

# Blockade of Multidrug Resistance-Associated Proteins Aggravates Acute Pancreatitis and Blunts Atrial Natriuretic Factor's Beneficial Effect in Rats: Role of MRP4 (ABCC4)

María Silvia Ventimiglia,<sup>1</sup> Ana Clara Najenson,<sup>1</sup> Juan Carlos Perazzo,<sup>1</sup> Alejandro Carozzo,<sup>3</sup> Marcelo S Vatta,<sup>2</sup> Carlos A Davio,<sup>3</sup> and Liliana G Bianciotti<sup>1</sup>

<sup>1</sup>Cátedra de Fisiopatología, Instituto de Inmunología, Genética y Metabolismo (INIGEM-CONICET); <sup>2</sup>Cátedra de Fisiología-Instituto de Química y Metabolismo del Fármaco (IQUIMEFA-CONICET); and <sup>3</sup>Laboratorio de Farmacología de Receptores, Cátedra de Química Medicinal, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

We previously reported that atrial natriuretic factor (ANF) stimulates secretin-evoked cAMP efflux through multidrug resistance-associated protein 4 (MRP4) in the exocrine pancreas. Here we sought to establish *in vivo* whether this mechanism was involved in acute pancreatitis onset in the rat. Rats pretreated with or without probenecid (MRPs general inhibitor) were infused with secretin alone or with ANF. A set of these animals were given repetitive cerulein injections to induce acute pancreatitis. Plasma amylase and intrapancreatic trypsin activities were measured and histological examination of the pancreas performed. Secretin alone activated trypsinogen but induced no pancreatic histological changes. Blockade by probenecid in secretin-treated rats increased trypsin and also induced vacuolization, a hallmark of acute pancreatitis. ANF prevented the secretin response but in the absence of probenecid. In rats with acute pancreatitis, pretreatment with secretin aggravated the disease, but ANF prevented secretin-induced changes. Blockade of MRPs in rats with acute pancreatitis induced trypsinogen activation and larger cytoplasmic vacuoles as well as larger areas of necrosis and edema that were aggravated by secretin but not prevented by ANF. The temporal resolution of intracellular cAMP levels seems critical in the onset of acute pancreatitis, since secretin-evoked cAMP in a context of MRP inhibition makes the pancreas prone to injury in normal rats and aggravates the onset of acute pancreatitis. Present findings support a protective role for ANF mediated by cAMP extrusion through MRP4 and further suggest that the regulation of MRP4 by ANF would be relevant to maintain pancreatic acinar cell homeostasis.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2014.00166

## INTRODUCTION

Atrial natriuretic factor (ANF) is a key regulator of cardiovascular and renal function, but compelling evidence supports its physiological role in the digestive system where it modulates digestive motility and secretions in a paracrine and/or autocrine manner (1–4). We previously reported that ANF through natri-

uretic peptide receptor type C (NPR-C) receptors coupled to phospholipase C/protein kinase C (PLC/PKC) negatively modulates secretin intracellular signaling by stimulating secretin-evoked cAMP out of the pancreatic acinar cells through multidrug resistance-associated protein 4 (MRP4) (5,6). MRP4 (ATP-binding cassette sub-family C member 4 [ABCC4])

is a member of the multidrug resistance proteins (MRPs) belonging to the C group of the ATP-binding cassette (ABC) protein superfamily, which functions as an energy-dependent, transmembrane efflux transporter. Compelling evidence supports that cAMP extrusion through MRP4 in several cell types provides a complementary mechanism to the phosphodiesterases (PDEs) as modulators of the intracellular concentration of the second messenger (6–10). In isolated pancreatic acini, we found that secretin-induced cAMP is extruded by ANF through NPR-C receptors coupled to the PLC/PKC pathway, thus restricting its intracellular accumulation. This mechanism operates in the presence of active PDEs supporting that cAMP extrusion through MRP4 might be a mechanism in addition to PDE action

**Address correspondence to** Liliana G Bianciotti, Cátedra de Fisiopatología, INIGEM-CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Piso 5 (1113AAD) Buenos Aires, Argentina. Phone: +54-11-4964-8296; Fax: +54-11-962-5341; E-mail: [lbianc@ffyb.uba.ar](mailto:lbianc@ffyb.uba.ar).

Submitted August 25, 2014; Accepted for publication January 5, 2015; Published Online ([www.molmed.org](http://www.molmed.org)) January 6, 2015.

The Feinstein Institute  
for Medical Research 

Empowering Imagination. Pioneering Discovery.®

to regulate intracellular cAMP levels in pancreatic acinar cells. In addition, we showed that *in vivo* cAMP is released in the bloodstream in response to secretin and that this effect is further enhanced and sustained by ANF supporting the physiological role of ANF in the modulation of intracellular cAMP levels in the exocrine pancreas (6).

Tight regulation of cAMP production within cells leads to avoidance of overstimulation of certain secretory events, deregulation of cell function or even cell toxicity. In pancreatic acinar cells, secretin as well as other agents that increase cAMP have little effect on zymogen activation alone but sensitize the response of agents that increase intracellular calcium such as cerulein or cholecystokinin (11,12). In pancreatic acini, it was reported that a cell-permeable cAMP inhibitor (Rp-8Br-cAMP) reduces the sensitization induced by vasoactive intestinal peptide and 8Br-cAMP to low and high doses of cerulein (11). It also reduces chymotrypsin activation induced by high doses of cerulein alone. It was proposed that agonists that augment cAMP formation in the acinar cell may predispose the cell to pathophysiological zymogen activation and contribute to the development of acute pancreatitis. Therefore, it is possible that ANF, by limiting cAMP intracellular levels, is somehow protecting eventual premature zymogen granule activation within the acini.

Acute pancreatitis is a sudden inflammatory disorder of the exocrine pancreas that, in its severe form, carries considerably morbidity and mortality. Pancreatic injury is caused by the premature activation of digestive enzymes that the pancreas normally produces, although the early events involved in the onset of the disease still remain obscure (13). Recent studies show that impaired autophagy mediates acinar cell vacuole formation and trypsinogen activation (14,15). The combined autophagic, lysosomal and mitochondrial dysfunctions are considered key to the pathogenesis of the disease (15).

In the present study, we sought to establish whether the extrusion of cAMP

by ANF would play a beneficial role *in vivo* by avoiding and/or attenuating the onset of acute pancreatitis in the rat.

## MATERIALS AND METHODS

### Animals and Reagents

Sprague Dawley rats (180–200 g) were housed in steel cages and maintained at 22–24°C in a controlled room with a 12-h light–dark cycle (light from 0700 to 1900). All experimental protocols were approved by the Animal Care Committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires. 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, ref. [16]; revised 1996, ref. [17]). All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

### Experimental Protocols

Animals were fed standard animal laboratory chow, given water *ad libitum*, fasted overnight and randomly assigned to control or experimental groups. First we addressed whether secretin and ANF affected pancreatic enzymatic activity and/or morphology in normal and pathophysiological conditions. For this purpose, animals were infused with saline (control), secretin (1 U/kg/h) or ANF (1 µg/kg/h) alone or combined for 60 min. The doses of secretin and ANF were obtained from previous dose-response curves (3). In another group of rats, acute pancreatitis was induced by four repetitive intraperitoneal injections of 40 µg/kg cerulein at hourly intervals and secretin infusion alone, or with ANF for 60 min starting 30 min before the first cerulein injection. Saline-administered animals served as controls. To evaluate the role of MRPs, a set of animals was pretreated with 0.5 mL probenecid (50 mg/kg intraperitoneally) (MRP inhibitor) 60 min before ANF and secretin infusion or acute pancreatitis induction. Probenecid was made up in a minimal volume of 1 mol/L NaOH until dis-

solved and was then diluted in saline, and the pH was adjusted to 7.4. Control animals received probenecid vehicle. Animals were killed by decapitation at 1 h after the last cerulein injection, and blood and pancreatic tissue samples were harvested for enzymatic determinations and histology.

Additional experiments were performed in isolated pancreatic acini pretreated with 50 µmol/L MK571 (MRPs inhibitor) or 1 mmol/L probenecid for 30 min and further exposed to 100 nmol/L secretin for 10 min. After incubation with secretin, intracellular cAMP and intrapancreatic trypsin were assayed as detailed below. Pancreatic acini were isolated by enzymatic digestion as previously reported (6,18).

### Enzymatic Determinations

Plasma amylase activity was measured by using chloro-p-nitrophenyl- $\alpha$ -D-maltotriose as substrate (Wiener Laboratories, Rosario, Argentina). Trypsin activity was fluorometrically determined as previously detailed by using the specific substrate Boc-Gln-Ala-Arg-MCA (Peptide International, Louisville, KY, USA). Briefly, pancreas was homogenized in cold (4°C) buffer (250 mmol/L sucrose, 5 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mmol/L MgSO<sub>4</sub>, pH 6.5) by using a Teflon-coated homogenizer and then centrifuged at 150g for 10 min at 4°C. Duplicate aliquots of each supernatant were added to a 48-well tissue culture plate containing 350 µL of trypsin assay buffer (50 mmol/L Tris [pH 8.1], 150 mmol/L NaCl, 1 mmol/L CaCl<sub>2</sub>, 0.01% bovine serum albumin [BSA]), and then 10 mmol/L enzyme substrate was added to each well. Accumulation of fluorescence was measured over 11 min by using a fluorescent microtiter plate reader (FlexStation III; Molecular Devices, Sunnyvale, CA, USA) at 380 nm excitation and 440 nm emission wavelengths. Enzymatic activity was expressed as percentage of control.

Histological examination of pancreatic tissue: For light microscopy studies, pancreatic tissue was fixed in 10% buffered

formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (17). The pancreas of at least five rats from each group was examined for vacuolization, inflammation, edema, necrotic areas and pyknotic nuclei (morphological marker of apoptosis) by a pathologist blinded to the experimental protocol. The quantitative evaluation was performed using a modified score previously described (17,18). The sections (a minimum of five fields with 400× magnification) were examined for each sample and scored on a scale of 0–4 based on the percentage of area with the presence of vacuoles, zymogen granules, edema and vascular congestion. The highest percentage was assigned as 4. The percentage of pyknotic nuclei and necrotic areas were evaluated per field (minimum of 12 fields) with 1,000× magnification. Cells with swollen cytoplasm, loss of plasma membrane integrity and absent nucleus were considered necrotic and evaluated per field (minimum of 12 fields) with 1,000 magnification.

### Measurement of cAMP

A set of animals pretreated with probenecid were infused with secretin alone or with ANF, and blood samples were collected at different time points (0, 3, 5, 10, 15 and 30 min) as previously detailed (6). Blood samples were rapidly centrifuged, and plasmas were collected in ethanol and stored at –20°C. Samples were dried and the remaining residue was suspended for cAMP determination, assessed by competition of [<sup>3</sup>H]cAMP for PKA as previously detailed (9,19). Results were expressed as picomoles per milliliter plasma. Determination of cAMP in pancreatic acini was expressed as percentage of control and performed as in previous studies (3,5,6).

### Immunofluorescence for MRP4

For MRP4 immunolocalization, pancreatic tissue sections in paraffin were soaked in xylol and hydrated with decreasing concentrations of alcohol (100% to 96% to 70%). Sections were then treated with 5% BSA in phosphate-

buffered saline (PBS) (blocking solution), followed by incubation with primary antibody against MRP4 (Alexis Biochemicals [Enzo Life Sciences Inc., Farmingdale, NY, USA]) (1:300). Slides were washed in PBS, incubated with secondary antibody Cy3-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a dilution of 1:500 and coverslipped with Vectashield (Vector, Burlingame, CA, USA). The stained samples were then examined by using a fluorescence microscope (Olympus BX50; Olympus Corporation, Tokyo, Japan) with appropriate filter sets.

### Statistical Analysis

Results are expressed as the mean ± standard error of the mean. Statistical analysis was performed by analysis of variance followed by the Student-Newman-Keuls test. A *p* value ≤0.05 was considered statistically significant.

*All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).*

## RESULTS

### MRP4 Is Localized to the Basolateral Membrane of Acinar Cells and Mediates cAMP Efflux *In Vivo*

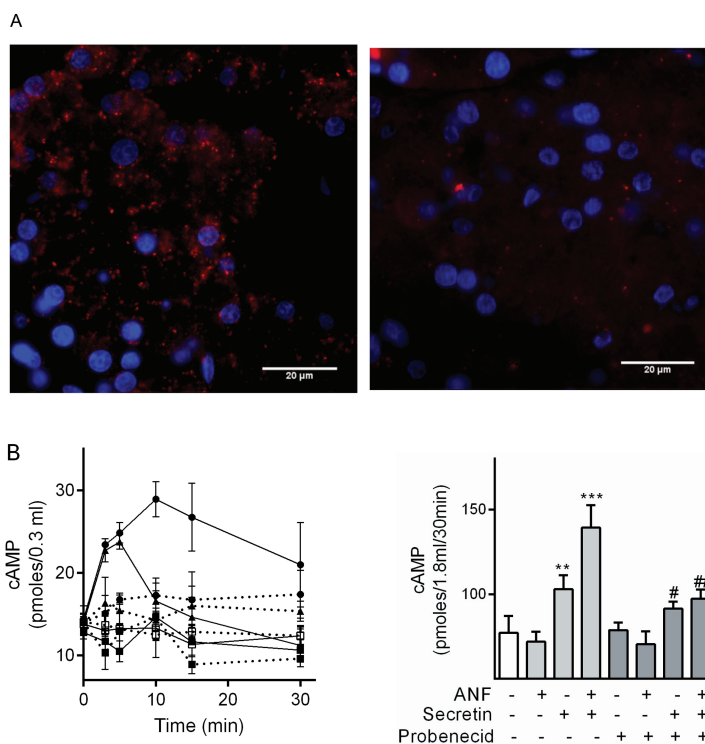
We previously reported that the infusion of secretin increases plasma cAMP and that this response is enhanced and sustained in the presence of ANF, suggesting that the cyclic nucleotide is released into the bloodstream presumably through MRP4, which is the major transporter for the second messenger in pancreas as revealed by siRNA studies (6). We also showed by reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blot analyses that MRP4 mRNA and protein are expressed in the rat pancreas; however the localization of MRP4 in the acinar cell was unknown. Immunofluorescence showed that the transporter was localized to the basolateral membrane of pancreatic acinar cells (Figure 1A).

To evaluate *in vivo* the role of MPRs, animals were pretreated with

probenecid and further infused with secretin alone or with ANF. Although probenecid is a general MRP inhibitor, previous studies silencing MRP4 by siRNA revealed that it is the major cAMP efflux transporter in the exocrine pancreas (6). Blockade of MRPs by probenecid prevented the increase in plasma cAMP induced by secretin alone or with ANF, suggesting that the release of the cyclic nucleotide to the bloodstream is mediated by MRPs, likely MRP4 (Figure 1B). The temporal course of cAMP in each experimental group is shown in the left panel of Figure 1B, whereas the area under each curve is presented in the right panel.

### Role of ANF and MRPs in Normal Rats

We next evaluated whether blockade by probenecid affected zymogen activation and pancreatic histology. Plasma amylase activity was not modified by probenecid alone or followed by the infusion of secretin alone or with ANF (Supplementary Figure S1). However, trypsin activity was enhanced by secretin alone and in probenecid-treated rats infused either with secretin or secretin and ANF (Figure 2A). Microscopy studies showed a higher density of apical zymogen granules in acinar cells from animals with ANF and secretin (Table 1 and Supplementary Figures S2A–C). Probenecid alone did not induce histological changes in the pancreas (Figure 3B), but in the presence of secretin alone or with ANF, vacuoles in acinar cells were observed (Table 1 and Figures 3D, E), which correlated well with intrapancreatic trypsin elevation (Figure 2A). In the absence of probenecid, secretin alone enhanced trypsin, although it was not accompanied by pancreatic histological changes (Supplementary Figure S2B). Secretin-induced trypsinogen activation was prevented by ANF (Figure 2A). An increase in pyknotic nuclei was observed in all ANF-treated rats including those pretreated with probenecid, suggesting that the atrial peptide stimulates apoptosis independently of its effect on cAMP efflux (Table 1).



**Figure 1.** (A) MRP4 is localized to the basolateral membrane of acinar cells. MRP4 was determined by immunofluorescence as detailed in Materials and Methods. Immunofluorescence was concentrated in the basolateral membrane of acinar cells (left panel); negative control is shown (right panel). (B) MRP4 inhibition abolishes plasma cAMP increase evoked by secretin and ANF. Animals were pretreated with or without probenecid followed by infusion with secretin alone or with ANF; blood samples were withdrawn at 3, 5, 10, 15 and 30 min and cAMP was assessed by radioimmunoassay as described in Materials and Methods. Right panel: time course of cAMP after the different treatments. □, Control; ■, ANF; ▲, secretin; ●, secretin + ANF. Dotted lines represent the same groups treated with probenecid. Left panel: Area under each temporal curve. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus control; # $p < 0.05$  versus secretin; ## $p < 0.01$  versus secretin + ANF. Scale bar, 20  $\mu\text{m}$ .

### Correlation between Intracellular cAMP and Trypsinogen Activation in Isolated Pancreatic Acini

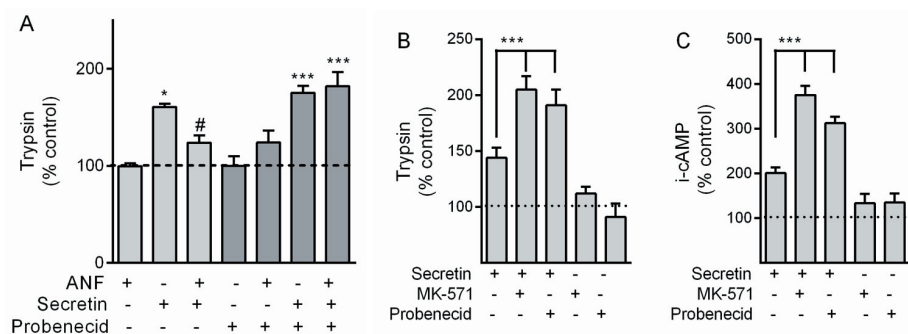
Because probenecid is a general MRP inhibitor and to further confirm the participation of MRP4, isolated pancreatic acini were pretreated with MK571, which is more specific than probenecid because it inhibits MRP4 and no other MRPs involved in cAMP efflux (22,23). MK571 mimicked probenecid response, and they both further enhanced the increase in intracellular cAMP and trypsin evoked by secretin, showing that higher intracellular cAMP correlates with higher trypsinogen activation as found in *in vivo* studies (Figures 2B, C). These findings support that increased intracellular cAMP induced by secretin or secretin and MRP4 blockade results in zymogen activation.

### Role of ANF in Rats with Acute Pancreatitis

We then evaluated the role of ANF in an animal model of acute pancreatitis induced by repetitive injections of cerulein. Results showed that cerulein-treated rats showed elevation of plasma amylase as well as intrapancreatic trypsin activity (Figures 4A, B) and histological features of acute pancreatitis such as vacuolization, edema and areas of necrosis as previously reported (Table 2 and Figure 5A). That secretin infusion aggravated acute pancreatitis was supported by the observation of a further increase in plasma amylase and trypsin activity as well as by the enhanced loss of acini architecture, higher vacuolization, more areas of necrosis and important interstitial edema (Table 2 and Figure 5C). The infusion of ANF ameliorated acute pancreatitis supported by reduced plasma amylase and trypsin activities (Figures 4A, B). ANF also enhanced apoptosis, and although it did not prevent vacuolization, less number of vacuoles were observed (Table 2 and Figure 5D).

### Role of ANF and MRP Blockade in Rats with Acute Pancreatitis

We next addressed the role of MRPs and ANF in probenecid-treated rats with



**Figure 2.** (A) MRP4 inhibition further increases secretin-induced zymogen activation. Intrapaneacric trypsin was fluorometrically assessed in animals pretreated with probenecid followed by the infusion of secretin alone or with ANF. \* $p < 0.05$  and \*\*\* $p < 0.001$  versus control; # $p = 0.05$  versus secretin. Number of experiments: 5. (B, C) Blockade of MRPs with MK571 mimics probenecid response in the presence of secretin. Isolated pancreatic acini were pretreated with probenecid or MK571 and further exposed to secretin. Intrapaneacric trypsin (B) and intracellular cAMP (C) were assayed as detailed in Materials and Methods. S, secretin.



**Table 1.** Histological examination of the exocrine pancreas with or without probenecid (P) pretreatment and infused with secretin (S) alone or with ANF.

	Apical granules	Edema	Vascular congestion	Vacuoles	Necrotic areas/field	Pyknotic nuclei/field
Control	2	0	0	0	0	0-1
ANF	2	0	0	0	0	1
Secretin	4	0	0	0	0	0-1
S + ANF	4	0	0-1	0	0	1
Probenecid	2	0	0	0	0	0-1
P + ANF	2	0	0-1	0-1	0-1	1
P + S	3	0-1	0	1	0-1	0-1
P + S + ANF	2-3	0-1	0	1	0-1	1-2

acute pancreatitis. When animals were pretreated with probenecid followed by cerulein injections, MRPs blockade resulted in further aggravation of the disease compared with cerulein-treated rats (Figures 5E-H and Table 2). Trypsin activity was elevated and further increased in the presence of secretin (Figure 4B). Histological studies supported aggravation of acute pancreatitis. Loss of pancreatic acini architecture was clearly observed in all groups but was further enhanced in those groups that received secretin alone or with ANF (Table 2). Increased areas of necrosis, edema and vascular congestion were evident together with enhanced vacuolization (Figures 5G and 6H). Larger vacuoles with cytoplasmic content and zymogen granules dispersed in the cytoplasm were observed (Table 2). ANF failed to inhibit secretin response or to ameliorate acute pancreatitis, suggesting that the beneficial effect of the peptide is likely mediated by stimulating cAMP efflux through MRPs. In ANF-treated animals, an increased number of pyknotic nuclei was also observed and did not depend on MRP blockade.

## DISCUSSION

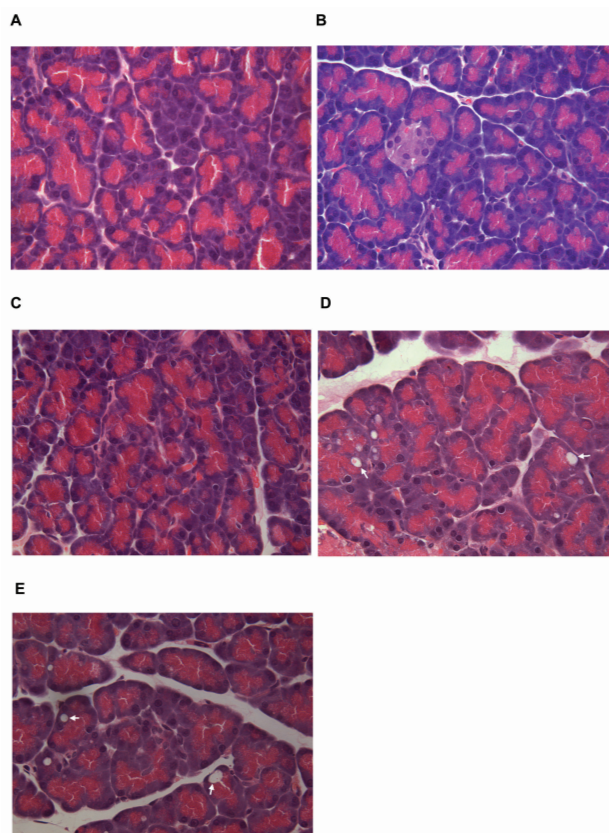
The major finding of the present work is that blockade of MRPs makes the exocrine pancreas of normal rats prone to injury; further, it aggravates acute pancreatitis as supported by biochemical and histological studies, suggesting that enhanced intracellular cAMP levels in the onset of the disease clearly favors the damage of the exocrine pancreas. ANF seems to have a beneficial effect by stim-

ulating cAMP extrusion and by presumably enhancing apoptosis.

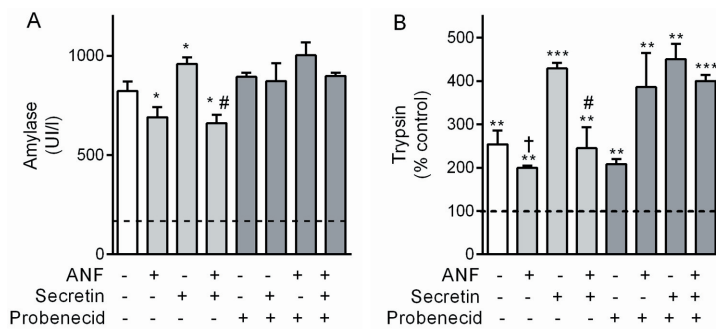
The present study confirms that MRP4 is localized to the basolateral membrane of acinar cells. MRP4 differs from other C subfamily members because of its dual localization in polarized cells. In hepato-

cytes, choroid plexus epithelium and prostate tubuloacinar cell MRP4 is localized to the basolateral membrane, whereas in renal proximal tubular cells, it is in the apical membrane (24,25). The basolateral localization of MRP4 in pancreatic acinar cells is in accordance with plasma cAMP elevation in response to secretin alone or with ANF. Secretin receptors are also expressed in duct cells, so it is likely that this cell type may contribute to the response and to a less extent other cells expressing both secretin receptors and MRP4. Although previous studies in human pancreas and pancreatic carcinoma show that MRP4 is also localized in duct cells, we failed to detect the transporter in this cell type in the rat (26).

We first evaluated *in vivo* the effect of ANF on intrapancreatic enzymatic acti-



**Figure 3.** Blockade of MRPs induces vacuole formation in acinar cells of secretin-treated rats. Hematoxylin and eosin sections of pancreas from control (A), probenecid (B), probenecid + ANF (C), probenecid + secretin (D) and probenecid + secretin + ANF (E) are shown. Small and large vacuoles were observed in groups D and E (arrowheads). Each is a representative photograph (400× magnification).



**Figure 4.** (A) MRPs inhibition prevents ANF-induced reduction of plasma amylase in the presence or absence of secretin in acute pancreatitis. Animals were infused with secretin alone or with ANF acute pancreatitis was induced by repetitive cerulein injections and amylase was measured in plasma as detailed in Materials and Methods. White bar: cerulein alone. \* $p < 0.05$  versus cerulein; # $p < 0.05$  versus secretin. Number of experiments: 6. The dotted line represents plasma amylase average value in normal rats. (B) MRP inhibition prevents ANF-induced reduction of zymogen activation in the presence or absence of secretin in acute pancreatitis. Trypsin activity was fluorometrically assessed in pancreas from animals with acute pancreatitis infused with secretin alone or with ANF, as detailed in Materials and Methods. White bar: cerulein alone. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus control; † $p < 0.05$  versus cerulein; # $p < 0.05$  versus secretin. Results are expressed as percentage of control. Number of experiments: 5.

vation and histological changes in the presence or absence of secretin. The hormone alone enhanced intrapancreatic trypsin activity, but no pancreatic histological changes were associated. ANF blunted secretin response, suggesting that the atrial peptide may play a protective role to avoid premature enzyme activation within the acinar cell and eventual gland damage. The activation of trypsinogen is one of the earliest events known to be one of the key steps of protease activation in acute pancreatitis. Inappropriate intracellular activation of

trypsinogen in turn activates a cascade of phospholipases, elastases and other mediators with increased neutrophil migration to the pancreas and subsequent release of inflammatory cytokines that propagate inflammation and tissue damage. Although enhanced trypsinogen activation was not accompanied by evident histological alterations in the present study, increased trypsin is a critical initiating event of acute pancreatitis.

To determine whether the ANF protective role was mediated by stimulated cAMP extrusion through MRP4, animals

were pretreated with a MRP inhibitor. Probenecid is a general MRP inhibitor, but our previous studies silencing MRP4 by siRNA supported that MRP4 was responsible for cAMP efflux in the exocrine pancreas, as shown in other tissues (6). Blockade of MRPs by probenecid prevented the increase in plasma cAMP after secretin infusion alone or with ANF, suggesting that the release of the second messenger to the bloodstream is mediated by MRP4. We previously reported that after secretin infusion alone, plasma cAMP increased by 60% over control values at 3 min, peaked at 5 min (168%) and then declined to control values at 15 min. However, when ANF was co-infused, the increase in plasma cAMP was higher and more sustained than with secretin alone, although the peak was reached later (10 versus 5 min) as well as control values (30 versus 15 min) (6).

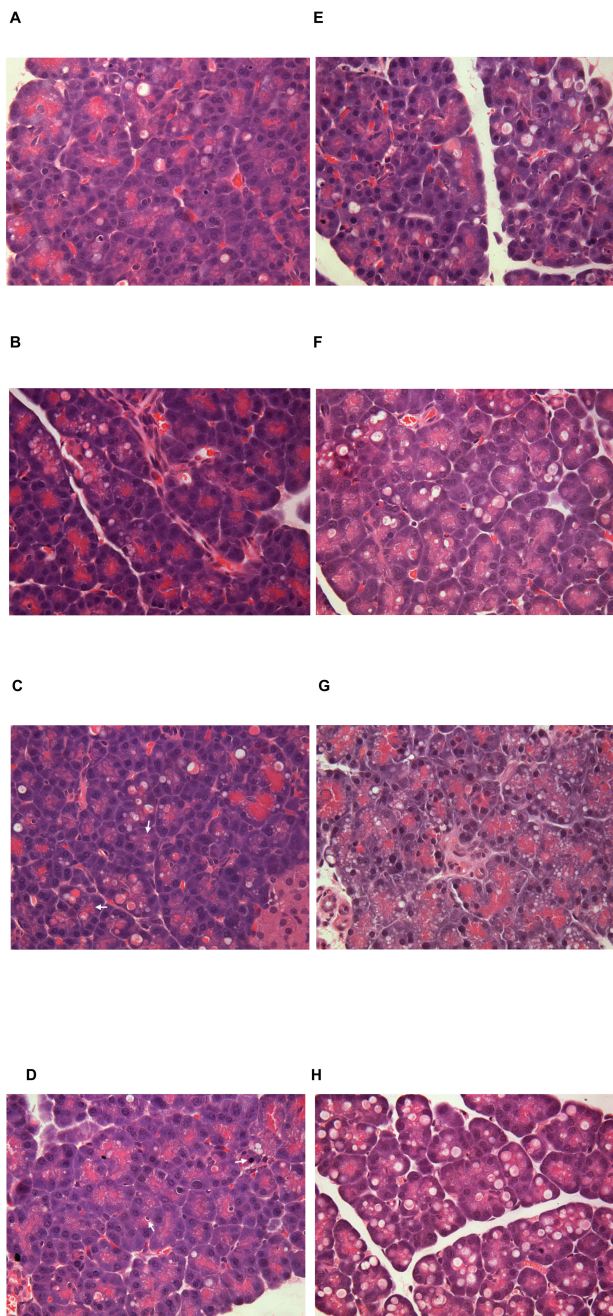
Blockade of MRPs in secretin-treated rats further increased trypsinogen activation, which correlated with the appearance of cytoplasmic vacuoles in pancreatic acinar cells, a hallmark of acute pancreatitis. In accordance, in isolated pancreatic acini, blockade of MRPs by probenecid or MK571 further enhanced secretin-evoked increases in intracellular cAMP and trypsinogen activation, supporting that elevated cAMP in acinar cells induce zymogen activation. Findings in the presence of MK571 supports the participation of MRP4 and excludes the contribution of MRP5 and MRP8, which is expressed in the exocrine pancreas and has been associated with cAMP efflux in other cell types.

ANF failed to inhibit secretin response in probenecid-pretreated rats, supporting that ANF protects the pancreas from injury by stimulating cAMP efflux. These findings further suggest that cAMP extrusion would play a relevant role in maintaining pancreatic acinar cell homeostasis.

We next evaluated the role of ANF and MRP blockade in acute pancreatitis. Cerulein-treated rats showed elevation of plasma amylase and intrapancreatic trypsin as well as morphological alter-

**Table 2.** Histological examination of the exocrine pancreas in cerulein-treated rats (C) pretreated with secretin (S) alone or with ANF in the absence or presence of probenecid (P).

	Apical granules	Edema	Vascular congestion	Vacuoles	Necrotic areas/field	Pyknotic nuclei/field
C	1-2	1	2	1-2	2	1-2
C + ANF	1-2	1-2	2-3	1-2	2	2-3
C + S	1-2	3	4	2	2-3	1-2
C + S + ANF	1-2	2	2-3	2	3	4
P + C	1-2	1	2	1-2	2	1-2
P + C + ANF	1	2	3	3	3-4	4
P + C + S	1	4	4	4	3-4	2
P + C + S + ANF	1	4	4	4	4	4



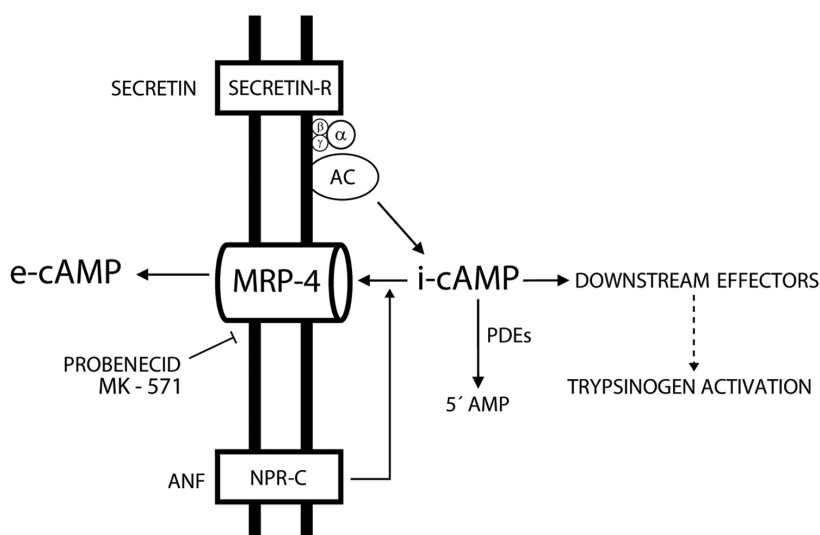
**Figure 5.** Secretin aggravates cerulein-induced acute pancreatitis and ANF reduces secretin response, but MRP inhibition further aggravates cerulein-induced acute pancreatitis in the presence of secretin and blunts ANF response. (A–D) Hematoxylin and eosin section of pancreas from rats with acute pancreatitis induced by cerulein and infused with secretin alone or with ANF. Control (A), ANF (B), secretin (C), and secretin + ANF (D) are shown. Higher vacuolization and areas of necrosis were observed in (C). Each is a representative photograph (400× magnification). (E–H) Hematoxylin and eosin section of pancreas from rats with acute pancreatitis induced by cerulein and infused with secretin alone or with ANF in the presence of probenecid pretreatment. Probenecid (E), probenecid + ANF (F), probenecid + secretin (G), and probenecid + secretin + ANF (H) are shown. Higher areas of necrosis and larger vacuoles were observed in (G) and (H). Each is a representative photograph (400× magnification).

ations of the disease such as vacuolization, areas of necrosis, pyknotic nuclei and distortion of the pancreatic acini architecture as previously reported (13,14). Secretin further enhanced enzymatic activity and induced more severe morphological changes in the pancreas. ANF blunted secretin response and attenuated acute pancreatitis. However, when animals were also pretreated with probenecid, acute pancreatitis was more severe, with larger necrotic areas, loss of acini architecture, increased vacuolization, edema and vascular congestion as well as marked distribution of zymogen granules from the apical membrane. Secretin further aggravated the morphological features of acute pancreatitis in rats with MRP blockade, since larger vacuoles with cytoplasmic content as well as more necrotic areas were clearly observed. However, ANF failed to prevent secretin response or attenuate the disease, suggesting that cAMP efflux partly mediates ANF beneficial effect.

Present results show that elevated intracellular cAMP levels in acinar cells induced by secretin and/or MRP blockade at the onset of acute pancreatitis would be deleterious. The morphological and biochemical features of acute pancreatitis are aggravated by blocking cAMP efflux. Further, MRP blockade alone in the presence of secretin resulted in vacuolization of the acinar cell and trypsinogen activation, revealing that intracellular elevation of cAMP makes the pancreas prone to injury. Although probenecid is not a specific MRP4 inhibitor, our previous studies and present findings with MK571 support the relevance of MRP4 in cAMP regulation.

Previous studies suggest a beneficial role for cAMP in cerulein-induced acute pancreatitis. It was reported that cAMP-stimulated pathway reduces cellular injury by causing secretion of active enzymes from pancreatic acinar cells (27). Furthermore, it was shown that exogenous secretin ameliorates acute pancreatitis in rats and dogs (28–31). The apparent discrepancy with our study may be related to the time course of cAMP in-





**Figure 6.** Schematic representation of the proposed action of ANF, secretin and MRP4 (MRP-4) in pancreatic acinar cells. Stimulation of secretin receptors leads to adenyl cyclase activation, and the rise of intracellular cAMP is regulated by the action of PDEs and the exclusion of the second messenger through MRP4. ANF by activating NRP-C receptors potentiates cAMP efflux. Marked cAMP elevation as induced by secretin and MRP4 blockade by probenecid or MK571 leads through downstream effectors to trypsinogen activation and eventual damage of acinar cells. AC, adenyl cyclase; e-cAMP, extracellular cAMP; i-cAMP, intracellular cAMP; SECRETIN-R, secretin receptor.

tracellular levels and a possible dual role of the second messenger. In our study secretin, ANF and/or probenecid were administered before the induction of acute pancreatitis, so that cells had elevated cAMP intracellular levels before the onset of the disease. In other studies, secretin and other drugs were simultaneously given. It is likely that increased intracellular cAMP may be deleterious when elevated in the onset of the disease but protective once acute pancreatitis is triggered.

A recent study show that ductal secretion protects the pancreas from acute pancreatitis, suggesting that improved ductal function may help to prevent and treat the disease (32). However, in our study, the sole infusion of secretin, which acts on receptors in acinar and duct cells, enhanced trypsinogen activation. Further, it aggravated acute pancreatitis, suggesting that secretin-enhanced ductal function does not prevent the disease.

An interesting finding of the present study was the observation of a higher number of pyknotic nuclei in all ANF-

treated animals despite other concomitant treatments. This increase was not prevented or reduced by probenecid, suggesting that it is independent of the ANF effect on MRP4 and cAMP intracellular levels. Stimulation of apoptosis in acute pancreatitis is beneficial because the extent of apoptosis has been shown to be inversely related to the severity of the disease (33). It has been shown that the milder forms of experimental pancreatitis are associated with more apoptosis, whereas the relatively severe forms are associated with more necrosis. Although diverse studies suggest that ANF stimulates apoptosis, further studies are needed to evaluate whether the increase in pyknotic nuclei induced by ANF represents an additional protective effect (34).

The role of MRP4 and ANF in acute pancreatitis may have further implications related to the inflammatory response involved in the pathogenesis of the disease. Activation of the transcription factor nuclear factor (NF)- $\kappa$ B promotes cytokine synthesis, which in turn

upregulates inducible enzymes such as COX2, leading to the increase in prostaglandins, responsible for the progression of inflammation. Accumulating evidence indicates the essential contribution of the COX2 pathway to cerulein-induced acute pancreatitis because its inhibition ameliorates the disease (35,36). MRP4 has been identified as a transporter for cAMP, but prostaglandins E1 and E2 are also physiological substrates for MRP4 (36–40). Furthermore, it was recently shown that MRP4 contributes to breast cancer progression by exporting PGE2 (41). Preliminary data from our laboratory show that intrapancreatic content of PGE2 is diminished in cerulein-treated rats infused with ANF, suggesting that the atrial peptide may not only stimulate cAMP efflux but also PGE2 extrusion, thus regulating the inflammatory response in acute pancreatitis.

Despite extensive studies, acute pancreatitis still remains a serious disease. In most cases, it has a benign course and is self-limiting and the pancreas fully recovers its functionality. However, in ~20% of the patients, it progresses to a more severe form that can lead to death due to multiple organ failure. The underlying mechanisms that lead to one form or the other still remain obscure. Our results support a deleterious effect of elevated cAMP in the onset of acute pancreatitis and a beneficial role for ANF by extruding the second messenger through MRP4. Whether elevated cAMP intracellular levels in the onset of acute pancreatitis conditions the course of the disease is presently unknown. Figure 6 depicts a schematic representation of the proposed actions of ANF, MRP4 and secretin in pancreatic acinar cells on the basis of previous and present findings.

Increasing evidence supports the participation of MRPs in diverse physiological and pathophysiological cellular events beyond the initially described property of these transporters linked to antidrug efficacy. Our findings highlight that the clinical use of drugs that modulate MRP activity should be carefully evaluated to avoid undesired effects given the wide



spectrum of differential biological actions where these transporters are involved.

## CONCLUSION

Given the physiological importance of MRP4 in the regulation of cAMP intracellular levels in pancreatic acinar cells homeostasis and its modulation by ANF, impaired activity or altered expression of MRP4 may affect the onset, course and/or outcome of acute pancreatitis. Besides cAMP, MRP4 also effluxes PGE<sub>2</sub>, a key player in the inflammatory response that features the disease. Because increasing reports in the literature show that MRP4 is emerging as a new therapeutic target for diverse diseases, it may represent a therapeutic target for acute pancreatitis.

## ACKNOWLEDGMENTS

The authors thank John A Williams (University of Michigan) for critical reading of the manuscript. Silvia Presmanes is also acknowledged for excellent technical assistance in histological studies. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET) (PIP-0370) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT2012-2755 and PICT2010-1571).

## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

## REFERENCES

- Gower WR Jr, et al. (2000) Regulation of atrial natriuretic peptide gene expression in gastric antrum by fasting. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 278:R770-80.
- Bianciotti LG, et al. (1998) Atrial natriuretic factor induced amylase output in the rat parotid gland appears to be mediated by the inositol phosphate pathway. *Biochem. Biophys. Res. Commun.* 247:123-28.
- Sabbatini ME, et al. (2003) Atrial natriuretic factor stimulates exocrine pancreatic secretion in the rat through NRP-C receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285:G929-37.
- Ventimiglia MS, et al. (2008) Atrial natriuretic factor intracellular signaling in the rat submandibular gland. *Regul. Pept.* 150:43-9.
- Sabbatini ME, Vatta MS, Davio CA, Bianciotti LG. (2007) Atrial natriuretic factor negatively modulates secretin intracellular signaling in the exocrine pancreas. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292:G349-57.
- Rodríguez MR, et al. (2011) Atrial natriuretic factor stimulates the efflux of secretin-evoked cAMP in the exocrine pancreas through multidrug resistance proteins. *Gastroenterology.* 140:1292-302.
- Sassi Y, et al. (2008) Multidrug resistance-associated protein 4 regulates cAMP-dependent signaling pathways and controls human and rat SMC proliferation. *J. Clin. Invest.* 118:2747-57.
- Godinho RO, Costa VL. (2003) Regulation of intracellular cyclic AMP in skeletal muscle cells involves the efflux of cyclic nucleotide to the extracellular compartment. *Br. J. Pharmacol.* 138:995-1003.
- Copsel S, et al. (2011) Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J. Biol. Chem.* 286:6979-88.
- Pattabiraman PP, Pecan PE, Rao PV. (2013) MRP4-mediated regulation of intracellular cAMP and cGMP levels in trabecular meshwork cells and homeostasis of intraocular pressure. *Invest. Ophthalmol. Vis. Sci.* 54:1636-49.
- Lu Z, Kolidecik TR, Karne S, Nyce M, Gorelick F. (2003). Effects of ligands that increase cAMP on caerulein-induced zymogen activation in pancreatic acini. *Am. J. Physiol. Gastrointest. Liver Physiol.* 85:G822-28.
- Perides G, et al. (2005) Secretin differentially sensitizes rat pancreatic acini to the effects of supra-maximal stimulation with caerulein. *Am. J. Physiol. Gastrointest. Liver Physiol.* 289:G713-21.
- Sah RP, Garg P, Saluja AK. Pathogenic mechanisms of acute pancreatitis. (2012) *Curr. Opin. Gastroenterol.* 28:507-15.
- Mareninova OA, et al. (2009) Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. *J. Clin. Invest.* 119:3340-55.
- Gukovsky I, Pandol SJ, Gukovskaya AS. (2012) Organellar dysfunction in the pathogenesis of pancreatitis. *Antioxid. Redox Signal.* 15:2699-710.
- Institute of Laboratory Animal Resources (U.S.), Committee on Care and Use of Laboratory Animals. (1985) *Guide for the Care and Use of Laboratory Animals*. Rev. 1985. Bethesda (MD): NIH. 83 pp. (NIH publication; no. 85-23).
- Institute of Laboratory Animal Resources; Commission on Life Sciences; National Research Council. (1996) *Guide for the Care and Use of Laboratory Animals*. Washington (DC): National Academy Press.
- Williams JA, Kore M, Dormer RL. (1978) Actions of secretagogues on a new preparation of functionally intact isolated pancreatic acini. *Am. J. Physiol. Endocrinol. Metab.* 235:E517-24.
- Dembinski A, et al. (2006) Effect of ischemic preconditioning on pancreatic regeneration and pancreatic expression of vascular endothelial growth factor and platelet-derived growth factor-A in ischemia/reperfusion-induced pancreatitis. *J. Physiol. Pharmacol.* 57:39-58.
- Seo SW, et al. (2007) Selective cyclooxygenase-2 inhibitor ameliorates cholecystokinin-octapeptide-induced acute pancreatitis in rats. *World J. Gastroenterol.* 19:2298-304.
- Davio CA, Cricco GP, Bergoc RM, Rivera E. (1995) H1 and H2 histamine receptors in NMU-induced carcinoma with atypical coupling to signal transducers. *Biochem. Pharmacol.* 50:91-6.
- van Aubele RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J. Am. Soc. Nephrol.* 13:595-603.
- Sinha C, et al. (2013) Multi-drug resistance protein 4 (MRP4)-mediated regulation of fibroblast cell migration reflects a dichotomous role of intracellular cyclic nucleotides. *J. Biol. Chem.* 288:3786-94.
- Kepler D. (2011) Multidrug resistance proteins (MRPs, ABCs): importance for pathophysiology and drug therapy. *Handb Exp Pharmacol.* 201:299-323.
- Russel FG, Koenderink JB, Masereeuw R. (2008) Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signaling molecules. *Trends Pharmacol. Sci.* 29:200-7.
- Konig J, et al. (2005) Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. *Int. J. Cancer.* 115:359-67.
- Chaudhuri A, Kolodecik TR, Gorelick FS. (2005) Effects of increased intracellular cAMP on carbachol stimulated zymogen activation, secretion and injury in the pancreatic acinar cell. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288:G235-43.
- Renner IG, Wisner Jr, Lavigne BC. (1986) Partial restoration of pancreatic function by exogenous secretin in rats with ceruletide-induced acute pancreatitis. *Dig. Dis. Sci.* 31:305-13.
- Renner IG, Wisner JR Jr, Rinderknecht H. (1983) Protective effects of exogenous secretin on ceruletide-induced acute pancreatitis in the rat. *J. Clin. Invest.* 72:1081-92.
- Renner IG, Wisner JR Jr. (1986) Ceruletide-induced acute pancreatitis in the dog and its amelioration of exogenous secretin. *Int. J. Pancreatol.* 1:39-49.
- Niederer C, Ferrell LD, Grendell JH. (1985) Caerulein-induced acute necrotizing pancreatitis in mice: protective effects of proglumide, benzotript, and secretin. *Gastroenterology.* 88:1192-204.
- Pallagi P, et al. (2014) The role of pancreatic ductal secretion in protection against acute pancreatitis in mice. *Crit. Care Med.* 42:177-88.
- Bhatia M. Apoptosis versus necrosis in acute pancreatitis. (2004) *Am. J. Physiol. Gastrointest. Liver Physiol.* 286:G189-96.
- Wu CF, Bishopric NH, Pratt RE. (1997) Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. *J. Biol. Chem.* 272:14860-66.

35. Ethridge RT, *et al.* (2002) Cyclooxygenase-2 gene disruption attenuates the severity of acute pancreatitis and pancreatitis-associated lung injury. *Gastroenterology*. 123:1311–22.
36. Song AM, *et al.* (2002) Inhibition of cyclooxygenase-2 ameliorates the severity of pancreatitis and associated lung injury. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283:G1166–74.
37. Reid G, *et al.* (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal anti-inflammatory drugs. *Proc. Natl. Acad. Sci. U. S. A.* 100:9244–49.
38. Sauna ZE, Nandigama K, Ambudkar SV. (2004) Multidrug resistance protein 4 (ABCC4)-mediated ATP hydrolysis: effect of transport substrates and characterization of the post-hydrolysis transition state. *J. Biol. Chem.* 279:48855–64.
39. Rius M, Thon WF, Keppler D, Nies AT. (2005). Prostanoid transport by multidrug resistance protein 4 (MRP4/ABCC4) localized in tissues of the human urogenital tract. *J. Urol.* 174:2409–14.
40. Lin ZP, *et al.* (2008) Disruption of cAMP and prostaglandin E2 transport by multidrug resistance protein 4 deficiency alters cAMP-mediated signaling and nociceptive response. *Mol. Pharmacol.* 73:243–51.
41. Kochel TJ, *et al.* (2013) Multiple drug resistance-associated protein 4 (MRP4) may contribute to breast cancer progression by exporting the COX-2 product PGE2. *Cancer Res.* 73 (Suppl. 1):Abstract 5119.

Cite this article as: Ventimiglia MS, *et al.* (2015) 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.* 21:58–67.