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ORIGINAL ARTICLE

Overexpression of spermidine/spermine N^1-acetyltransferase or treatment with N^1-N^{11}-diethylnorspermine attenuates the severity of zinc-induced pancreatitis in mouse

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Abstract Depletion of pancreatic intracellular polyamine pools has been observed in acute pancreatitis both in the animal models and in humans. In this study, the wild-type mice, polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase overexpressing (SSAT mice) and SSAT-deficient mice were used to characterize the new zinc-induced acute pancreatitis mouse model and study the role of polyamines and polyamine catabolism in this model. Intraperitoneal zinc injection induced acute necrotizing pancreatitis in wild-type mice as well as in SSAT-overexpressing and SSAT-deficient mice. Serum α-amylase activity was significantly increased in all zinc-treated mice compared with the untreated controls. However, the α-amylase activities in SSAT mice were constantly lower than those in the other groups. Histopathological examination of pancreatic tissue revealed edema, acinar cell necrosis and necrotizing inflammation, typical for acute pancreatitis. Compared with the other zinc-treated mice less damage according to the histopathological analysis was observed in the pancreatic tissue of SSAT mice. Levels of intracellular spermidine, and occasionally spermine, were significantly decreased in pancreases of all zinc-treated animals and SSAT enzyme activity was enhanced both in wild-type and SSAT mice. Interestingly, a spermine analog, N^1, N^{11}-diethylnorspermine (DENSpm), enhanced the proliferation of pancreatic cells and reduced the severity of zinc-induced pancreatitis in wild-type mice. The results show that in mice a single intraperitoneal zinc injection causes acute necrotizing pancreatitis accompanied by decrease of intracellular polyamine pools. The study supports the important role of polyamines for the integrity and function of the pancreas. In addition, the study suggests that whole body overexpression of SSAT obtained in SSAT mice reduces inflammatory pancreatic cell injury.

Keywords Spermidine/spermine N^1-acetyltransferase (SSAT) · Pancreatitis · Spermine analog · Polyamines

Abbreviations

SSAT	Spermidine/spermine N^1-acetyltranferase
DENSpm	N^1, N^{11}-Bis(ethyl)norspermine
ODC	Ornithine decarboxylase
SAMDC	*S*-Adenosylmethionine decarboxylase
MT	Metallothionein
BSA	Bovine serum albumin
PCNA	Proliferating cell nuclear antigen
HRP	Horseradish peroxidase
DBA	Diaminobenzidine
ALAT	L-Alanine aminotrasferase
NF-κB	Nuclear factor-κB
TNF-α	Tumor necrosis factor-alpha
ROS	Reactive oxygen species
HIF-1α	Hypoxia-inducible factor-1α

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Introduction

Acute pancreatitis is a necrotizing inflammation of the pancreas. It is initiated when the digestive enzymes produced by the pancreas are prematurely activated while still in the pancreas. As a consequence, the autolysis of pancreatic tissue and acute inflammatory response occurs. Under normal conditions, the digestive enzymes are secreted from pancreas as zymogens, inactive enzyme precursors, in zymogen granules and released and activated in the small intestine. However, co-localization of lysosomal and digestive enzymes in pancreas results in premature enzyme activation. The central early process of acute pancreatitis is the intracellular cleavage of trypsinogen to active trypsin in pancreatic parenchyma leading to tissue destruction and thus further release of digestive enzymes (Harper and Cheslyn-Curtis 2011). Conditions that disturb the normal digestive enzyme secretion and activation pathways potentially initiate pancreatitis. The most common causes of acute pancreatitis are gallstones and heavy alcohol use, but several other reasons, like infections, medication, tumors and genetic susceptibility can also induce the disease (Harper and Cheslyn-Curtis 2011). Among the rare causes of acute pancreatitis is hypercalcemia, an elevated blood concentration of Ca^{2+} ions. Several potential underlying mechanisms are connected with hypercalcemic pancreatitis including calcium precipitation in the pancreatic duct, mimicking the effect of gallstones, as well as calcium-facilitated premature activation of trypsinogen (Harper and Cheslyn-Curtis 2011; Mithofer et al. 1995; Ward et al. 1995).

The annual incidence of acute pancreatitis in western countries is 4–45 patients per 100,000 population, of which around 20% are severe cases associated with organ failure and significant mortality and morbidity (Yadav and Lowenfels 2006; Sandzen et al. 2009; Lund et al. 2006). In severe acute pancreatitis the death is caused by systemic inflammatory response syndrome, pancreatic necrosis and multi-organ failure. Understanding the pathology of acute pancreatitis is important not only for developing medication but also for characterization and evaluation of the prognostic factors and scoring systems to assess the severity of the disease. Several rodent models of pancreatitis have been developed, including the L-arginine, caerulein, taurodeoxycholate, choline deficient ethionine-supplemented diet and L-ornithine induced rat models (Tani et al. 1990; Niederau et al. 1990; Lankisch and Ihse 1987; Lombardi et al. 1975; Rakonczay et al. 2008). These models differ in several aspects from each other, but they also share the common characteristics of pancreatitis (e.g. ascites, pancreatic edema, cellular necrosis, leukocyte infiltration and destruction of pancreatic acini).

Putrescine, spermidine and spermine are positively charged polyamines essential for several cellular functions including cell proliferation and differentiation. Polyamine metabolism is strictly regulated by multiple factors affecting largely through the three key enzymes, ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (SAMDC) and spermidine/spermine N^1-acetyltransferase (SSAT) (Persson 2009). ODC and SAMDC are biosynthetic enzymes while SSAT is controlling the catabolic pathway. SSAT acetylates spermine and spermidine producing N^1-acetylspermine and N^1-acetylspermidine, respectively. In the next step, polyamine oxidase converts N^1-acetylspermine and N^1-acetylspermidine to spermidine and putrescine, respectively. Recently, depletion of spermidine and spermine has been connected with the pathology of acute pancreatitis both in animal models and in human samples of acute pancreatitis (Hyvonen et al. 2006, 2007; Jin et al. 2008b; Biczo et al. 2010). In our transgenic MT-SSAT rat model, in which the expression of SSAT was governed by the metallothionein promoter, zinc-induced overexpression of SSAT led to severe acute necrotizing pancreatitis and death of the animals (Alhonen et al. 2000). Accumulation of zinc in the pancreas induced especially high overexpression of SSAT leading to drastic depletion of pancreatic intracellular spermidine and spermine pools in the transgenic rats. Importantly, the wild-type control rats had normal polyamine pools and were unaffected by the non-toxic dose of zinc. The importance of intrapancreatic polyamines was evident by treatment of MT-SSAT animals with metabolically stable methylated polyamine analogs which decreased the severity of pancreatitis and reduced the pancreatitis-associated mortality (Hyvonen et al. 2006; Rasanen et al. 2002).

Zinc is a structural constituent of a great number of proteins, including enzymes of cellular signaling pathways and transcription factors, thus affecting the basic cellular functions, e.g. cell proliferation, differentiation and apoptosis (Beyersmann and Haase 2001). Zinc homeostasis is controlled by uptake, intracellular sequestration in zinc storing vesicles as well as nucleocytoplasmic distribution and elimination. In a cell most of the zinc is bound to either metallothionein, the major zinc binding protein, or to other proteins or anionic molecules, meaning that the amount of free intracellular zinc is low. In addition to being an essential mineral, zinc is also a toxic heavy metal. Acute pancreatitis caused by overexposure to zinc has been reported in case of, e.g. trumpet swans, dogs, humans and laboratory mice (Carpenter et al. 2004; Mikszewski et al. 2003; Chobanian 1981; Sarma and Narula 1996; Minami et al. 2001; Onosaka et al. 2002). The mechanism of zinc-associated pancreatitis is not known. In normal physiology, zinc accumulates in the pancreas and is secreted into the digestive tract. Under high exposure to zinc, the overload

of the toxic heavy metal in the pancreas may disturb the normal function of the cells and partly explain the inflammation and damage of the tissue.

In this paper, we introduce the zinc-induced pancreatitis as a new mouse model for acute necrotizing pancreatitis and characterize the role of polyamine metabolism in pancreatic inflammation. In this model, the pancreatic higher polyamines are depleted in the course of pancreatitis, irrespective of the genetically modified polyamine catabolism. In addition, intrapancreatic accumulation of a stable spermine-analog, N^1, N^{11}-diethylnorspermine (DENSpm), attenuates the severity of the inflammatory response. The study also suggests that whole body SSAT overexpression in SSAT mice has a protective function against zinc-induced pancreatic damage in mice.

Materials and methods

Materials

Transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase (SSAT) gene (Pietila et al. 1997), SSAT-deficient mice (Niiranen et al. 2006) and their wild-type littermates in C57BL/6J background at age of 5–7 months were used. Animals of same sex and age were included in each separate experiment. Induction of pancreatitis was accomplished by intraperitoneal injections of zinc sulphate (20 mg/kg) in physiological saline (zinc dose 4.55 mg/kg). Control animals received intraperitoneal saline at a dose of 10 ml/kg. DENSpm was synthesized essentially as described in (Rehse et al. 1990). The intraperitoneal dose of DENSpm (in saline) was 125 mg/kg. All animal experiments were approved by the National Animal Experiment Board.

Quantification of SSAT enzyme activity, intracellular polyamine content and serum α-amylase and ALAT activities

The activity of SSAT was measured as previously described (Bernacki et al. 1992). Natural polyamines were determined by HPLC as described previously (Hyvonen et al. 1992). Polyamine analogs were measured using a previously described method (Kabra et al. 1986). Serum α-amylase and L-alanine aminotrasferase activities (ALAT) were measured photometrically with Microlab 200 analyzer system (Merck, Darmstadt, Germany) using assay reagents from DiaSys (Holzheim, Germany) according to the manufacturers' instructions.

Histopathological scoring of the severity of pancreatitis

Animals were killed with CO_2 and the pancreatic tissue samples were immediately formalin-fixed for 24 h at room temperature. The fixed tissue was embedded in paraffin, cut into 4-μm-thick slices and stained with hematoxylin and eosin. The histopathological analyses were made by an experienced histopathologist (RS) as previously described (Schmidt et al. 1992). Shortly, the scoring criteria consisted of six variables determining the severity of injury. Thus, tissue edema, acinar cell necrosis, hemorrhage, fat necrosis, intralobular inflammation and perivascular neutrophilic infiltrates were scored by the standardized scoring system. Each variable was able to have values between 0 and 4 (two slides per animal were studied) and the overall severity of pancreatitis was determined by the sum of these scores.

Immunohistochemistry

For immunohistochemistry, the formalin-fixed and paraffin-embedded 4-μm-thick slices were deparaffinized and rehydrated in decreasing alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10 min, after which the slides were washed three times, 5 min each, with TBST (0.2 M Tris–HCl, pH 7.4, 1.5 M NaCl, 0.01% Tween 20) solution. For antigen retrieval the slides were cooked in 0.01 M citric acid, pH 6.0, in microwave oven twice for 5 min followed by three 5-min washes in TBST. Nonspecific protein binding was blocked by preincubating the slides with 1.5% BSA, 0.05 M Tris–HCl, pH 7.4, for 1 h at room temperature. For the detection of proliferating cell nuclear antigen (PCNA), the slides were incubated with 1:100 dilution of the mouse monoclonal PCNA-HRP antibody (sc-56, Santa Cruz, CA) for 1 h at room temperature, and for caspase-3 detection the slides were incubated with 1:500 diluted polyclonal rabbit anti-cleaved caspase-3 antibody (#9661, Cell Signaling Technology, MA) over night at +4°C, washed as indicated above and incubated with PowerVision™ Poly-HRP anti-rabbit IgG secondary antibody (ImmunoVision Technologies Co, Brisbane, CA) for 1 h at room temperature. The antibody-treated slides were washed as before, incubated for 3 min with DAB (ImmunoVision Technologies Co, Brisbane, CA) solution and washed three times for 5 min with distilled water. The slides were counterstained by dipping in Mayer's hematoxylin for five times, rinsed with tap water for 5 min, dehydrated in increasing alcohol series, cleared in xylem and mounted with Depex (BDH, Poole, UK).

Analysis of tissue zinc content

Tissue samples of 100 mg were homogenized with 300 μl of buffer containing 25 mM Tris–HCl, pH 7.4, 0.1 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol using Tissuelyser II (Qiagen, Germantown, MD).

Homogenized samples (100 µl) were digested in 500 µl of concentrated nitric acid in a CEM 81D microwave digestion system (Scientific Support, Inc, Hayward, CA) and diluted into 3 ml with 0.01% Triton X-100 in Milli-Q-water. Zinc determinations were carried out with a 5100 atomic absorption spectrometer (Perkin Elmer, Waltham, MA) with a zinc hollow cathode lamp at wavelength 213.9 nm using air-acetylene flame.

Statistical analysis

Data are expressed as mean ± SD where applicable. GraphPad Prism 5.03 software package (GraphPad Software Inc., LaJolla, CA) was used to perform *t* test, one way ANOVA and Dunnett's multiple comparison test.

Results

Low-dose administration of intraperitoneal zinc induces acute necrotizing pancreatitis in mice

We evaluated the usability of zinc-induced pancreatic inflammation as a relevant model for acute necrotizing pancreatitis. In addition, the role of polyamine metabolism in the development of acute pancreatitis was evaluated. Based on the previous studies describing zinc as an inducer of pancreatitis in mice (Minami et al. 2001), we tested a low-dose administration of zinc both in the wild-type and SSAT mice. In both animal lines pancreatic zinc content increased significantly within 24 h and stayed high (1.6–2.2 times the zinc level in the untreated animals) for the next 7 days the animals were followed (Table 1). Within 24 h after the zinc injection, the animals showed typical macroscopic, enzymatic and histological findings of acute pancreatitis. Thus, the mice had ascites, pancreatic edema and fat necrosis in the pancreata and abdominal adipose tissues. The zinc-treatment significantly increased the activity of α-amylase in the sera of both mouse lines and L-alanine aminotransferase activity in the sera of wild-type mice (Table 1). Histopathological analyses revealed the presence of edema, necrosis and infiltration of inflammatory cells in pancreatic tissues of zinc-treated wild-type and SSAT mice (Fig. 1a–f). Only few apoptotic cells, indicated by the caspase-3 immunopositivity, were detected in tissue samples of untreated mice and a few more were present in the pancreatic tissue samples of zinc-treated mice (Fig. 1g–l). The animals were followed for 7 days during which time the zinc-treated animals become comparable with the control mice in terms of appearance and serum α-amylase activity. The histological examination revealed compensatory acinar cell proliferation which started already within 24 h after the zinc administration (Fig. 1m–r).

Effect of zinc on pancreatic polyamine metabolism in wild-type and SSAT mice

The depletion of intracellular higher polyamines is a common phenomenon in various animal models of acute necrotizing pancreatitis. To analyze the effect of zinc-induced pancreatitis on the pancreatic polyamine levels and on the expression of SSAT gene, we subjected the wild-type and SSAT mice to zinc-induced pancreatitis as described in the aforementioned pilot study. Samples were taken 24 h and 4–5 days after the zinc administration to see the short- and long-term effects. As in the pilot study, the serum α-amylase activities in the zinc-treated mice of both lines were significantly increased 24 h after induction of the disease compared with the controls (Table 2). As well, L-alanine aminotransferase activities in sera of wild-type mice were significantly elevated after 24 h from the zinc administration. The SSAT activity in the pancreases of SSAT mice was significantly increased after 24 h of zinc injection compared with the controls (Table 2). In the wild-type mice pancreatic SSAT activity was significantly increased by zinc but only in the samples taken 4–5 days after the zinc treatment. Pancreatic spermidine and spermine contents in zinc-treated animals decreased significantly within 24 h after the zinc injection in both lines (Table 2). In wild-type mice the spermidine and spermine levels stayed significantly lower than in the control animals for the next 4–5 days after the zinc treatment. Based on these results, the pool of higher polyamines in the pancreas is strongly reduced in the zinc-induced pancreatitis model.

The severity of pancreatitis is attenuated in SSAT mice

The zinc treatment induced acute necrotizing pancreatitis both in the wild-type and SSAT mice. However, the serum α-amylase and L-alanine aminotransferase enzyme activities obtained in zinc-treated mice 24 h after the zinc administration were significantly lower in SSAT mice compared with the wild-type animals (Tables 1, 2). Furthermore, the histopathological analyses of the pancreatic tissue revealed less severe acinar cell necrosis, inflammation and perivascular neutrophilic response in the transgenic animals (Table 3; Fig. 2). Hemorrhage was not detected in any samples. After 4–5 days there was no difference in the extent of pancreatic damage between the lines. Similar results were obtained in repeated experiments. Thus, the results suggest that SSAT overexpression obtained in SSAT mice attenuates the severity of zinc-induced pancreatitis.

Polyamine depletion in zinc-induced pancreatitis is independent of SSAT activity

Since SSAT is the main enzyme involved in the catabolic pathway of higher polyamines, we induced pancreatitis in

Table 1 Pancreatic Zn content and serum α-amylase and L-alanine aminotransferase (ALAT) activities in wild-type mice (Wt) and SSAT mice (Tg) in Zn-induced pancreatitis

Animal and treatment	α-Amylase (U/l)	ALAT (U/l)	Zinc (ng/mg prot)
Wt	2,985 ± 285	31 ± 2	220 ± 26
Tg	2,675 ± 321	39 ± 10	212 ± 6
Wt + zinc 24 h	132,400 ± 49,460***	392 ± 197**	516 ± 52***
Tg + zinc 24 h	37,010 ± 29,150*†	83 ± 56	466 ± 78***
Wt + zinc 7 days	2,288 ± 410	30 ± 5	360 ± 53**
Tg + zinc 7 days	2,230 ± 390	44 ± 9	343 ± 44**

Data are the means ± SD; $n = 3$–4

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the untreated animals with corresponding genotype

† $p < 0.05$ as compared with wild-type mice with corresponding treatment

wild-type and SSAT-deficient mice to reveal the role of SSAT in the development of pancreatitis and depletion of intracellular polyamines. Within 24 h, equally increased serum α-amylase and L-alanine aminotransferase enzyme activities were evident in the wild-type and SSAT-deficient mice (Table 4). Spermidine and spermine levels in the wild-type and SSAT-deficient mice significantly decreased within 24 and 48 h after the zinc injection when compared with the corresponding controls. There were no significant differences between the polyamine levels observed in SSAT-deficient and wild-type mice. Thus, during zinc-induced inflammation the higher intracellular polyamines in pancreas are depleted by mechanisms other than the action of SSAT enzyme and the absence of the basal endogenous SSAT expression does not affect the outcome of the disease.

Spermine analog affects the severity of zinc-induced pancreatitis

N^1-N^{11}-Diethylnorspermine (DENSpm) is a widely characterized spermine analog which is able to induce SSAT activity. The analog is stable in the animal cells and thus, its accumulation increases the total intracellular spermine and polyamine content. In order to further analyze the role of polyamine metabolism in the development of zinc-induced pancreatitis, we treated the animals with DENSpm at the time of disease induction. DENSpm treatment did not affect the accumulation of zinc in the pancreas, but interestingly, the serum α-amylase and L-alanine aminotransferase activities in the mice receiving DENSpm together with zinc were significantly lower compared with the mice injected with zinc alone (Table 5). Similar pancreatic accumulation of DENSpm was detected in the mice treated either with DENSpm or with DENSpm and zinc. Pancreatic SSAT activity was not significantly increased in the treated animals. Interestingly, the pancreatic spermidine level decreased significantly only in the mice treated with zinc alone. In addition, as shown in the Fig. 3a–d, the histopathological examination revealed significantly increased acinar cell necrosis in the samples from zinc-treated mice compared with the samples from mice treated with zinc and DENSpm (2.0 ± 0.2 vs. 0.88 ± 0.3; $p = 0.011$). Also in SSAT overexpressing and SSAT-deficient mice the injury associated with zinc-induced pancreatitis was less severe when DENSpm was included in the treatment (data not shown).

DENSpm enhances acinar cell proliferation

It is known that different forms of cell death have at least partially restricting pathways towards each other (Challa and Chan 2010). Since DENSpm treatment decreased the amount of acinar cell necrosis in zinc-injected animals, we wanted to study the amount of apoptosis in the samples of the DENSpm experiment described above. We also examined the proliferation of acinar cells. In the samples of zinc-treated animals a few more caspase-3 immunopositive cells were observed compared with the control samples (Fig. 3e, f, h). However, DENSpm-treatment did not affect the amount of apoptosis (Fig. 3g). Similarly, as in the pilot study (Fig. 1) zinc-treatment induced proliferation of acinar cells within 24 h after the zinc administration (Fig. 3i, j, l). However, an interesting finding was that DENSpm alone enhanced the proliferation of pancreatic cells in the analog-treated animals (Fig. 3k).

Discussion

The animal models available for acute pancreatitis use various methods to induce the disease. The methods replicating the suggested events of acute clinical pancreatitis, e.g. bile reflux into the pancreatic duct, are based on retrograde infusion of bile acids into the pancreatic duct and involve surgery of the laboratory animals. For technical reasons, these surgical models, like taurodeoxycholate-approach, have involved studies with rats or larger

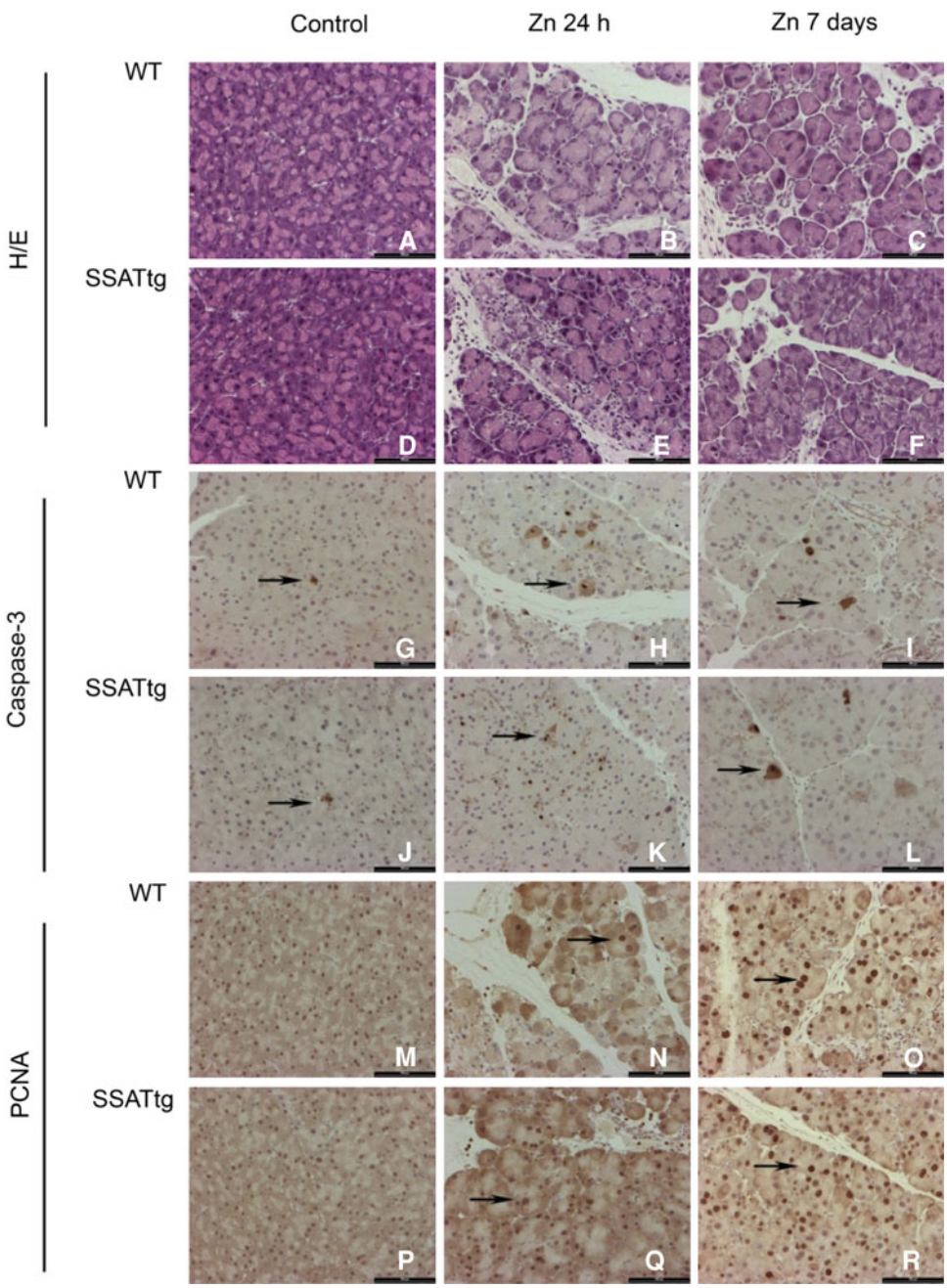

Fig. 1 Histopathological findings of zinc-induced pancreatitis. Pancreatic samples of wild-type (WT) and SSAT (SSATtg) mice were stained with hematoxylin and eosin (**a**–**f**). The zinc induced intra- and interlobular edema, neutrophilic inflammation and acinar cell necrosis (**b**, **c**, **e**, **f**) typical for acute pancreatitis. Apoptosis and cell proliferation were detected by immunohistochemical demonstration of caspase-3 (**g**-**l**) and PCNA (**m**–**r**), respectively. A few more apoptotic cells were present in zinc-treated mice (**h**, **i**, **k**, **l**) than in control mice (**g**, **j**) and proliferation of acinar cells was increased by zinc administration (**n**, **o**, **q**, **r**) compared with the untreated controls (**m**, **p**). Samples from control mice (**a**, **d**, **g**, **j**, **m**, **p**) and from mice after 24 h (**b**, **e**, **h**, **k**, **n**, **q**) and 7 days (**c**, **f**, **i**, **l**, **o**, **r**) of the zinc injection are shown. The *arrows* indicate immunopositive cells. Original magnification of ×200, *scale bar* 100 μm

experimental animals. Only recently, the technique has also been applied to the mice (Laukkarinen et al. 2007). Several other animal models of acute pancreatitis are based on observations that certain compounds, like L-arginine and L-ornithine, elicit inflammation in the pancreas. The exact mechanisms leading to inflammation are in most cases unknown. Although none of the present models is completely satisfactory in terms of human disease, they all have increased the knowledge of certain aspects of acute pancreatitis (Su et al. 2006). The zinc-induced model used in this study was performed with mice and was based on intraperitoneal injection of the heavy metal. The consequential pancreatic inflammation was characterized as acute necrotizing pancreatitis with no mortality. However, higher doses of zinc have been shown to cause mortality (Onosaka et al. 2002) and also in our study, some mice were occasionally more sensitive to the used zinc dose and, based on the poor appearance, these animals were killed and eliminated from the experiment. In addition, as indicated by the changes in the ALAT activity, zinc also disturbed the normal function of the liver. In several models of acute pancreatitis, including taurodeoxycholate, caerulein and L-ornithine models, the liver response indicated by enhanced levels of serum ALAT and aspartate

Table 2 Serum α-amylase and L-alanine aminotransferase (ALAT) activities and pancreatic polyamine metabolism in wild-type mice (Wt) and SSAT mice (Tg) in Zn-induced pancreatitis

Animal and treatment	α-Amylase (U/l)	ALAT (U/l)	SSAT activity (pmol/10 min/mg protein)	Polyamine pools (pmol/μg protein)		
				Put	Spd	Spm
Wt	3,404 ± 239	29 ± 6	12 ± 6	ND	29.0 ± 3.1	6.1 ± 0.5
Tg	3,238 ± 400	36 ± 5	29 ± 11	10.4 ± 2.9	24.1 ± 6.0	2.9 ± 0.7
Wt + zinc 24 h	121,800 ± 28,200***	878 ± 255***	39 ± 16	ND	6.4 ± 6.6***	1.6 ± 1.5***
Tg + zinc 24 h	81,740 ± 18,490***†	277 ± 286	258 ± 134**	17.6 ± 8.2	5.2 ± 1.5**	1.1 ± 0.3***
Wt + zinc 4–5 days	2,618 ± 313	183 ± 247	139 ± 99**	ND	9.5 ± 8.0***	3.0 ± 0.9**
Tg + zinc 4–5 days	2,603 ± 702	38 ± 21	119 ± 84	10.1 ± 2.2	15.3 ± 10.0	2.2 ± 0.5

Data are the means ± SD; $n = 5$

ND not detectable

** $p < 0.01$, *** $p < 0.001$ as compared with the untreated animals with corresponding genotype

† $p < 0.05$ as compared with wild-type mice with corresponding treatment

Table 3 Histopathological analysis of pancreatic tissue 24 h and 4–5 days after the zinc treatment in wild-type (Wt) and SSAT mice (Tg)

Animal and treatment	Edema	Acinar cell necrosis	Fat necrosis/max per MPF	Inflammation and perivascular infiltrate	Score sum
Wt + zinc 24 h	2.9 ± 0.2	3.7 ± 0.7	4.0 ± 0	3.0 ± 1.4	13.6 ± 2.3
Tg + zinc 24 h	2.7 ± 0.3	2.4 ± 1.1	4.0 ± 0	1.4 ± 0.7	10.5 ± 1.7*
Wt + zinc 4–5 days	2.7 ± 0.7	3.5 ± 1.1	3.4 ± 0.4	1.8 ± 1.2	11.4 ± 2.8
Tg + zinc 4–5 days	2.1 ± 1.4	2.5 ± 1.8	3.3 ± 1.5	1.4 ± 1.3	9.3 ± 5.7

For details of the scoring, see "Materials and methods"

Data are the means ± SD; $n = 5$

* $p < 0.05$, as compared with the wild-type animals

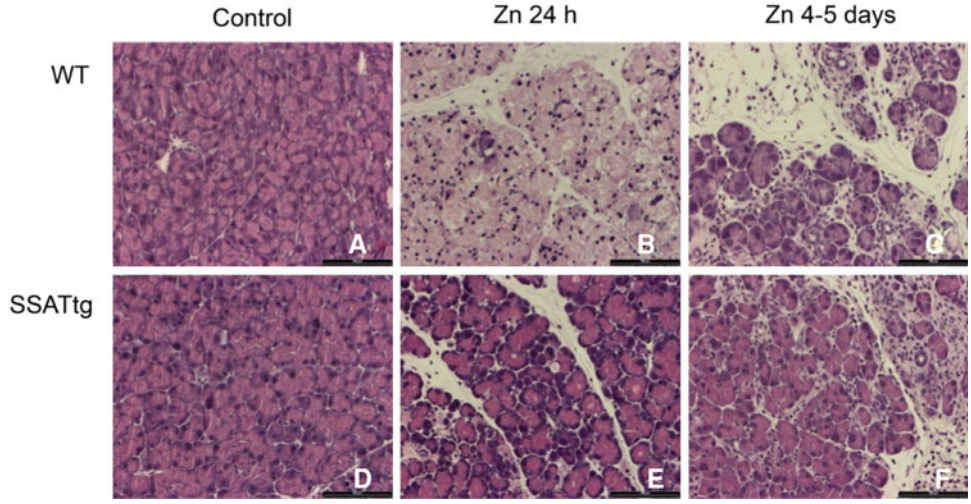

Fig. 2 Histopathology of pancreases from wild-type (WT) and SSAT (SSATtg) mice. Pancreatic samples stained with hematoxylin and eosin from the control mice (**a**, **d**) and zinc-treated animals 24 h (**b**, **e**) and 4–5 days (**c**, **f**) after the zinc-injection. Original magnification of ×200, *scale bar* 100 μm

aminotransferase (ASAT) activities is observed (Sha et al. 2008; Turkyilmaz et al. 2007; Rakonczay et al. 2008).

In this study, wild-type mice, ubiquitously SSAT over-expressing mice and SSAT-deficient mice were used. While the response of wild-type mice and SSAT-deficient mice to the zinc was comparable to each other, SSAT mice constantly showed significantly lower serum α-amylase levels and less severe histopathological evidence of tissue injury. The SSAT mice have several copies of the genomic SSAT gene driven by its own promoter in their genomes. During the development of pancreatitis, pancreatic SSAT activity of wild-type and transgenic animals increased significantly when compared with the control mice. However, the increase in SSAT activity in the transgenic mice

Table 4 Serum α-amylase and L-alanine aminotransferase (ALAT) activities and pancreatic polyamine levels in wild-type (Wt) and SSAT-deficient (Ko) mice in Zn-induced pancreatitis

Animal and treatment	α-Amylase (U/l)	ALAT (U/l)	Polyamine pools (pmol/μg protein)	
			Spd	Spm
Wt	2,874 ± 178	32 ± 2	27.4 ± 1.2	4.7 ± 0.5
Ko	3,292 ± 245	42 ± 7	28.0 ± 1.4	5.1 ± 0.3
Wt + zinc 24 h	202,600 ± 64,300***	1,340 ± 51***	3.0 ± 1.5***	1.3 ± 0.5***
Ko + zinc 24 h	168,800 ± 80,400***	1,440 ± 308***	4.4 ± 4.2***	1.4 ± 1.0***
Wt + zinc 48 h	6,636 ± 5,067	333 ± 222**	10.5 ± 9.3***	2.6 ± 1.2**
Ko + zinc 48 h	11,850 ± 11,630	336 ± 117	8.2 ± 3.0***	2.7 ± 0.9***

Data are the means ± SD; $n = 5$

** $p < 0.01$, *** $p < 0.001$ as compared with the untreated animals with corresponding genotype

Table 5 Effect of DENSpm on serum α-amylase and L-alanine aminotransferase (ALAT) activities, pancreatic zinc content and polyamine metabolism in wild-type mice in Zn-induced pancreatitis

Treatment	α-Amylase (U/l)	ALAT (U/l)	Zinc (ng/mg protein)	SSAT activity (pmol/10 min/mg protein)	Polyamine pools (pmol/μg protein)			
					Put	Spd	Spm	DENSpm
No treatment	2,953 ± 221	42 ± 6	374 ± 75	11 ± 8	ND	35.0 ± 2.8	5.5 ± 0.7	NA
Zinc 24 h	50,150 ± 39,360*	195 ± 113*	741 ± 24***	69 ± 24	7.3 ± 1.5	26.9 ± 4.2*	4.3 ± 0.3	NA
DENSpm 24 h	2,338 ± 161	36 ± 3	454 ± 74	107 ± 91	ND	35.5 ± 0.7	4.0 ± 0.8	3.6 ± 0.2
Zinc + DENSpm 24 h	12,590 ± 13,320	120 ± 45	896 ± 152***	69 ± 34	10.6 ± 10.7*	35.1 ± 5.1	4.3 ± 1.4	4.4 ± 3.3

Data are the means ± SD; $n = 5$

ND not detectable, *NA* not applicable

* $p < 0.05$, *** $p < 0.001$ as compared with the untreated animals

occurred within 24 h after the zinc administration but was evident in the wild-type mice in the later detected time points (4–5 days). SSAT has been addressed as a stress response gene whose expression can be induced by multiple endogenous and environmental factors (Casero and Pegg 1993), like the inflammatory mediators nuclear factor-κB (NF-κB), tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS). During inflammation, the action of these mediators and concomitantly increased SSAT activity are suggested to protect the tissue from extensive damage (Babbar et al. 2007).

In our earlier studies where rats overexpressing SSAT under the metallothionein promoter (MT-SSAT rats) were given a low dose of zinc to induce the transgene expression, massive induction of SSAT activity and consequent polyamine depletion were detected in pancreas leading to acute necrotizing pancreatitis and death of the transgenic rats (Alhonen et al. 2000). The wild-type control rats did not respond to the given zinc dose, and hence zinc-toxicity was not the primary cause of pancreatitis in the MT-SSAT rats. We also showed that pancreatitis-associated mortality in MT-SSAT rats was due to polyamine depletion-mediated coagulopathy that was alleviated by treatment with α-methylated spermine analog (Hyvonen et al. 2010). We also subjected mice, carrying the same MT-SSAT transgene as the MT-SSAT rats, to the zinc-induced pancreatitis described in this paper. Contrary to the wild-type and SSAT mice, these animals died within a few days after the zinc injection and in addition of acute necrotizing pancreatitis showed liver damage and occasional internal bleeding (data not shown). After zinc treatment the MT-SSAT mice had 200-fold increased SSAT activity in pancreas compared with the untreated MT-SSAT mice (data not shown). Thus, in MT-SSAT animals the zinc-inducible metallothionein promoter directs massive SSAT overexpression in certain tissues able to activate MT promoter, including pancreas, and leads to mortality. Based on these observations, the level and localization of SSAT expression are critical determinants of the resultant outcome of the function of the enzyme. In addition, the studies show that the MT-SSAT transgenic model of acute pancreatitis based on enhanced SSAT expression is different than the zinc-induced genotype-independent mouse model introduced in this paper. Recently, SSAT has been connected with some interesting new functions. For example, SSAT participates in the migration of $\alpha 9\beta 1$-integrin

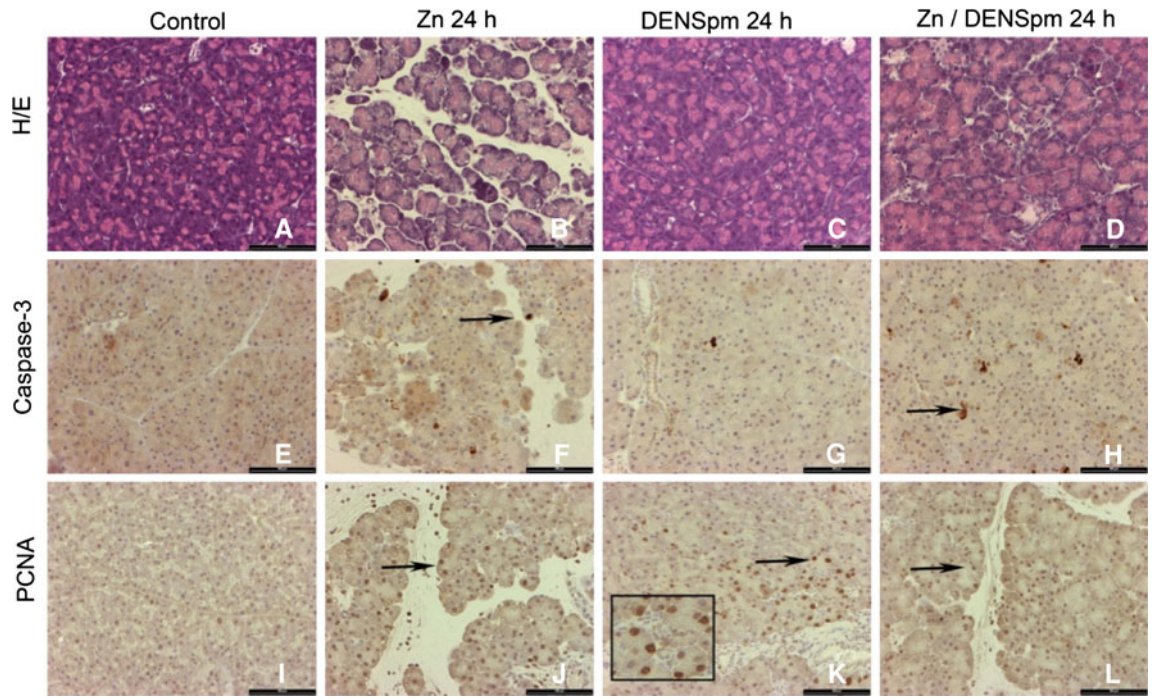

Fig. 3 Effects of DENSpm on zinc-induced pancreatitis. Wild-type mice were subjected to zinc-induced pancreatitis and pancreatic samples were stained with hematoxylin and eosin (**a**–**d**) or detected by immunohistochemistry for apoptosis with caspase-3 (**e**–**h**) or for cell proliferation with PCNA (**i**–**l**) antibodies. DENSpm protected against zinc-induced cell damage (**d**) and induced proliferation of acinar cells (**k**). Samples from control mice (**a**, **e**, **i**) and samples from mice 24 h after zinc (**b**, **f**, **j**), DENSpm (**c**, **g**, **k**) and zinc + DENSpm (**d**, **h**, **l**) are shown. The *arrows* indicate the immunopositive cells. Original magnification of ×200, *scale bar* 100 μm

expressing cells, e.g. neutrophils, by interacting with the α9-cytoplasmic domain (Chen et al. 2004; deHart et al. 2008). SSAT also indirectly regulates the expression of hypoxia-affected genes by affecting the ubiquitination and degradation of hypoxia-inducible factor-1α (HIF-1α) which regulate gene transcription in response to changes in O_2 availability in the cell (Baek et al. 2007). Thus, further studies are needed to uncover the partly controversial role of SSAT in association with pancreatitis.

Polyamines are essential for all living cells and they are associated with multiple different functions. Cationic polyamines bind negatively charged cellular macromolecules and through these interactions modulate chromatin confirmation, gene expression and membrane stability. The amount of free polyamines in the cell is low and whole-cell polyamine homoeostasis is tightly regulated through biosynthesis, catabolism and membrane transport. Polyamine catabolism and concomitant changes of intracellular polyamine pools have been seen to participate in pathologies of several human diseases, including cancer and pancreatitis (Casero and Pegg 2009). In case of pancreatitis, the loss of polyamines has been suggested to impair membrane stability and thus, increase the cellular damage. Since MT-SSAT rat model the changes in polyamine metabolism has been observed in taurodeoxycholate- and L-ornithine-induced experimental pancreatitis (Biczo et al. 2010; Jin et al. 2008a). Also in zinc-induced pancreatitis the depletion of pancreatic spermidine and spermine pools was evident. Although the SSAT activity in pancreas increased, it was apparently not the primarily cause for intracellular polyamine depletion since the pancreatic polyamines decreased during pancreatitis also in SSAT-deficient mice. One reason for the depletion could be the action of the polyamine metabolic enzyme spermine oxidase that can catabolize spermine to spermidine and thus affect the spermine concentration. Enhanced export can likewise diminish the intracellular polyamine pools.

The stable spermine analog DENSpm attenuated the symptoms of zinc-induced pancreatitis. In the acinar cells, DENSpm may be able to fulfill some of the functions of natural polyamines and thus diminish the cellular damage during pancreatitis. We also observed increased proliferation of pancreatic cells in DENSpm-treated mice. Accumulation of the analog into the cells increases the total amount of intracellular polyamines and this may contribute to cell growth by offering essential components for cell proliferation. In this study, the potential ability of DENSpm to induce factors involved in proliferation may protect the cells against pancreatitis associated necrosis. In general, DENSpm is considered as a novel anti-cancer drug and has progressed to phase II clinical trials (Streiff and Bender 2001; Wolff et al. 2003). However, it was observed

recently that DENSpm increased cell proliferation at low concentrations in human melanoma cells resistant to the antiproliferative function of DENSpm (Minchin et al. 2006). This result supports our finding of DENSpm-enhanced proliferation detected in a living organism.

In the present study, we show that intraperitoneal injection of heavy metal zinc can induce acute necrotizing pancreatitis offering a new putative mouse model for the disease. Pancreatic spermidine and occasionally spermine polyamines were depleted during the zinc-induced pancreatitis without the catabolic activity of SSAT. Stable spermine analog DENSpm enhanced acinar cell proliferation in mouse and during zinc-induced pancreatitis the replacement of natural spermine pool with DENSpm attenuated the severity of the disease. In addition, whole-body SSAT overexpression in SSAT mice seemed to possess a partially protective role against the zinc-caused pancreatic damage with a mechanism to be characterized in the future. These results highlight the significance of polyamine metabolism in acute necrotizing pancreatitis.

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