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Atrial natriuretic peptide reduces inflammation and enhances apoptosis in rat acute pancreatitis

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Abstract

Aim: We previously reported that atrial natriuretic peptide (ANP) reduces serum amylase and intrapancreatic trypsinogen activation in the onset of acute pancreatitis whereas secretin increases them. In the present work, we sought to establish the effect of ANP and secretin on the inflammatory response and cell death in experimental acute pancreatitis.

Methods: The expression and activity of key inflammatory mediators and apoptosis were evaluated in the presence or absence of the atrial peptide, secretin or both in cerulein-induced acute pancreatitis in rats. Also, ultrastructural changes in pancreatic acinar cells were assessed by transmission electron microscopy.

Results: ANP significantly reduced NF-κB activation and TNF-α intrapancreatic levels. Furthermore, it decreased inducible nitric oxide synthase and cyclooxygenase 2 expression and activity while it diminished myeloperoxidase activity. ANP also stimulated apoptosis as shown by caspase-3 expression and activation as well as TUNEL assay. These findings correlated well with the ultrastructural changes observed in the exocrine pancreas. Although secretin reduced various inflammatory markers, it also diminished caspase-3 activation and the overall response was the aggravation of the disease as reflected by the ultrastructural alterations of pancreatic acinar cells. In the presence of ANP, various effects evoked by secretin were antagonized.

Conclusion: Present findings show that ANP significantly attenuated the severity of acute pancreatitis in the rat by inducing apoptosis and reducing the inflammatory response and further suggest that ANP may have eventual therapeutic implications in the disease and/or in medical interventions at risk of its developing like endoscopic retrograde cholangiopancreatography.

KEYWORDS
cell death, inflammation, natriuretic peptides, pancreatitis

1 | INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with significant morbidity that unfortunately has no specific therapy. It is a potentially life-threatening condition with varying severity of presentation (mild, moderate or severe, according to the updated Atlanta classification). Most patients (approx. 80%) suffer a mild and self-limiting disease that resolves without serious complications, but about 20% of the cases develop multiple organ dysfunction syndrome, associated with high mortality rate. The course and severity of AP can fluctuate rapidly and unpredictably, and unfortunately, there is currently no ideal predictor of severity.[1]

For many years, cathepsin B-mediated intrapancreatic trypsinogen activation was considered causally responsible for the pathogenesis of AP.[2] However, clinical studies showed that protease inhibitors were not completely effective in the disease.[3] Recent findings support that although premature trypsinogen activation is a major event leading to cell death during early pancreatitis and responsible for half of eventual pancreatic injury in the disease, nuclear factor kappa B (NF-κB) activation accounts for local inflammation contributing to pancreatic injury and the eventual widespread systemic inflammatory response.[4] Interventions preventing NF-κB activation reduce the severity of AP as shown in different experimental models.[5] Therapies targeting the inhibition of both trypsinogen and NF-κB activation would be desirable.

In AP, the acinar cell dies by necrosis and apoptosis and the magnitude of each type of cell death conditions the course of the disease. Acinar cell necrosis exacerbates inflammation, and favours distant organ failure, thus correlating with poor prognosis.[6] Apoptosis instead limits inflammation because the cytoplasmic content is packaged in apoptotic bodies and these membrane-bound cell fragments are rapidly degraded, and so it is associated with a better outcome.[7] In AP, apoptosis is mediated by both the extrinsic and the intrinsic pathways, being the former initiated by caspase-8 following tumour necrosis factor (TNF)-α or Fas receptor activation whereas the latter involves mitochondrial permeabilization followed by the release of cytochrome-c and other pro-apoptotic factors. Both pathways then activate the effector caspase-3, which drives the apoptotic machinery by the cleavage of various substrates, such as poly (ADP-ribose) polymerase 1 (PARP-1). Various studies show that in AP, caspases not only stimulate apoptosis but also inhibit necrosis, leading to a better prognosis of the disease.[7] Switching necrosis to apoptosis would be a beneficial therapeutic approach.

Atrial natriuretic peptide (ANP), a member of the natriuretic peptide family, plays a relevant role in the regulation of cardiovascular and renal function but increasing evidence supports that it also modulates digestive physiology.[8,9] We previously reported that ANP through the natriuretic peptide type C receptor (NPR-C) coupled to the phospholipase C/protein kinase C (PLC/PKC) pathway stimulates pancreatic secretion, and negatively regulates cAMP intracellular levels in the pancreas evoked by pancreatic secretagogues like secretin and vasoactive intestinal peptide.[10-12] ANP stimulates cAMP efflux of the acinar cell through multidrug resistance-associated protein type 4 (MRP4) as a regulatory mechanism in addition to phosphodiesterase activity to restrict the intracellular accumulation of the cyclic nucleotide within the acinar cell to prevent cell damage.[13] In this sense, we reported that early AP is aggravated by enhanced intracellular cAMP, but pre-treatment with ANP through NPR-C activation attenuates the severity of the disease by extruding the second messenger.[14] In addition, ANP reduces serum amylase and intrapancreatic trypsin content, suggesting diminished acinar cell injury, which was further confirmed by light microscopy studies showing reduced acinar cell vacuolization and necrotic areas.[14] Further, we showed that secretin, which signals through cAMP, aggravates the course of AP as it significantly increases trypsinogen activation and amylase activity, but ANP ameliorates in part secretin action.[14] The protective effect of ANP at early stages of AP is partly mediated by ANP-induced cAMP efflux through MRP4.

Studies in macrophages and other immune cells show that ANP displays anti-inflammatory properties given that it inhibits the synthesis and release of pro-inflammatory cytokines and reduces the expression of several adhesion molecules with a pivotal role during the inflammation process.[15] Several immune cells synthetize ANP and express natriuretic peptide receptors.[16-18] In addition, various reports show that ANP exerts cryoprotective effects in cardiomyocytes, hepatocytes and vascular smooth muscle cells, endothelial and renal cells in different pathophysiological situations.[19] Therefore, in this study, we sought to establish the role of ANP and secretin in the inflammatory response and acinar cell death in cerulein-induced AP in the rat.

2 | RESULTS

2.1 | ANP reduces NF-κB and ERK 1/2 activation

NF-κB (p65 subunit) translocates to the nucleus to promote the synthesis and expression of cytokines and pro-inflammatory factors.[20] The expression of p65 was assessed in nuclear pancreatic extracts and results showed that in cerulein-treated animals, p65 was enhanced, but it was

significantly reduced by ANP (Figure 1A). Surprisingly, secretin also decreased p65 nuclear translocation, and further in the presence of both peptides, p65 expression was similar to control. The level of NF-κB activation was further confirmed by the expression of the inhibitor κB (IκB)-α, which normally represses p65 nuclear translocation.[5] Both ANP and secretin increased IκB-α expression, in accordance with reduced NF-κB activation (Figure 1B).

ERK 1/2 activation is an initial event in AP and is one of the pathways involved in NF-kB activation and cytokine release.[21] ERK 1/2 phosphorylation was enhanced in AP, but pre-treatment with ANP reduced it to control values (Figure 1C). Secretin did not modify ERK 1/2 activation in AP, but when co-infused with ANP, ERK 1/2 phosphorylation was significantly reduced.

2.2 | ANP reduces intrapancreatic TNF-α and myeloperoxidase (MPO) activity

NF-κB activation leads to enhanced TNF-α production.[22] Intrapancreatic TNF-α was increased in cerulein-treated rats, but it was reduced to control values by ANP (Figure 2A). Although secretin reduced NF-κB activation, the hormone failed to reduce TNF-α, supporting that factors others than NF-κB stimulate its production in AP.

Neutrophil infiltration, stimulated by cytokines and chemokines, is a main cause of tissue damage in AP.[6] In the present study, neutrophil infiltration was estimated by the activity of intrapancreatic MPO, the most abundant pro-inflammatory enzyme stored in these cells. Both secretin and ANP reduced MPO activity in AP, although ANP effect was more prominent. Combined administration of both peptides failed to further decrease MPO activity as compared with ANP (Figure 2B). Unexpectedly, the sole administration of ANP to control rats induced a small but significant increase in MPO activity.

2.3 | ANP reduces inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) expression and activity

Several studies support a deleterious effect mediated by nitric oxide overproduction derived from iNOS activation in AP.[23] The expression of iNOS increased in AP, but it was reduced to control values in the presence of secretin, ANP or both peptides (Figure 3A). Accordingly, iNOS activity correlated well with the enzyme expression (Figure 3B). The increase in COX-2 expression at early stages of AP is associated with poor clinical prognosis.[24] COX-2 expression was significantly diminished by ANP or secretin, although the effect of ANP was more prominent (Figure 3C). When both peptides were co-infused, the reduction of COX-2 was even lower. The activity of COX-2, estimated through the assessment of prostaglandin (PG) E_2 intrapancreatic content, followed COX-2 expression pattern (Figure 3D).

2.4 | ANP stimulates apoptosis

TUNEL assay studies showed that ANP stimulated apoptosis not only in AP but also in control animals (Figure 4A). Secretin also increased apoptotic cells in normal animals but not in rats with AP. Although an increasing tendency was observed in rats with AP in the presence of secretin, it is not statistically significant as compared with animals with AP alone. Assessment of caspase-3 expression showed that ANP significantly enhanced it, although secretin clearly diminished it in rats with AP (Figure 4B). These findings were in accordance with caspase-3 activity as shown in Figure 4C. The enzyme activity was also indirectly assessed by cleavage of PARP-1 and results further confirmed caspase-3 activity (Figure 4D). Altogether, these findings support that ANP but not secretin stimulates apoptosis in AP.

2.5 | ANP attenuates the major ultrastructural pancreatic features in AP

Ultrastructural changes in the exocrine pancreas were assessed to determine whether they correlated with the biochemical changes observed in the different experimental groups. Control animals showed regular acinus, well organized with intact gap junctions (GJ) (Figure 5A). Nucleus (not seen in this micrograph) was localized at the basolateral area surrounded by abundant rough endoplasmic reticulum (ER) with regular parallel arrangement of the stacks and numerous zymogen granules (ZG) at the apical zone close to the acinar lumen (L) (Figure 5A). Animals with cerulein-induced pancreatitis showed clear features of AP like loss of gap junctions, cytoplasm vacuolization (V), oedema (E), few zymogen granules dispersed in the cytoplasm, endoplasmic reticulum (ER) swelling, nuclear interdigitation and disrupted basement acinar cell membranes indicative of necrosis (Figure 5B). In addition, autophagosomes (A) containing disintegrating organelle membranes and nuclear fragments were also observed. The presence of nuclear fragments in these organelles was consistent with the observation of vacuoles positive for TUNNEL assay (data not shown). Mitochondria (M) showed swelling and deformed cristae whereas nuclei peripheral condensation of chromatin is indicative of apoptosis (Figure 5B).

In the presence of secretin, animals with AP showed enhanced cytoplasmic vacuolization (V) and oedema (E), endoplasmic reticulum (ER) swelling with loss of ribosomes, mitochondria (M) with dilated and abnormal cristae as well as increased myelin-like bodies (MLB) indicative

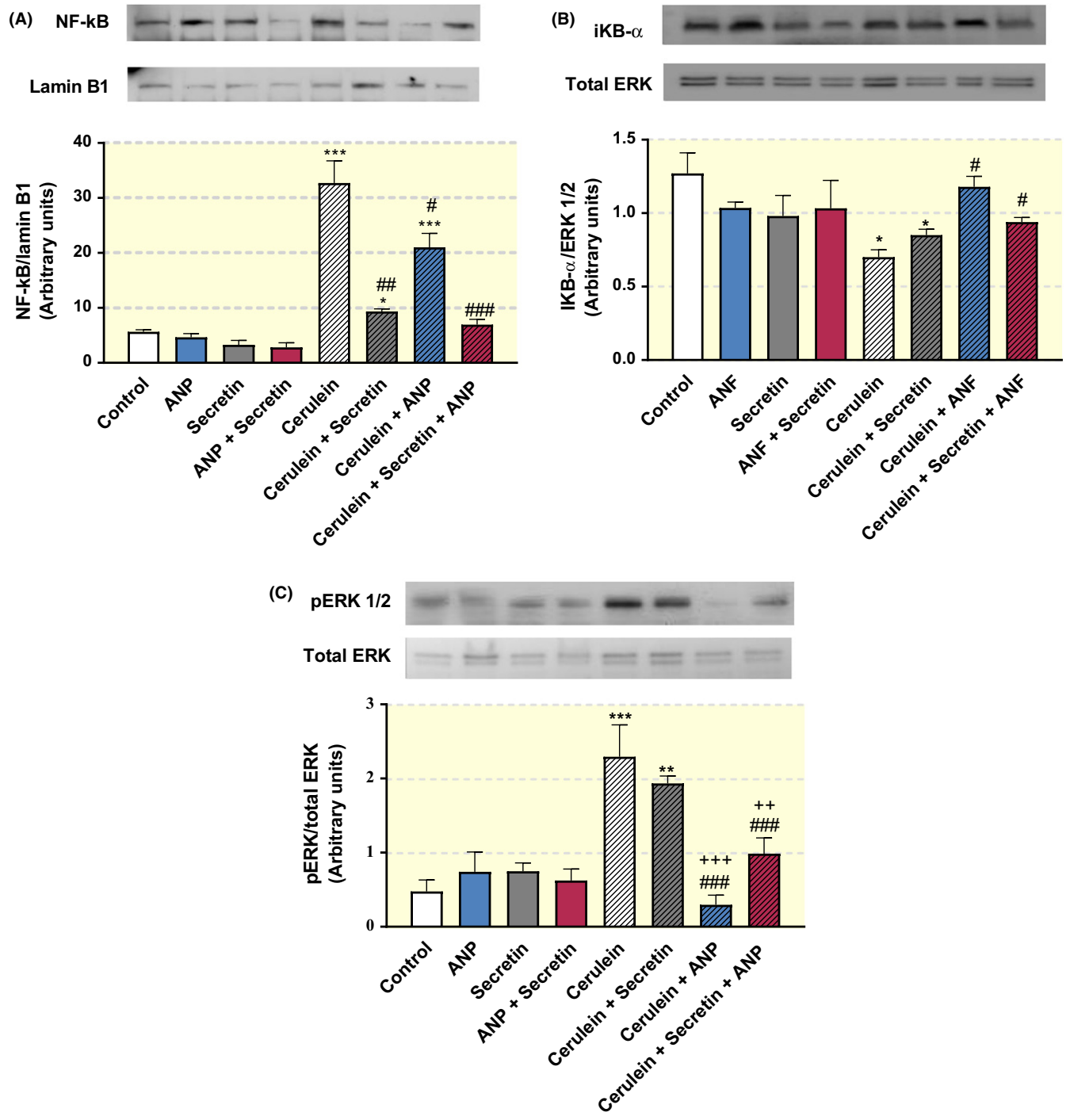

FIGURE 1 ANP decreases NF-κB activation and ERK 1/2 phosphorylation in acute pancreatitis. The expression of NF-κB (p65 subunit) (A), IκB-α (B) and pERK 1/2 (C) was determined by Western blot as detailed in Materials and Methods. Representative Western blots and the densitometric analysis expressed in arbitrary units are shown. *: $P < .05$, **: $P < .01$ and ***: $P < .001$ vs control; #: $P < .05$ and ###: $P < .001$ vs cerulein; ++: $P < .01$ and +++: $P < .001$ vs cerulein + secretin. Number of cases 4-6

of necrosis (Figure 5C). Furthermore, increased acinar cells with disrupted basement membrane were also observed. These findings show that although secretin reduced some inflammatory mediators, these changes failed to impact on the ultrastructure of acinar cells (Figure 5C). When animals with AP were treated with ANP, a significant improvement of exocrine pancreatic morphology was observed. Cytoplasmic vacuolization (V), endoplasmic reticulum (ER) swelling and necrotic areas were significantly reduced, whereas cellular gap junctions (GJ) within acini were preserved (Figure 5D). In addition, apoptotic nuclei were significantly enhanced as compared with the other experimental groups.

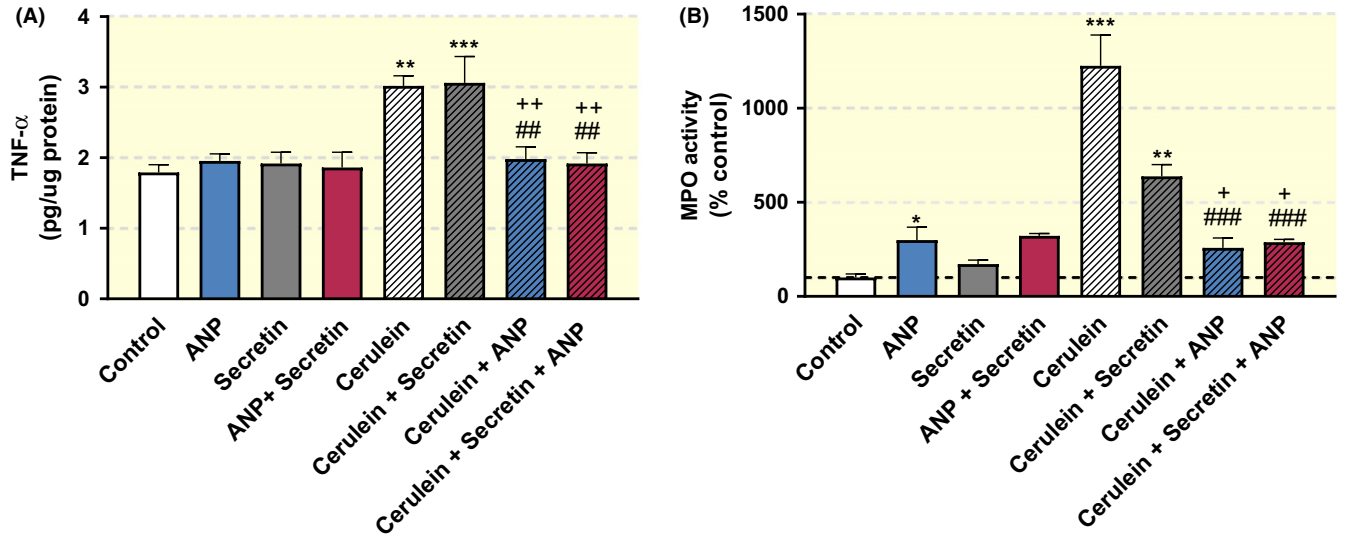

FIGURE 2 ANP decreases TNF-α and MPO activity in acute pancreatitis. TNF-α intrapancreatic content (A) and MPO activity (B) were assessed as detailed in Materials and Methods. *: $P < .05$, **: $P < .01$ and ***: $P < .001$ vs control; ##: $P < .01$ and ###: $P < .001$ vs cerulein; +: $P < .05$ and ++: $P < .01$ vs cerulein + secretin. Number of cases 4-6

Furthermore, ANP also ameliorated the morphological changes induced by secretin in AP (Figure 5E). Lower power micrographs are shown as Figure S1.

3 | DISCUSSION

The major finding of the present work was that ANP significantly attenuated the course of AP by reducing the inflammatory response and stimulating apoptosis, suggesting a potential therapeutic and/or prophylactic effect in medical interventions like endoscopic retrograde cholangiopancreatography (ERCP). Despite numerous studies over the past years, the inner aspects of AP pathogenesis remain elusive, and although different theories have been proposed, they can only explain certain aspects of the pathogenesis.

Cathepsin B-induced trypsinogen activation was for the last decades the most accepted theory in the pathogenesis of AP. However, studies performed in trypsin or cathepsin B deficient animals revealed that although trypsinogen activation within the pancreas is a major event leading to acinar cell death in AP, it is not sufficient to explain the whole pathogenesis.[25] Several studies show that NF-κB activation is a key event for the initiation of the disease independent of trypsinogen activation.[2] NF-κB activation occurs at early stages of AP and is responsible for the development and propagation of the local inflammatory response through the synthesis and expression of cytokines and inducible enzymes like iNOS and COX-2. The level of NF-κB activation correlates with the severity of AP.[26]

We show here that ANP decreased the nuclear translocation of NF-κB (p65 subunit) and TNF-α intrapancreatic content in AP, consistent with previous studies in immune cells and other cell types.[15,27] The reduction of NF-κB activation induced by ANP in AP was higher in the presence of secretin. Previous studies showed that ANP prevents liver injury induced by ischaemia-reperfusion by reducing NF-κB and TNF-α synthesis and release through NPR-A receptors coupled to guanylyl cyclase activation.[15] Furthermore, ANP also displays anti-inflammatory effects in the kidney, heart and lung.[27]

In the exocrine pancreas, ANP signals preferentially through NRP-C receptors coupled to Gi activation, and regulates intracellular cAMP levels to prevent acinar cell injury.[13,14] Various studies reveal that cAMP is a modulator of NF-κB action, although the positive or negative effect on the transcription factor appears to be highly cell type- and context-dependent, as it is its role in programmed cell death.[28] Although secretin reduced NF-κB activation, it failed to diminish mediators like TNF-α or ERK 1/2, which exert positive feedback on the local inflammatory response.

In the onset of AP, nitric oxide and PGs are released in large amounts due to the activation of iNOS and COX-2, enzymes playing a pivotal role in the inflammatory response. The overproduction of nitric oxide and PGs may be detrimental for cell survival and different studies show that a constant crosstalk between both pathways exists at different levels.[29] The regulation of iNOS activity is rather complex and involves different mediators. The synthesis is partially controlled by NF-κB, but ANP can reduce iNOS mRNA stability and L-arginine uptake in macrophages.[30] Further, COX derivatives also regulate the nitric oxide pathway. During inflammation, iNOS activation generates

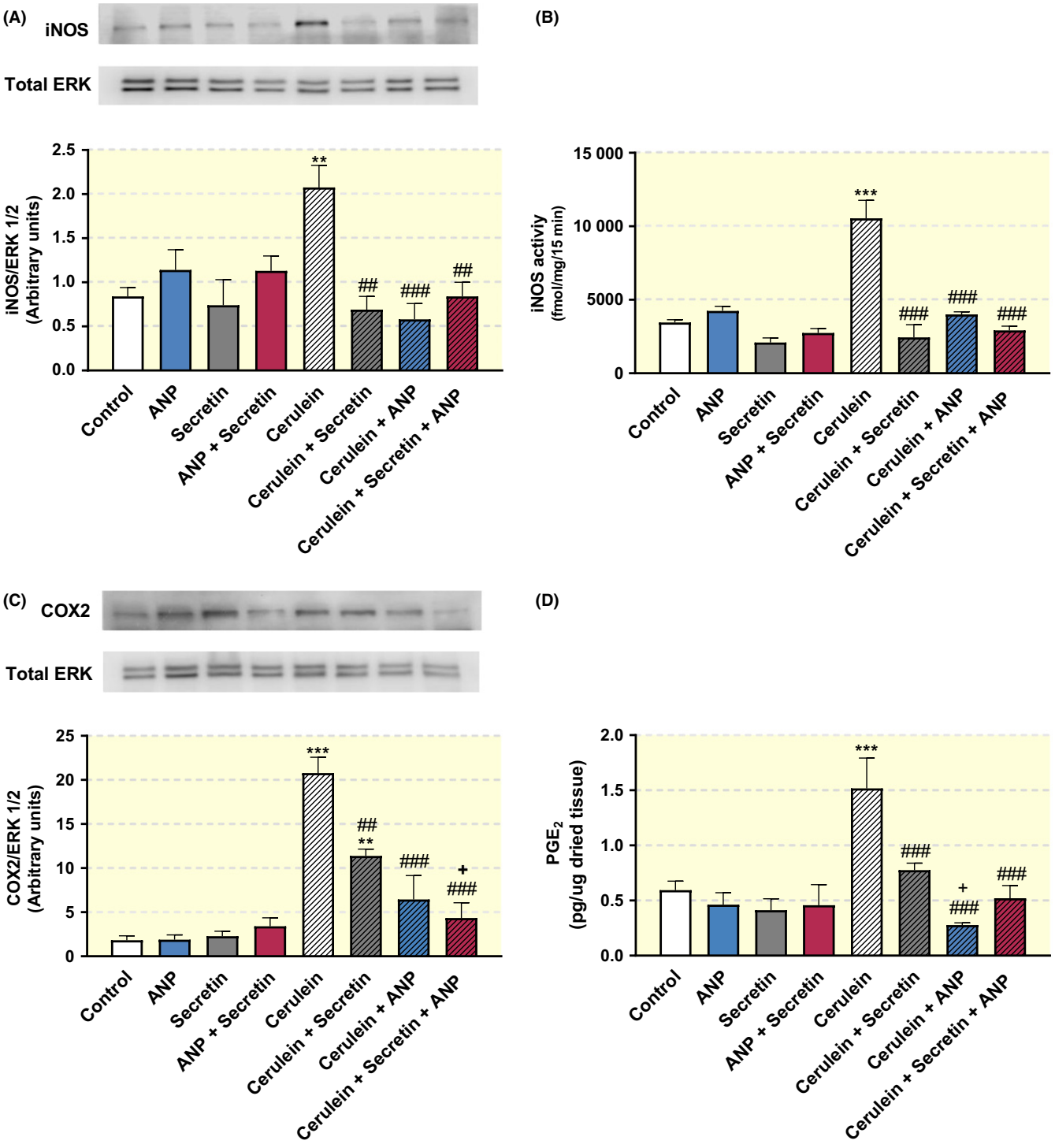

FIGURE 3 ANP diminishes iNOS and COX-2 activity and expression in acute pancreatitis. The expression of iNOS (A) and COX-2 (C) was determined by Western blot as detailed in Materials and Methods. Representative immunoblots and the densitometric analysis expressed in arbitrary units are shown (A, B). The activity of iNOS activity was assessed by the conversion of [14C]-L-arginine to [14C]-L-citrulline in the presence of EGTA (B) and COX2 by PGE_2 production (D) as detailed in Materials and Methods. **: $P < .01$ and ***: $P < .001$ vs control; ##: $P < .01$ and ###: $P < .001$ vs cerulein; +: $P < .05$ vs cerulein + secretin. Number of cases 4-6

high amounts of nitric oxide which is deleterious for the cells.[23] In AP, ANP reduced both the expression and activity of iNOS, which correlated with a significantly lesser degree of necrosis as shown by ultrastructural studies. Furthermore, ANP also reduced COX-2 expression and PGE_2 levels. Accumulating evidence indicates the essential contribution of the COX-2 pathway to cerulein-induced acute pancreatitis because its inhibition ameliorates the

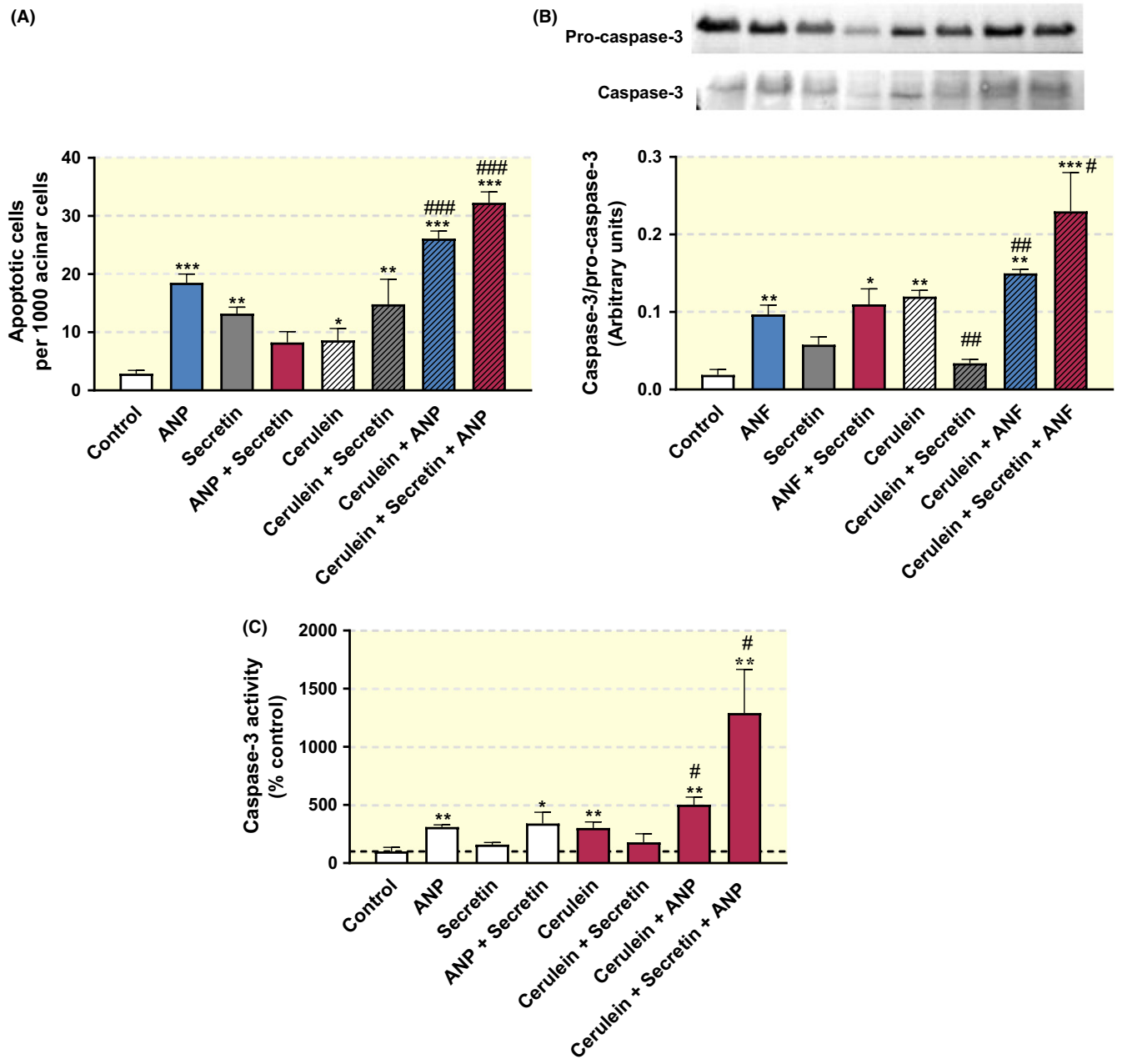

FIGURE 4 ANP stimulates apoptosis in acute pancreatitis. A, Quantification of apoptotic nuclei in the different experimental groups as measured by TUNEL assay as described in Materials and Methods. Apoptotic nuclei were expressed per 1000 acinar cells. B, Caspase-3 expression was determined by Western blot as detailed in Materials and Methods. Representative Western blots and the densitometric analysis expressed as the ratio between caspase-3 and procaspase-3 are shown. C, Caspase 3 activity as determined by a fluorometric assay as detailed in Materials and Methods and expressed as percentage over control. D, PARP-1 expression was determined by Western blot. Representative immunoblot and densitometric analysis expressed as cleaved PARP-1 over PARP-1 are shown. *:$P < .05$, **: $P < .01$ and ***: $P < .001$ vs control; ##: $P < .01$ and ###: $P < .001$ vs cerulein. Number of cases 4-6

disease.[24,31] Upregulation of COX-2 expression is mediated by NF-κB, TNF-α and pERK 1/2, so the reduced expression of the enzyme might result from the attenuation of such mediators induced by ANP.[32] However, a direct effect cannot be excluded, given that previous studies show that ANP inhibits COX-2 expression in macrophages through NPR-C receptors.[33] In addition, the activity of COX-2 is regulated by nitric oxide but also by iNOS, which binds to COX-2 and s-nitrosylates it, thus enhancing its activity.[34] PGE_2, the major product of COX-2, propagates the inflammatory response, so ANP-induced PGE_2 decrease is in line with the reduced expression of the enzyme. However, ANP also stimulates MRP4, which is a transporter for cAMP, but for which PGE_2 is also physiological substrate.[14,35]

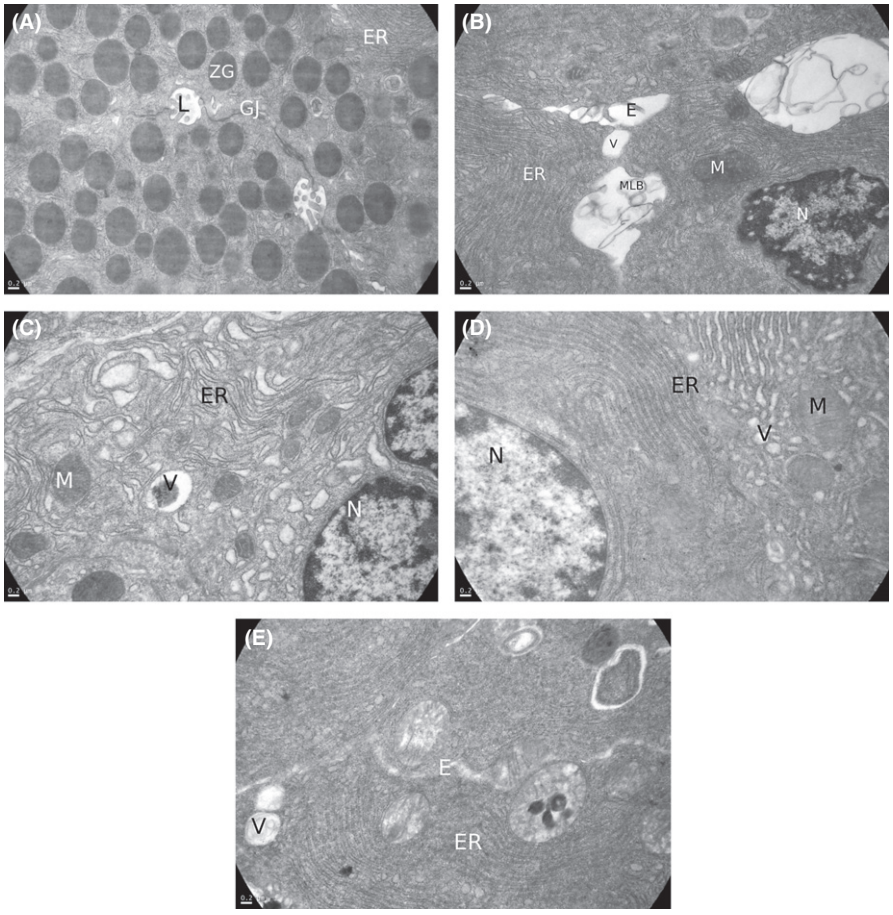

FIGURE 5 ANP prevents most of the ultrastructural changes of the acinar cells in the early stages of acute pancreatitis. Representative transmission electron micrographs of pancreatic acinar cells derived from control animals (A) and with acute pancreatitis (B) infused with secretin (C), ANP (D) or secretin + ANP (E). E, oedema; ER, endoplasmic reticulum; F, phagolysosome; GJ, gap junction; L, lumen; M, mitochondria; N, nucleus; V, vacuole; ZG, zymogen granule; MLB, myelin-like body. See text for details

Thus, the contribution of MRP4 stimulated by ANP to reduce intrapancreatic PGE_2 is likely to also occur.

In the onset of AP, the release of inflammatory signals from acinar cells promotes the recruitment and activation of neutrophils, which contribute to induce an intense local inflammatory response that may eventually become systemic. Recent advances show that neutrophils are central to the evolution of severe AP, mediating local tissue damage as well as distant organ injury.[6] In this sense, diverse interventions targeting neutrophils have been effective in reducing tissue damage. ANP decreased intrapancreatic MPO activity in AP suggesting less neutrophil infiltration which was supported by less acinar cell damage at the ultrastructural level.

The intense local inflammatory response in AP induces cell death mainly by necrosis which helps to propagate inflammation. Apoptosis was clearly stimulated in the presence of ANP and diminished in the presence of secretin. Several studies support that the severity of AP correlates directly with necrosis and inversely with apoptosis.[7,36] Necrosis is associated with poor prognosis, potential multiorgan failure and death whereas apoptosis with a better outcome given that the inflammatory response is limited because cells preserve the structural integrity of the plasma membrane. A recent report shows that during AP, cathepsin B leaks to the cytosol from colocalized organelles and is responsible for the stimulation of apoptosis, although when excessively released it causes necrosis.[37] Switching from necrosis to apoptosis is one of the big issues in the development of therapies for AP.[38] We previously reported that ANP reduces the extent of necrosis in AP so we evaluated whether it favoured apoptosis.[14] The atrial peptide stimulated caspase-3 activity and expression as well as cleaved PARP-1, an early substrate of the enzyme, which is able to switch the type of cell death from apoptosis to necrosis.[39] Furthermore, it also significantly increased the number of apoptotic nuclei as revealed by TUNEL assay. These findings show that ANP switches necrosis to apoptosis. Secretin diminished apoptosis as reflected on caspase-3 activation and expression and increased necrosis. Interestingly, in the presence of secretin that aggravates AP, the effect of ANP on apoptosis was increased as shown by caspase-3 activity and expression, and increased apoptotic nuclei. This finding suggests that the stimulation of apoptosis by ANP would be higher when the disease is more severe.

It is worth noting that the nuclear protein PARP-1 is not only involved in DNA repair and cell death by necrosis, but it also plays a significant role in inflammatory disorders as it influences the expression of pro-inflammatory

cytokines like TNF-α, iNOS, COX-2 and inflammation adhesion molecules.[40] It has been reported that PARP-1 promotes NF-κB activation and enhanced inflammatory mediator's gene expression.[40] Genetic or pharmacological blockade of PARP-1 significantly attenuates the severity of AP.[41]

Present findings support that ANP attenuated the inflammatory response and stimulated apoptosis in cerulein-induced AP. The beneficial effect of ANP was reflected on the ultrastructure of the pancreatic acinar cells. In the presence of ANP, ER and mitochondrial swelling, cytoplasmic vacuoles and necrotic areas were significantly reduced, whereas cellular gap junctions within acini were preserved. Although secretin also reduced some inflammatory mediators, it was not sufficient to protect the exocrine pancreas from severe acinar cell damage as shown by the ultrastructural changes observed. Secretin clearly aggravated AP reflected on diminished apoptosis, increased number of vacuoles, autophagosomes and myelin-like bodies and deformed ER with loss of ribosomes and mitochondria with dilated cristae. It is worth noting that ANP reduces premature trypsinogen activation whereas secretin significantly enhances it.[14]

ERCP is the first therapeutic choice for many biliary and pancreatic disorders. The most frequently occurring complication of this endoscopic procedure is AP, and various strategies have been used to reduce the incidence of AP particularly pharmacological and endoscopic methods.[42,43] Prophylactic administration of protease inhibitors and non-steroidal anti-inflammatory drugs has been shown effective in reducing the risk of post-ERCP pancreatitis.[44] Present and previous findings support that ANP attenuates the severity of AP in the rat. The atrial peptide downregulates the activation of the two major players involved in AP pathogenesis, trypsinogen and NF-κB, and further, it switches cell death from necrosis to apoptosis. Previous studies have also shown that ANP displays cytoprotective effects in cardiovascular, hepatic and renal diseases as shown by experimental and clinical studies.[19] It is important to point out that although ANP displays vasodilation properties, the dose of the peptide used in the present study does not induce haemodynamic changes. The course of AP was significantly ameliorated in the presence of ANP, suggesting that the atrial peptide may have eventual therapeutic implications in the disease and/or medical interventions at risk of the disease development like ERCP.

4 | MATERIALS AND METHODS

4.1 | Animals and reagents

Female Sprague Dawley rats (180-210 g) were housed in steel cages and maintained at 22-24°C in a controlled room with a 12-hour light-dark cycle (light from 07:00 to 19:00 hour). Experimental protocols were approved by the Animal Care Committee of the Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires (CICUAL-FFYB #4107/15). All procedures complied with the recommendations of the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication N85-23, 1985; revised 1996). Most reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

4.2 | Experimental protocol

The experimental protocol in the present study was the same used in previous studies aiming to unveil the role of ANP in AP.[14] Animals were fed standard animal laboratory chow (Gepsa Feeds, Córdoba, Argentina), given water ad libitum, fasted overnight and randomly assigned to control or experimental groups. Animals were anaesthetized with ethyl urethane (1.25 g/kg ip), and AP was induced by four repetitive intraperitoneal injections of 40 μg/kg cerulein dissolved in saline.[14]ANP (1 μg/kg/h) (American Peptide Company, Sunnyvale, CA, USA) alone or with secretin (1 U/kg/h) (American Peptide Company) was infused by a cannula at the left jugular vein for 60 minutes starting 30 minutes before the first cerulein injection.[10,14] Saline-infused animals served as controls. Animals were killed by decapitation at 1 hour after the last cerulein injection, and blood and pancreatic tissue samples were harvested for biochemical determinations, Western blot assays and transmission electron microscopy studies. Animals with AP showed plasma amylase values threefold over control, whereas the other groups exhibited values as previously reported.[14]

4.3 | Western blot assays

Protein expression was assessed by immunoblotting in pancreatic homogenates except for NF-κB (p65 subunit) that was determined in pancreatic nuclear cell extracts as detailed below. Experimental procedures for Western blot were as previously detailed.[14] Samples separated on SDS-PAGE and electro-transferred to PVDF membranes were then exposed to primary antibodies overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated anti-rabbit (W4011) (Promega, Madison, WI, USA) or anti-mouse (sc-2005) (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies for 1 hour at room temperature. Membranes were developed by a bioluminescent Western blotting detection system (Kalium Technologies, Buenos Aires, Argentina) and exposed to X-ray films or assessed by a digital system (GeneGnome XRQ; Syngene, Maryland, MD, USA). The following primary antibodies were used: anti-p65 (610868) (BD Biosciences, San Jose, CA, USA);

anti-COX-2 (ab-15191) and anti-iNOS (ab-15323) (Abcam, Cambridge, UK); and anti-caspase-3 (sc-7148), anti-IκB-α (sc-371), PARP-1 (sc-7150), anti-lamin B1 (sc-377000), anti-ERK 1/2 (sc-94), anti-pERK 1/2 (sc-7383) and α-tubulin (sc-58666) (Santa Cruz Biotechnology). ERK 1/2 was used as loading control except for NF-κB expression where lamin B was used.

4.4 | Preparation of nuclear extracts

Nuclear protein extracts were obtained as previously described with minor modifications.[20] Pancreatic tissue was homogenized in sucrose-Hepes buffer containing protease inhibitor cocktail. Following centrifugation at 4000 *g* for 10 minutes, pellets were suspended in 10 mmol/L Hepes (pH 7.9), 10 mmol/L KCl and 1.5 mmol/L $MgCl_2$ with 1% NP-40 and protease inhibitor cocktail and incubated on ice for 3 minutes. Samples were then centrifuged at 4000 *g* for 10 minutes, and pellets washed, resuspended in 10 mmol/L Hepes, 2 mmol/L EDTA, 0.42 mmol/L KCl, 1.5 MgCl2 and 20% (vol/vol) glycerol and further incubated on ice for 20 minutes. Following centrifugation at 18 000 *g* for 20 minutes, supernatants were diluted in 10 mmol/L Hepes (pH 7.9), 0.25 mmol/L EDTA, 60 mmol/L KCl, 0.125 mmol/L EGTA and 20% (vol/vol) glycerol and stored at −80°C. The purity of nuclear extracts was evaluated by α-tubulin and lamin B expression. NF-κB (p65 subunit) expression was determined in nuclear extracts and lamin B used as loading control.

4.5 | Intrapancreatic TNF-α and PGE2 levels

Pancreatic tissue was homogenized in lysis buffer (200 mmol/L Tris-Cl, 5 mmol/L EDTA, 25 mmol/L NaF, 1% Triton X-100), centrifuged at 13 000 *g* for 10 minutes at 4°C and TNF-α assayed in supernatants by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). Results were expressed as pg TNF-α per μg protein. PGE_2 was assessed by radioimmunoassay as previously reported.[45] Pancreatic tissue was homogenized in 1 mL ice-cold ethanol and centrifuged at 10 000 *g* for 15 minutes at 4°C. Supernatants were dried in a speed-vac at room temperature and residues resuspended in radioimmunoassay buffer. PGE_2 intrapancreatic content was expressed as pg per μg dried tissue.

4.6 | MPO and iNOS activity

Neutrophil infiltration was estimated by measuring tissue MPO activity as previously detailed.[46] The absorbance was read at 405 nm and corrected for the DNA content in each tissue sample. Results were expressed as fold increase over the control group. Inducible NOS enzyme activity was assessed by measuring the conversion of [14C]-L-arginine to [14C]-L-citrulline as described by Bredt and Snyder with minor changes.[47] Radioactivity was measured by usual liquid scintillation counting methods and enzyme activity expressed as fmol [14C]-citrulline per mg of protein in 15 minutes.

4.7 | Caspase-3 activity

Caspase activity was assessed by a fluorometric assay kit (Clontech, Mountain View, CA, USA) containing a fluorogenic substrate specific for caspase-3 (Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin). The activity was determined by fluorometric detection (excitation, 380 nm; emission, 460 nm), and the negative control (blank, without sample) was subtracted from all samples. Results were expressed as fold increase over control.

4.8 | Quantification of apoptosis

Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labelling (TUNEL) assay (Promega). Pancreatic tissue was fixed in 10% buffered formaldehyde and embedded in paraffin and 4-μm-thick sections were obtained. Sections were deparaffinized by washing in xylene and hydrated by transferring through graded ethanol and stained for breaks in DNA. At least three sections per animal were counted and three animals per group. Results were expressed as apoptotic cells per 1000 acinar cells.

4.9 | Transmission electron microscopy studies

For ultrastructural analysis, pancreatic tissue was fixed by immersion in 4% wt/v paraformaldehyde plus 2.5% v/v glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS) pH 7.4 for 24 hours at 4°C. After washing in PBS with 0.32 mol/L sucrose, sections were post-fixed with 1.5% wt/v osmium tetroxide in 0.1 mmol/L PBS pH 7.4 for 2 hours at 4°C. After washing in PBS, sections were contrasted with 2% w/v uranyl acetate, dehydrated and embedded in Spurr medium kit (Ted Pella, Redding, CA, USA). Ultrathin sections were obtained with an ultramicrotome Porter Bloom MT 1 and collected in 300 mesh copper grids, contrasted with uranyl acetate and stained with Reynolds solution, and then photographed in a Gatam 1000 V coupled to a Zeiss EM109T Electron Microscope at different magnification (3000 × to 30 000×).

4.10 | Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) followed by the Student-Newman-

Keuls test. A *P*-value of .05 or less was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

1. Forsmark CE, Vege SS, Wilcox CM. Acute pancreatitis. *N Engl J Med*. 2016;375:1972-1981.
2. Sah RP, Dawra RK, Saluja AK. New insights into the pathogenesis of pancreatitis. *Curr Opin Gastroenterol*. 2013;29:523-530.
3. Singh VP, Chari ST. Protease inhibitors in acute pancreatitis: lessons from the bench and failed clinical trials. *Gastroenterology*. 2005;128:2172-2174.
4. Rakonczay Z, Hegyi P, Takacs T, McCarroll J, Saluja AK. The role of NF- B activation in the pathogenesis of acute pancreatitis. *Gut*. 2008;57:259-267.
5. Jakkampudi A, Jangala R, Reddy BR, Mitnala S, Reddy DN, Talukdar R. NF-κB in acute pancreatitis: mechanisms and therapeutic potential. *Pancreatology*. 2016;16:477-488.
6. Kang R, Lotze MT, Zeh HJ, Billiar TR, Tang D. Cell death and DAMPs in acute pancreatitis. *Mol Med*. 2014;20:466-477.
7. Mareninova OA, Sung K-F, Hong P, et al. Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. *J Biol Chem*. 2006;281:3370-3381.
8. Kerkelä R, Ulvila J, Magga J. Natriuretic peptides in the regulation of cardiovascular physiology and metabolic events. *J Am Heart Assoc*. 2015;4:e002423.
9. Sabbatini ME. Natriuretic peptides as regulatory mediators of secretory activity in the digestive system. *Regul Pept*. 2009;154:5-15.
10. Sabbatini ME, Villagra A, Davio CA, Vatta MS, Fernández BE, Bianciotti LG. Atrial natriuretic factor stimulates exocrine pancreatic secretion in the rat through NPR-C receptors. *Am J Physiol Gastrointest Liver Physiol*. 2003;285:G929-G937.
11. Sabbatini ME, Rodriguez M, di Carlo MB, Davio CA, Vatta MS, Bianciotti LG. C-type natriuretic peptide enhances amylase release through NPR-C receptors in the exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol*. 2007;293:G987-G994.
12. Sabbatini ME, Vatta MS, Davio CA, Bianciotti LG. Atrial natriuretic factor negatively modulates secretin intracellular signaling in the exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol*. 2006;292:G349-G357.
13. Rodríguez MR, Diez F, Ventimiglia MS, et al. Atrial natriuretic factor stimulates efflux of cAMP in rat exocrine pancreas via multidrug resistance-associated proteins. *Gastroenterology*. 2011;140:1292-1302.
14. Ventimiglia MS, Najenson AC, Perazzo JC, et al. Blockade of multidrug resistance-associated proteins aggravates acute pancreatitis and blunts atrial natriuretic factor's beneficial effect in rats: role of MRP4 (ABCC4). *Mol Med*. 2014;21:58-67.
15. De Vito P. Atrial natriuretic peptide: an old hormone or a new cytokine? *Peptides*. 2014;58:108-116.
16. Throsby M, Lee D, Huang W, Yang Z, Copolov DL, Lim AT. Evidence for atrial natriuretic peptide-(5–28) production by macrophages of the rat spleen: an immunochemical and nonradioactive in situ hybridization approach. *Endocrinology*. 1991;129:991-1000.
17. Vollmar AM, Schulz R. Expression and differential regulation of natriuretic peptides in mouse macrophages. *J Clin Invest*. 1995;95:2442-2450.
18. Morita R, Ukyo N, Furuya M, Uchiyama T, Hori T. Atrial natriuretic peptide polarizes human dendritic cells toward a Th2-promoting phenotype through its receptor guanylyl cyclase-coupled receptor A. *J Immunol*. 2003;170:5869-5875.
19. Saito Y. Roles of atrial natriuretic peptide and its therapeutic use. *J Cardiol*. 2010;56:262-270.
20. Gukovsky I, Gukovskaya AS, Blinman TA, Zaninovic V, Pandol SJ. Early NF-κB activation is associated with hormone-induced pancreatitis. *Am J Physiol*. 1998;275:G1402-G1414.
21. Irrera N, Bitto A, Interdonato M, Squadrito F, Altavilla D. Evidence for a role of mitogen-activated protein kinases in the treatment of experimental acute pancreatitis. *World J Gastroenterol*. 2014;20:16535-16543.
22. Gukovskaya AS, Gukovsky I, Zaninovic V, et al. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. *J Clin Invest*. 1997;100:1853-1862.
23. Ang AD, Adhikari S, Ng SW, Bhatia M. Expression of nitric oxide synthase isoforms and nitric oxide production in acute pancreatitis and associated lung injury. *Pancreatology*. 2009;9:150-159.
24. Ethridge RT, Chung DH, Slogoff M, et al. Cyclooxygenase-2 gene disruption attenuates the severity of acute pancreatitis and pancreatitis-associated lung injury. *Gastroenterology*. 2002;123:1311-1322.
25. Dawra R, Sah RP, Dudeja V, et al. Intra-acinar trypsinogen activation mediates early stages of pancreatic injury but not inflammation in mice with acute pancreatitis. *Gastroenterology*. 2011;141:2210-2217.e2.
26. Huang H, Liu Y, Daniluk J, et al. Activation of nuclear factor-κB in acinar cells increases the severity of pancreatitis in mice. *Gastroenterology*. 2013;144:202-210.
27. Mitaka C, Si MKH, Tulafu M, et al. Effects of atrial natriuretic peptide on inter-organ crosstalk among the kidney, lung, and

heart in a rat model of renal ischemia-reperfusion injury. *Intensive Care Med Exp*. 2014;2:28.
28. Gerlo S, Kooijman R, Beck IM, Kolmus K, Spooren A, Haegeman G. Cyclic AMP: a selective modulator of NF-κB action. *Cell Mol Life Sci*. 2011;68:3823-3841.
29. Salvemini D, Kim SF, Mollace V. Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. *Am J Physiol Regul Integr Comp Physiol*. 2013;304:R473-R487.
30. Kiemer AK, Vollmar AM. Autocrine regulation of inducible nitric-oxide synthase in macrophages by atrial natriuretic peptide. *J Biol Chem*. 1998;273:13444-13451.
31. Song AM, Bhagat L, Singh VP, Van Acker GGD, Steer ML, Saluja AK. Inhibition of cyclooxygenase-2 ameliorates the severity of pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol*. 2002;283:G1166-G1174.
32. Harper KA, Tyson-Capper AJ. Complexity of COX – 2 gene regulation. *Biochem Soc Trans*. 2008;36:543-545.
33. Kiemer AK, Lehner MD, Hartung T, Vollmar AM. Inhibition of cyclooxygenase-2 by natriuretic peptides. *Endocrinology*. 2002; 143:846-852.
34. Tsatsanis C, Androulidaki A, Venihaki M, Margioris AN. Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol*. 2006;38:1654-1661.
35. Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci USA*. 2003;100:9244-9249.
36. Kaiser AM, Saluja AK, Sengupta A, Saluja M, Steer ML. Relationship between severity, necrosis, and apoptosis in five models of experimental acute pancreatitis. *Am J Physiol*. 1995;269: C1295-C1304.
37. Talukdar R, Sareen A, Zhu H, et al. Release of cathepsin B in cytosol causes cell death in acute pancreatitis. *Gastroenterology*. 2016;151:747-758.e5.
38. Wang G, Qu F-Z, Li L, Lv J-C, Sun B. Necroptosis: a potential, promising target and switch in acute pancreatitis. *Apoptosis*. 2016;21:121-129.
39. Soldani C, Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis*. 2002;7:321-328.
40. Ba X, Garg NJ. Signaling mechanism of poly(ADP-ribose) polymerase-1 (PARP-1) in inflammatory diseases. *Am J Pathol*. 2011;178:946-955.
41. Mota RA, Sánchez-Bueno F, Saenz L, et al. Inhibition of poly (ADP-ribose) polymerase attenuates the severity of acute pancreatitis and associated lung injury. *Lab Invest*. 2005;85:1250-1262.
42. Talukdar R. Complications of ERCP. *Best Pract Res Clin Gastroenterol*. 2016;30:793-805.
43. Wang AY, Strand DS, Shami VM. Prevention of post–endoscopic retrograde cholangiopancreatography pancreatitis: medications and techniques. *Clin Gastroenterol Hepatol*. 2016;14:1521-1532.e3.
44. Yuhara H, Ogawa M, Kawaguchi Y, Igarashi M, Shimosegawa T, Mine T. Pharmacologic prophylaxis of post-endoscopic retrograde cholangiopancreatography pancreatitis: protease inhibitors and NSAIDs in a meta-analysis. *J Gastroenterol*. 2014;49:388-399.
45. Mohn CE, Fernandez-Solari J, De Laurentiis A, et al. The rapid release of corticosterone from the adrenal induced by ACTH is mediated by nitric oxide acting by prostaglandin E2. *Proc Natl Acad Sci USA*. 2005;102:6213-6218.
46. Sidhapuriwala JN, Hegde A, Ang AD, Zhu YZ, Bhatia M. Effects of S-propargyl-cysteine (SPRC) in caerulein-induced acute pancreatitis in mice. *PLoS ONE*. 2012;7:e32574.
47. Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc Natl Acad Sci USA*. 1989;86:9030-9033.

SUPPORTING INFORMATION

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