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Nancy Sarmiento, Carmen Sánchez-Bernal, Manuel Ayra, Nieves Pérez, Angel Hernández-Hernández, et al.. Changes in the expression and dynamics of SHP-1 and SHP-2 during cerulein-induced acute pancreatitis in rats. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2008, 1782 (4), pp.271. 10.1016/j.bbadis.2008.01.005 . hal-00501564

HAL Id: hal-00501564

<https://hal.science/hal-00501564>

Submitted on 12 Jul 2010

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Accepted Manuscript

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PII: S0925-4439(08)00030-6
DOI: doi: [10.1016/j.bbadis.2008.01.005](https://doi.org/10.1016/j.bbadis.2008.01.005)
Reference: BBADIS 62783

To appear in: *BBA - Molecular Basis of Disease*

Received date: 11 October 2007
Revised date: 23 January 2008
Accepted date: 23 January 2008



Please cite this article as: Nancy Sarmiento, Carmen Sánchez-Bernal, Manuel Ayra, Nieves Pérez, Angel Hernández-Hernández, José J. Calvo, Jesús Sánchez-Yagüe, Changes in the expression and dynamics of SHP-1 and SHP-2 during cerulein-induced acute pancreatitis in rats, *BBA - Molecular Basis of Disease* (2008), doi: [10.1016/j.bbadis.2008.01.005](https://doi.org/10.1016/j.bbadis.2008.01.005)

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Revised manuscript N° BBADIS-07-194R1

**Changes in the expression and dynamics of SHP-1 and SHP-2
during cerulein-induced acute pancreatitis in rats**

Nancy Sarmiento^a, Carmen Sánchez-Bernal^a, Manuel Ayra^a, Nieves Pérez^a,
Angel Hernández-Hernández^a, José J. Calvo^b, Jesús Sánchez-Yagüe^{a,*}

^a*Department of Biochemistry and Molecular Biology, University of Salamanca,
Spain*

^b*Department of Physiology and Pharmacology, University of Salamanca, Spain*

*Corresponding author. Departamento de Bioquímica y Biología Molecular,
Edificio Departamental. Lab. 106, Plaza Doctores de la Reina s/n, 37007
Salamanca, Spain.

E-mail address: sanyaj@usal.es (J. Sánchez-Yagüe)

Keywords: SHP-1; SHP-2; acute pancreatitis; cerulein; SP600125; rolipram

Abstract

Protein tyrosine phosphatases (PTPs) are important regulators of cell functions but data on different PTP expression and dynamics in acute pancreatitis (AP) are very scarce. Additionally, both c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK1/2), together with intracellular cAMP levels in inflammatory cells, play an essential role in AP. In this study we have detected an increase in PTP SHP-1 and SHP-2 in the pancreas at the level of both protein and mRNA as an early event during the development of Cerulein (Cer)-induced AP in rats. Nevertheless, while SHP-2 protein returned to baseline levels in the intermediate or later phases of AP, SHP-1 protein expression remained increased throughout the development of the disease. The increase in SHP-2 protein expression was associated with changes in its subcellular distribution, with higher percentages located in the fractions enriched in lysosomes+mitochondria or microsomes. Furthermore, while the increase in SHP-2 protein was also observed in sodium-taurocholate duct infusion or biliarypancreatic duct obstruction AP, that of SHP-1 was specific to the Cer-induced model. Neutrophil infiltration did not affect the increase in SHP-1 protein, but favoured the return of SHP-2 protein to control levels, as indicated when rats were rendered neutropenic by the administration of vinblastine sulfate. Inhibition of JNK and ERK1/2 with SP600125 pre-treatment further increased the expression of both SHP-1 and SHP-2 proteins in the early phase of Cer-induced AP, while the inhibition of type IV phosphodiesterase with rolipram only suppressed the increase in SHP-2 protein expression during the same phase. Our results show that AP is associated with increases in the expression of SHP-1 and SHP-2 and changes in the dynamics of SHP-2

subcellular distribution in the early phase of Cer-induced AP. Finally, both JNK and ERK1/2 and intracellular cAMP levels are able to modulate the expression of these PTPs.

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1. Introduction

In the pathogenesis of acute pancreatitis (AP) many factors, including activated pancreatic enzymes, cytokines, chemokines, free radicals, blood flow, and neurogenic factors, have been reported. Nevertheless, the pathophysiology of the disease remains incompletely understood, especially the early acinar events. It has been suggested that it would be important to detect rapid early events and signalling mechanisms in AP because these events would probably be translated into long term responses that would determine the development of pancreatitis [1].

One of the animal models of AP is that induced by supramaximal secretagogue stimulation. Usually, the trophic agent cerulein [Cer, an analogue of cholecystokinin (CCK)] is used in this model. Different doses of Cer (ranging from 5 to even 100 $\mu\text{g}/\text{Kg}$) have been used to induce AP in rats. Under the conditions used here [20 $\mu\text{g}/\text{Kg}$ subcutaneous (s.c.) injection] the manifestations of pancreatitis include hyperamylasemia, interstitial edema, increase pancreatic cell size, increased pancreatic weight, histological damages, including increased vacuolization and other morphological derangements, and neutrophil infiltration [2-5]. With current ongoing research, the intracellular mechanisms by which CCK or Cer regulate pancreatic acinar function appear to be increasingly complex. Through Gq proteins, CCK and Cer lead to an increase in cytosolic free Ca^{2+} . Ca^{2+} , diacylglycerol and cAMP activate exocytosis processes. Moreover, It is known that both CCK and Cer activate pancreatic protein tyrosine kinases (PTK) [6,7] and that tyrosine phosphorylation plays important roles in the regulation of many cell functions. Cer-induced AP also activates the mitogen-activated protein kinase (MAPKs) cascade, especially extracellular

signal-regulated kinase (ERK1/2) and c-jun N-terminal kinase (JNK), whose activation requires the phosphorylation of both tyrosine and threonine residues [8,9]. p38MAPK is also activated by CCK and Cer. Other signalling pathways are also activated in AP, e.g. the adenosine A1-receptor pathway [10], which decreases intracellular cAMP levels. In this regard, it is known that type IV phosphodiesterase inhibitors ameliorate Cer-induced AP [11]. It is also clear that pancreatitis requires the dissociation of cell-cell contacts [12] and adherens junctions [13], and that tyrosine phosphorylation is important for the maintenance of an intact cell adhesion complex [14].

The discovery of the role of PTK in the regulation of the levels of tyrosine phosphorylation has led phosphotyrosine phosphatases (PTP) to become better appreciated as important regulators of cell functions. The structurally very similar SH2-domain containing PTPs SHP-1 and SHP-2 have been proposed to have different roles in signal transduction: SHP-1 is expressed highly in hematopoietic cells and at a moderate level in many other types of cells and plays a largely negative signalling role in hematopoietic cells. SHP-2 is more widely expressed and plays a largely positive role in the cell signalling leading to cell activation. Nevertheless, it seems that the functional nature of SHP-1 and SHP-2 depends on the systems involved. SHP-1 has been implicated in the Jak-Stat and MAPK pathways [15]. SHP-2 has compound signalling functions and it appears to be involved in a variety of signal transduction processes, such as the Ras-Raf-MAPK, Jak-Stat, PI3 kinase and NF- κ B pathways [16]. Even within a single signalling pathway, SHP-2 may act at multiple sites to participate in signal transduction. All the above SHP-1 and SHP-2 signalling pathways have been implicated in the development of AP.

Data on PTP in AP are very scarce although it is well documented that these signalling enzymes are inactivated by reactive oxygen species (ROS) [17] or secondary products of oxidation [18,19] that may form during AP development [20,21]. Essentially, only one function of SHP-1 and PTP κ in the regulation of cell adhesion in the pancreas after mild experimental Cer-induced pancreatitis in rats has been described [12]. No data are available about changes in the expression of SH₂-domain PTPs either at the level of RNA or protein, and neither is the dynamics of their subcellular location along the development of pancreatitis known. Considering the importance of determining molecules or genes whose expression changes in the early phase of AP development, together with the signalling mechanisms that may modulate such expression, here we studied the expression and dynamics of the subcellular location of SHP-1 and SHP-2 in the development of Cer-induced AP, as well as the effect of SP600125 (anthrax[1,9cd]pyrazol-6(2H)-one 1,9 pyrazoloanthrone), a new JNK and ERK 1/2 inhibitor, and rolipram, a type IV phosphodiesterase inhibitor, on SHP-1 and SHP-2 expression levels in the early phase of AP development.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), Cer, dithiothreitol (DTT), Percoll, phenylmethylsulfonyl fluoride (PMSF), Protein Inhibitor Cocktail, rolipram, sodium taurocholate, soybean trypsin inhibitor, SP600125, Trizol Reagent, vinblastine sulfate were purchased from Sigma Chemical Co. (St. Louis, MO,

USA). Polyvinylidene difluoride (PVDF) membranes were obtained from Amersham Biosciences, Spain. Monoclonal antibody anti-PTP-1 (D-11) was obtained from Santa Cruz Biotechnology, Inc, CA, USA. Monoclonal antibody anti-PTP-2 was obtained from BD Biosciences Pharmingen, San Diego, CA, USA. Reverse Transcriptase (RevertAid M-MULV), Taq polymerase, dNTP's and the Ribolock Ribonuclease inhibitor used in the reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from Fermentas Life Sciences, Germany. Oligonucleotides were obtained from Isogen life Sciences, The Netherlands. Isopropanol was purchased from Panreac, Spain. The Myeloperoxidase (MPO) Fluorimetric Detection Kit was purchased from Assay Desigs, Inc, USA.

2.2. *Animals*

Male Wistar rats weighing 250-280 g were used. The animals were given a standard rat chow diet and were fasted overnight before experiments with free access to water. Care was provided in accordance with the procedures outlined in European Community guidelines on ethical animal research (86/609/EEC), and protocols were approved by the Animal Care Committee of the University of Salamanca.

2.3. *Induction of AP and preparation of samples*

Rats received 4 s.c. injections of 20 µg Cer/kg body weight or its vehicle (0.9% NaCl) at hourly intervals. At two, four or nine hours after the first injection, the animals were killed by cervical dislocation. The pancreases were rapidly

harvested and immediately used for experiments. Serum samples were stored at -80°C until amylase determination. In some cases, AP was also induced by sodium-taurocholate duct infusion or bile-pancreatic duct obstruction (BPDO) [22, 23, respectively]. Pancreases were dissected out from the surrounding fat tissue and then homogenized with a Potter Elvehjem device in 10 volumes (v/w) of homogenization buffer (3 mM imidazole buffer, pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 100 $\mu\text{g/ml}$ trypsin inhibitor and 2 $\mu\text{l/ml}$ Protease Inhibitor Cocktail). The subcellular fractionation was done in a postnuclear homogenate from 4 pancreases as described before [21]. Four subcellular fractions were obtained: the zymogen (Z), lysosomes plus mitochondria (L+M), microsomes (Mic) and soluble (S) fractions.

Serum amylase was measured in the Roche Modular Analyzer by the method of Lorentz [24]. In brief, the oligosaccharide 4,6-ethylidene-(G7) p-nitrophenyl-(G1)- α ,D-maltoheptaoside (ethylidene-G₇PNP) was cleaved under the catalytic action of α -amylases. The G₂PNP, G₃PNP y G₄PNP fragments so formed were then completely hydrolysed to p-nitrophenol and glucose by α -glucosidase. The color intensity of the p-nitrophenol formed is directly proportional to the α -amylase activity and was measured photometrically. Protein concentrations were assayed by the method of Bradford [25], using BSA as standard.

2.4. Induction of neutropenia

Vinblastine sulfate was dissolved in 10mM sodium phosphate buffer, 147 mM NaCl, 2.7 mM KCl (pH 7.4) and administered to rats intravenously (i.v.) at a

dose of 0.75 mg/kg on day 1, as previously described [26]. At this dose, animals become neutropenic between days 4 and 6 [26]. On day 5 following vinblastine sulfate or saline administration, the animals were treated with 2 doses of Cer (20 µg Cer/kg, administered at hourly intervals) to induce AP.

2.5. Inhibition of JNK- and ERK1/2 by SP600125 in Cer-induced AP

Rats received intraperitoneal (i.p.) injections of SP600125 (15 mg/kg) or its vehicle (1 ml/kg of a 10% DMSO/NaCl solution) both 2 h before and 30 min after the first Cer injection [9]. Two hours after the first injection of SP600125, the rats were injected subcutaneously with Cer (20 µg Cer/kg) or its vehicle (0.9% NaCl) at hourly intervals. The animals were sacrificed 2 h after the first Cer injection (early phase of Cer-induced AP).

2.6. Inhibition of type IV phosphodiesterase by rolipram in Cer-induced AP

Rats received i.p. injections of rolipram (5 mg/kg) or its vehicle (1 ml/kg of a 10% DMSO/NaCl solution) both 30 min before and 30 min after the first Cer injection [27]. Thirty minutes after the first injection of rolipram, the rats were injected subcutaneously with Cer (20 µg Cer/kg) or its vehicle (0.9% NaCl) at hourly intervals. The animals were sacrificed 2 h after the first Cer injection (early phase of Cer-induced AP).

2.7. RNA preparation and RT-PCR

Total RNA was isolated from the pancreas and brain of the same rat by immediate solubilization in Trizol Reagent and isopropanol purification. RNA concentrations were determined by absorbance, and RNA integrity was checked by agarose electrophoresis. Reverse transcription was completed by incubating 2 µg of total RNA with 0.5 µg of a 18-mer oligo(dT) primer at 42 °C for 1 h, using 200 units of RevertAid M-MuLV Reverse Transcriptase in a solution containing 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 1mM individual dNTPs and 20 units of Ribolock Ribonuclease inhibitor in a final volume of 20 µl. The reaction was stopped by heating at 70 °C for 10 min. Four-µl of this reaction was then used as a template for the polymerase chain reaction, which was performed with 1 unit of Taq polymerase in a final volume of 40 µl with specific oligos at a 0.4 µM final concentration. The annealing temperature was 54 °C. Five-µl samples were taken at 25, 29, 32 and 35 cycles to ensure that we were working within the semi-quantitative range. The oligonucleotide sequences were as follows: *SHP-1*: forward oligo: AGGCCGGCTTCTGGGAGGAGTT; reverse oligo: CCAGTGGGGAGATCTGCAATGTTC. *SHP-2*: forward oligo: TGACCTCTATGGCGGGGAAAAGTT; reverse oligo: TCAGCGGCATTAATACGAGTTGTG. Product lengths were 400 and 486 pb for *SHP-1* and *SHP-2*, respectively.

2.8. Myeloperoxidase (MPO) assay

Excised pancreatic tissue samples were rinsed with saline, blotted dry, snap-frozen in liquid nitrogen, and stored at -80 °C. MPO activity was detected

by using the MPO Fluorimetric Detection Kit following the manufacturer's instructions.

2.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SHP-1 and SHP-2 were analysed by SDS-PAGE using 10% gels [28]. The proteins present in the gels were transferred to PVDF membranes. After blocking non-specific binding with 5% non-fat dry milk (dissolved in buffer 10mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5), Western blots were probed with anti- SHP-1 or anti-SHP-2 monoclonal antibodies diluted 1:150 and 1:2,500, respectively, in the blocking solution. Blots were visualized by chemiluminescence. To analyse the spots on all the blots, film images were scanned using the Adobe Photoshop program (version 2.5.1.), and the images were then analysed with the MacBas v 2.5. program, as described previously [29]. The images shown in the figures are from blots whose films were exposed for optimal reproduction rather than for the linearity of band densities.

2.10. Statistical analysis

Data are expressed as means \pm SD. They were analysed using the non-parametric Mann-Whitney *U* test. Statistical significance was considered for a *p* value <0.05 . Analyses were implemented using the SPSS program for MS Windows (version 15.0).

3. Results

3.1. SHP-1 and SHP-2 expression during the development of AP

The upper parts of Figs. 1A and 1B show representative Western blot analyses indicating both SHP-1 and SHP-2 proteins in whole postnuclear pancreatic homogenates obtained from control or pancreatic rats at different times after the first injection of Cer. The lower parts of Figs. 1A and 1B indicate quantitative data related to the whole pancreas. We considered it more appropriate to express the data in relation to the whole pancreas because pancreatitis is associated with the neutrophil infiltration and cell death that make the cell composition of the pancreases of control or pancreatic rats different. We observed an increase in the expression of both SHP-1 and SHP-2 in the early phase of AP (2 h after the first injection of Cer) (6.1 ± 2.4 - and 3.3 ± 1.6 -fold that of controls for SHP-1 and SHP-2, respectively). For SHP-1, this early increase in expression remained in the intermediate and later phases of AP, although to a lower extent (i.e., 3.0 ± 0.8 -fold that of the control at 9 h). By contrast, SHP-2 expression fell to levels similar to those of the controls from 4 h onwards. Fig. 2A and 2B depict representative gels highlighting mRNA expression for both SHP-1 and SHP-2 in the early phase of AP (2 h). It may be observed that the induction of AP by Cer resulted in an increase in SHP-1 and SHP-2 mRNAs in pancreas but not in a control organ with Cer receptors (brain), in which the expression of SHP-1 and SHP-2 proteins remained constant (data not shown). Cer-induced AP is thus associated with increases in pancreatic SHP-1 and SHP-2 at the level of both protein and mRNA. Additionally, as proof

of the establishment of AP, a significant increase in serum amylase activity was observed along the development of AP, Fig 1C).

To analyse whether the increase in SHP-1 and SHP-2 protein expression was specific to the Cer AP model, we next assayed the taurocholate and BPDO models of AP. For this, the pancreases were studied once AP was fully developed. As depicted in Fig. 3B, an increase in the expression of SHP-2 was also observed in the other two models of AP, mainly in the BPDO model (4.5 ± 1.4 - and 11.9 ± 5.6 - fold that of controls for the taurocholate and BPDO models, respectively). By contrast, an increase in SHP-1 protein expression was not detected either in the taurocholate or in the BPDO model of AP; instead, a decrease was observed, especially in the BPDO model. (Fig. 3A).

3.2. Influence of neutrophil infiltration

Neutrophil infiltration is an important component in the development of both inflammation and cell death in pancreatitis. Thus, in an attempt to see whether infiltrating neutrophils were responsible for, or could somehow influence, the increase in the protein expression of both PTPs in Cer-induced AP, rats were rendered neutropenic with vinblastine sulfate before treatment. The rats were administered vinblastine on day 1, and the experiment with Cer was conducted on day 5, at which point it has been reported that animals have no remaining neutrophils [26]. As expected, a differential count of leukocytes in the blood of the rats used in our experiments afforded zero percent of neutrophils (39 ± 5 and 0% neutrophils in blood from Cer-induced AP (4 h) rats non-pretreated or pretreated with vinblastine sulfate, respectively, n=3: data not shown). Furthermore, leukocyte accumulation in the pancreas was investigated

by measuring MPO activity. We chose to study the intermediate phase of AP (4h) because it has been reported that at this time the inflammatory cell infiltration has already taken place [30]. In Fig. 4A it may be seen that while Cer treatment (4h) caused leukocyte accumulation in the pancreas, the administration of vinblastine resulted in blunted pancreatic MPO levels. With respect to serum amylase, vinblastine treatment alone did not affect its activity, and Cer treatment in vinblastine-treated rats did not decrease serum amylase levels (Fig. 4B), in agreement with previously reported data [26]. Fig. 4C shows that the depletion of neutrophils in the rats did not significantly affect the increase in SHP-1 protein expression at 4 h after Cer treatment (5.7 ± 1.8 - and 3.6 ± 1.6 -fold above the controls in pancreatitic rats non-pretreated or pretreated with vinblastine sulfate, respectively). In the case of SHP-2 (Fig. 4D), neutrophil depletion prevented the decrease in PTP expression down to basal levels at 2 h after Cer treatment, because in the intermediate phase of AP (4 h) SHP-2 protein expression was still significantly increased (1.1 ± 0.1 - and 4.1 ± 1.7 -fold that of controls in pancreatitic rats non-pretreated or pretreated with vinblastine sulfate, respectively).

3.3. Effect of JNK and ERK1/2 kinase inhibition by SP600125

It has been proposed that MAP kinases play a pivotal role in the development of hyperstimulation-induced pancreatitis, in part due to an amelioration of the severity of Cer-induced AP after the inhibition of JNK and ERK1/2 kinases [9]. To check whether these two MAP kinases might play a role in the increase of the expression of both PTPs in the early phase (2 h) of AP, we next investigated the effect of SP600125, a new inhibitor of JNK and

ERK1/2, on SHP-1 and SHP-2 protein expression. In Fig. 5, it can be observed that SP600125 pretreatment did not reduce the increase in the expression of either PTP after Cer treatment. By contrast, the expression of both SHP-1 (Fig. 5A) and SHP-2 (Fig. 5B) was significantly increased in the pancreases of Cer-injected rats pretreated with SP600125 in comparison with the non-pretreated animals (6.2 ± 1.6 - and 3.1 ± 0.7 -fold, and 7.1 ± 2.5 - and 2.1 ± 1.1 -fold as compared with control rats for SHP-1 and SHP-2, respectively). Moreover, JNK and ERK1/2 inhibition in the absence of Cer stimulation was also able to increase SHP2 protein expression to a similar extent to Cer (2.6 ± 0.8 - and 2.1 ± 1.1 -, 2.3 ± 0.7 -fold that of control rats injected with saline or saline+vehicle (DMSO), for Cer and Cer+vehicle (DMSO) or saline+SP600125 injected rats, respectively). This latter effect was not observed for SHP-1. Regarding serum amylase activity in the early phase (2 h) of AP, SP600125 was not able to reduce the significant increase observed in the animals given Cer alone (1603 ± 10 , 1710 ± 56 , 4800 ± 300 , 5500 ± 400 U/l, $n=3$, for rats given saline+DMSO, saline+SP600125, Cer+DMSO and Cer+SP600125, respectively, data not shown).

3.4. Effect of type IV phosphodiesterase inhibition by rolipram

We next decided to analyse the role of cAMP levels in the increase in the expression of both PTPs in the early phase (2 h) of AP, since it has recently been proposed that intracellular cAMP levels in inflammatory cells might also play an essential role in the pathogenesis of AP [27]. In Fig. 6A it can be observed that rolipram, an inhibitor of type IV phosphodiesterase, did not affect

the increase in SHP-1 protein expression due to Cer, since in comparison with the non-pretreated animals PTP protein expression was not modified in the pancreases obtained from Cer-injected rats pretreated with rolipram (4.9 ± 1.5 - vs 6.6 ± 3.5 -fold above control rats, respectively). Unlike SHP-1, the increase in SHP-2 protein expression due to Cer was significantly suppressed in rolipram-pretreated rats (Fig. 6B) (1.3 ± 0.3 - vs 5.0 ± 0.3 -fold above control rats (injected with saline+vehicle) for Cer+rolipram and Cer+vehicle rats, respectively). Rolipram did not change SHP-2 protein expression in the absence of Cer stimulation [1.2 ± 0.1 -fold in saline+rolipram-injected rats as compared with saline+vehicle-injected rats]. Regarding serum amylase activity in the early phase (2 h) of AP, rolipram showed a tendency (although not significant) to reduce such activity in rats given Cer+rolipram (1450 ± 30 , 1600 ± 50 , 5500 ± 250 and 4700 ± 400 U/l, n=3, for rats given saline+DMSO, saline+rolipram, Cer+DMSO and Cer+rolipram, respectively, data not shown).

3.5. SHP-1 and SHP-2 subcellular distribution during the development of AP

Finally we analysed the subcellular distribution and dynamics of both PTPs. As expected, both PTPs were completely (SHP-1, data not shown) or mainly cytosolic (located in the S fraction, Table 1). SHP-1 remained as a cytosolic enzyme along the development of pancreatitis development and was never seen to become associated with any subcellular membrane-fraction (data not shown). By contrast, we observed that in the early phase of AP (2 h), the increase in the expression of SHP-2 protein was associated with significant increases in its location in the L+M fraction, but mainly in the Mic subcellular

fraction. This redistribution was less marked at 4 h after the first injection of Cer, and it disappeared at 9 h.

4. Discussion

AP involves a complex cascade of local and systemic events that are initiated as a response to stress by the pancreatic acinar cell, but the cellular and molecular mechanisms responsible of the initiation of pancreatitis are not well understood. Acinar cell responses in AP include not only rapid protein activation by posttranslational modifications, but also alterations in gene and/or protein expression that may convert the rapid activation of signalling mechanisms in acinar cells into long-term responses [1]. All these events, as a whole, will determine the ultimate severity of AP.

It has been proposed that alterations in gene expression within the initiation phase of AP play an important role in its development [1]. Taking this into account, in this study we report an increase in SHP-1 and SHP-2 in the pancreas at the level of both protein and mRNA as an early event during the development of Cer-induced AP. Especially significant was the increase in the expression of SHP-2 protein, because it was observed commonly in all three different in vivo models of AP. This highlights the general importance of the increase in the expression of this PTP in AP. By contrast, the specificity of the increase in SHP-1 protein in the Cer-induced AP model could reflect a specific response to a specific insult.

It is known that both SHP-1 and SHP-2 are expressed in the rat exocrine pancreas [12] and that both PTPs are scarcely detectable in human neutrophils [30], which, together with the data obtained here with vinblastine sulfate,

indicates that the increase in the expression of both PTPs most likely arises from pancreatic acinar cells. Nevertheless, it is still possible that other minor pancreatic cell types could account for some of the observed changes. In this context, further studies are currently being designed to fully determine the cellular origins of these PTPs.

It has been reported that there is a common set of genes expressed in at least Cer- and taurocholate-induced AP [1], which suggests the possibility of a common set of upstream signalling mechanisms. From previous studies, it is known that the different AP models used here induce the activation of stress kinases, including JNK [32]. Both JNK and ERK1/2 have been proposed to be important mediators during the early phase (first 1- 1.5 h) of AP [9, 26, 33]. Many of the transcription factors (TFs) induced early on in the onset of AP are known to be regulated by the activation of stress kinase pathways [1]. Thus, the activation of stress kinases provides a potential link between the earliest known signalling events in AP and the long-term consequences that stem from changes in gene expression. Furthermore, stress kinases also regulate protein translation [34]. Since these enzymes have been reported to promote gene and protein expression early on in the development of Cer-induced AP [9,26], we decided to analyse their potential influence in the observed increase of both SHP-1 and SHP-2 proteins. We used SP600125, a new MAPK inhibitor that, under the conditions used here, has been described to almost totally inhibit Cer-induced pancreatic JNK activation and partially inhibit ERK1/2 activation [9]. Our results demonstrated that SP600125 pretreatment was able to positively modulate both SHP-1 and SHP-2 protein expression in the early phase of Cer-induced AP. Currently it is difficult to envisage the mechanism by which the inhibition of MAPK would afford further increases in both PTP proteins. It is

possible that the observed effect could be mediated through an effect on the half-life of early-activated TFs. It has been proposed that the activation of TFs also facilitates their immediate degradation, thus ensuring the end of translation activation [35]. It is possible that an inhibition of the MAPK pathway that activates specific TFs might influence the concomitant degradation rate of such TFs, thus modifying their half-life. Another possibility would be an effect of JNK and/or ERK1/2 on the half-life of both SHP-1 or SHP-2 proteins or messages. Further specific research would be necessary to analyse this surprising effect. Considering that MAPKs have also been shown to up-regulate the expression of inflammatory cytokines, such as TNF- α [9], which also primes cell infiltration in the pancreas, and that SP600125 pre-treatment results in a blunted leukocyte accumulation in the pancreases of Cer-induced AP rats [9], the positive effects of SP600125 and the data on neutropenic rats seem to be in good agreement. We believe that analyses of infiltration as a regulator of at least SHP-2 protein expression merit further investigation. The lack of a significant reduction in amylase activity after SP600125 pre-treatment seems to be in disagreement with previous results [9]. Nevertheless, it is important to recall that we were analysing the early phase of Cer-induced AP and that those previous results [9] were found in rats with fully developed AP. Therefore, the reduction in amylase activity after JNK and ERK1/2 inhibition would occur later on in the development of AP.

Excessive ATP catabolism has been reported to occur during AP [20] and increases in cAMP levels in inflammatory cells by rolipram have been reported to attenuate some inflammatory diseases, including AP [27]. Rolipram is a specific and strong inhibitor of type IV phosphodiesterase, a key enzyme in the metabolism of intracellular cAMP that is abundantly expressed in

inflammatory cells such as neutrophils and that also exerts anti-inflammatory effects by increasing the level of intracellular cAMP by blocking its catalysis. Thus, rolipram has a similar action to that of adenosine A_{2a} receptors, a receptor type mainly expressed in inflammatory cells [10]. Increases in intracellular cAMP levels enhance the activity of cAMP-dependent protein kinase A and reduce the production of proinflammatory cytokines such as TNF- α [36]. Importantly, acinar cells can behave as true inflammatory cells, which also produce such proinflammatory cytokines [37]. Accordingly, although we are not aware of any report describing the presence of type IV phosphodiesterase in acinar cells, rolipram could be a useful tool for investigating the role of intracellular cAMP levels in cell infiltration, and probably also in acinar cells, during the development of AP. Accordingly, analysis of the putative influence of rolipram not only on infiltrating neutrophils but also on acinar cells as a cause of the specific sensitivity of SHP-2 expression to rolipram deserves further investigation. Future *in vitro* experiments with isolated acinar cells in the presence or absence of neutrophils will be necessary to further increase our understanding of the *in vivo* effect of rolipram on SHP-2 protein expression in Cer-induced AP rats.

SHP-1 remained as a cytosolic enzyme during Cer-induced AP development (this work), which is in agreement with previous results [12]. By contrast, the increase in SHP-2 in the early phase of Cer-induced AP was associated with a redistribution of its subcellular location. Preliminary data with three different lysosomal populations obtained by centrifugation of the pancreatic L+M subcellular fraction in Percoll density gradients revealed that SHP-2 significantly increases its location by more than 100% (reaching up to 50% of total SHP-2 protein in the L+M fraction) in the two densest populations

(unpublished results). Electron microscopic observations of these reveal that they are composed of vesicles enclosing membrane fragments and amorphous membrane-bound material of varying sizes and shapes (unpublished results). Data on the intracellular proteolytic systems used for PTP degradation are very scarce, although it is generally accepted that most regulatory molecules involved in highly regulated cellular processes such as signal transduction are substrates of the proteasome. Thus, the meaning of the association of SHP-2 with lysosomes in AP is unknown. Despite this, it should be noted that the lysosomal/vacuolar system is a discontinuous and heterogeneous digestive system that also includes structures that are devoid of hydrolases and that carry out processes of heterophagy and autophagy [38], and that autophagy has been reported to occur in AP [39]. It is possible that as a result of autophagy, membranous and/or non-membranous material carrying SHP-2 protein could reach the densest lysosomal populations. Moreover, SHP-2 protein was also increased in the Mic subcellular fraction. At this point it is not possible to know what this would be due to. One possibility is the recruitment of SHP-2 to the plasma membrane by activated receptor protein tyrosine kinases or other phosphotyrosine-containing ligands that occurs early on in the development of AP [40]. It should be noticed that most of the plasma membrane would be present in the Mic fraction in our fractionation method. Finally, it is known that AP is associated with the disassembly of the acinar cell cytoskeleton [41] and several lines of in vitro and in vivo evidence suggest that SHP-2 might be involved in the control of cytoskeletal architecture [40].

In sum, in this study we have detected an increase in SHP-1 and SHP-2 in the pancreas at the level of both protein and mRNA as an early event during the development of Cer-induced AP. The increase in SHP-2 protein (which was

associated with changes in its subcellular distribution) in two other different *in vivo* pancreatic models points towards the general importance of this phosphatase in AP. The inhibition of JNK and ERK1/2 further increased the expression of both SHP-1 and SHP-2 proteins, while the inhibition of type IV phosphodiesterase only suppressed the increase in SHP-2 protein expression in the early phase of AP.

Acknowledgements

This work has been supported in part by MCYT (BFU2006-10362/BMC) and Junta de Castilla y León (SA033A05 and SA126A07). The authors wish to thank Drs. M. Manso and JM González de Buitrago (University of Salamanca) for their help with the BPDO model and amylase determination, respectively.

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Figure legends

Fig. 1. Expression of SHP-1 (A) and SHP-2 (B) proteins and serum amylase activity (C) during the development of Cer-induced AP. Rats were either administered saline (0.9% NaCl) (control, C) or treated with Cer, as indicated in Materials and methods, and then killed 2, 4 or 9 h after the first injection of Cer. In (A) and (B), quantification (graphic) of expression (Western blots in whole postnuclear pancreatic homogenates -a representative for each time is shown) was carried out considering the whole pancreas. Each lane in the blots contained 30 μ g of proteins. Data are means \pm S.D. of 5 animals.

Fig. 2. SHP-1 (A) and SHP-2 (B) mRNA expression in the early phase of Cer-induced AP. Rats were either administered saline (0.9% NaCl) (control, C) or treated with Cer (P), as indicated in Materials and methods, and then killed 2 h after the first injection of Cer. mRNA expression was analysed in pancreas or brain by RT-PCR. A representative RT-PCR is shown.

Fig. 3. Expression of SHP-1 (A) and SHP-2 (B) proteins in different models of AP. Three different AP models were used: Cer-induced (Cer), sodium-taurocholate duct infusion (Tau) or bile-pancreatic duct obstruction (BPDO) pancreatitis. Saline (0.9% NaCl)- injected rats are also shown (control, C). Cer represents 9 h treatment with Cer, as indicated in Materials and methods. Quantification (graphic) of expression (Western blots in whole postnuclear pancreatic homogenates -a representative is shown) was carried out considering the whole pancreas. Each lane in the blots contained 30 μ g of proteins. Data are means \pm S.D. of 3 animals.

Fig. 4. Effect of neutropenia on MPO (A) and serum amylase (B) activities, and SHP-1 (C) and SHP-2 (D) protein expression during the development of Cer-induced AP. Rats were given 0.75 mg/kg i.v. vinblastine sulfate to induce neutropenia. Four days after vinblastine administration, these neutropenic rats were given 4 injections of saline (0.9% NaCl) or Cer hourly over 4 h

(representing the intermediate phase of AP) as indicated in Methods. The same treatments were given to control animals (C) (saline instead of vinblastine pretreatment on day 1). In (C) and (D), quantification (graphic) of expression (Western blots in whole postnuclear pancreatic homogenates -a representative is shown) was carried out considering the whole pancreas. Each lane in the blots contained 30 μ g of proteins. Data are means \pm S.D. of 3 animals. N.S., not significant.

Fig. 5. Effect of SP600125 pretreatment on SHP-1 (A) and SHP-2 (B) protein expression in the early phase of Cer-induced AP. Rats were pretreated with SP600125 or its vehicle (15 mg/kg or 1ml/kg of 10% DMSO/NaCl solution i.p. administered 2 h before and 30 min after the first injection of Cer, respectively). Then, the animals were injected subcutaneously with either Cer or its vehicle [20 mg/kg or saline (0.9% NaCl, Sal) for each of two injections at hourly intervals, respectively]. As a positive control of the experiment, non-pretreated rats (Sal and Cer) were also included. Quantification (graphics) of expression (Western blots in whole postnuclear pancreatic homogenates -a representative is shown) was carried out considering the whole pancreas. Each lane in the blots contained 30 μ g of proteins. Data are means \pm S.D. of 2 experiments with 3 rats per group in each.

Fig. 6. Effect of rolipram pre-treatment on SHP-1 (A) and SHP-2 (B) protein expression in the early phase of Cer-induced AP. Rats were pretreated with rolipram or its vehicle (5 mg/kg or 1ml/kg of 10% DMSO/NaCl solution i.p. administered 30 min before and 30 min after the first injection of Cer, respectively). Then, the animals were injected subcutaneously with either Cer or its vehicle [20 mg/kg or saline (0.9% NaCl, Sal) for each of two injections at hourly intervals, respectively]. As a positive control of the experiment, non-pretreated rats (Sal and Cer) were also included. Quantification (graphics) of expression (Western blots in whole postnuclear pancreatic homogenates -a representative is shown) was carried out considering the whole pancreas. Each lane in the blots contained 30 μ g of proteins. Data are means \pm S.D. of 2 experiments with 3 rats per group in each.

Table 1

Percentage distribution of SHP-2 protein in subcellular fractions from pancreas in control or pancreatic rats

Subcellular fraction (%)	Time after first injection of saline or Cae (h)					
	2		4		9	
	C	P	C	P	C	P
L + M	1.8 ± 0.8	8.1 ± 5.8 ^a	0.7 ± 0.2	4.4 ± 1.2	3.1 ± 2.8	4.0 ± 3.0
Mic	3.3 ± 1.0	21.8 ± 8.2 ^b	1.9 ± 1.0	7.1 ± 0.4	2.7 ± 2.6	3.0 ± 1.9
Z	1.0 ± 0.7	2.6 ± 2.5	0.4 ± 0.06	0.6 ± 0.2	0.7 ± 0.9	1.5 ± 1.1
S	93.9 ± 1.4	67.5 ± 5.4 ^a	97.1 ± 1.3	87.9 ± 0.6	93.5 ± 5.6	91.5 ± 5.4

Subcellular fractions: L+M, lysosomes+mitochondria; Mic, microsomes; Z, zymogens; S, soluble (cytosolic fraction). Rats: C, control (saline-injected); P, pancreatic (Cer-injected).

Values were obtained from quantification of Western blots as indicated in Materials and methods, and are expressed as relative percentages considering the sum of the integrated densities for all subcellular fractions in the whole pancreases as 100%.

Data are means ± S.D. of 3 experiments with 4 rats in each.

^a $p < 0.021$, ^b $p < 0.020$ with respect to C at 2 h.











