regulation of many cellular processes, and specifically it has been shown to play a central role in controlling the secretion of digestive enzymes in pancreatic acinar cells.[5] Evidence from several studies has indicated that $[Ca^{2+}]_{cyt}$ overload is a key event in the early pathogenesis of pancreatitis.[23–26] In pancreatic acinar cells, abnormal Ca^{2+} signaling can be stimulated by bile and metabolites of alcohol, among other causes, which triggers a prolonged, global elevation in Ca^{2+}, leading to activation of trypsin, and subsequently contributing to the initiation of cell injury.[11] To counter this process during AP, specific calcium channel blockers have been used in combination with drugs.[6,27,28]

First reported in squid giant nerve axons and guinea pig heart,[29,30] NCX1 is a bidirectional transport protein capable of rapidly translocating a high number Ca^{2+} ions across the cell membrane.[16] Normally, NCX1 is the main channel for calcium efflux such as occurs during the physiological processes of insulin secretion, and myocardial and neuronal action potential.[31,32] However, during the occurrence and development of most diseases, the function of NCX1 reverses, changing the direction of ion exchange, resulting in elevated accumulation of intracellular Ca^{2+}.[19] In this work, we found that NCX1 was significantly upregulated in cerulein-treated rat and cell culture models of AP,

and that intracellular Ca^{2+} increased when AR42J cells were given this treatment. Knockdown of NCX1 by shRNA also led to stable, unchanging levels of intracellular Ca^{2+} after cerulein treatment, thus confirming that NCX1 mediates the flow of external calcium into AR42J cells, and suggesting that this protein may be an essential contributor to Ca^{2+} overload leading to pancreatitis.

In light of these findings, we next explored the relevant mechanisms by which NCX1 is regulated during pancreatitis to better understand the role of NCX1 in AP and to subsequently develop more effective strategies for AP treatment. The PI3K/AKT-NF-κB pathway has been well studied in AP, and the roles of this signaling pathway have been confirmed in previous research.[32–35] There is a substantial body of evidence showing that the development and progression of AP depend on NF-κB expression, which is also crucial for the transcriptional regulation of inflammatory mediators.[36–38] We found several NF-κB binding sites in the NCX1 promoter, and that pP65, the activated form of P65, a component of NF-κB, was increased in both AR42J cell culture and in vivo rat AP models. The NF-κB agonist PMA directly activated the NCX1 promoter, although transcription driven by the NCX1 promoter was repressed if cells were pretreated with the inhibitor of NF-κB, Bay11-7082. In addition, the expression of both NF-κB and NCX1 were increased during L-arginine-induced AP disease progression. In agreement with this study, recent review has also shown that NCX1 is an essential factor mediating the pathogenesis of digestive system, while chemical inhibition of NCX1 can alleviate the symptoms of pancreatitis.[39] In our study, we found that the NCX1-targeted inhibitor KB-R7943 mitigated the symptoms of edema, exudation, necrosis, and inflammatory cell infiltration during induced AP in vivo, thus confirming the role of NCX1 in this disease.

In summary, our data indicated a link between NF-κB and NCX1 in AP pathogenesis. We propose that cerulein treatment induced AP given our findings that the early transcription factor NF-κB was activated by this treatment, which in turn promoted NCX1 expression and increased intracellular levels of Ca^{2+}, resulting in a Ca^{2+} overload that exacerbated pancreatitis development. These data provide a preliminary, mechanistic insight into the function of NCX1 in development and progression of AP and its possible involvement in the regulatory cascade leading to AP. This study therefore provides a novel, potential target for treatment and prevention of AP.

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