Attenuation of Zinc-Induced Acute Pancreatitis by Zinc Pretreatment: Dependence on Induction of Metallothionein Synthesis

Noriko Tetsuchikawahara, Kyong-Son Min,* and Satomi Onosaka

Faculty of Nutrition, Kobe Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan

(Received January 31, 2005; Accepted February 5, 2005; Published online February 18, 2005)

Pretreatment with zinc (Zn) is known to produce tolerance to several of the toxic effects of heavy metals and pro-oxidants with or without the induction of metallothionein (MT) synthesis. We found previously that injection of high-dose Zn caused acute pancreatitis in mice. An injection of Zn (50–75 mg/kg) resulted in a significant increase in the plasma activities of exocrine enzymes, indicating acute pancreatitis, and no greater increase in Zn concentration in the pancreatic MT fraction with Zn doses. To clarify the role of MT in the pancreatic toxicity of Zn, we examined the effects of pretreatment with nontoxic doses of Zn on the induction of acute pancreatitis by Zn challenge. Pretreatment with Zn (10 mg/kg) 24 hr before Zn challenge attenuated Zn-induced acute pancreatitis. However, pretreatment with Zn only 2 hr before challenge was ineffective. Pretreatment with Zn 24 hr before resulted in more Zn in the MT fraction of pancreatic cytosol and less Zn in the low-molecular-weight fraction compared with pretreatment 2 hr before. These data indicate that pancreatic MT may diminish the toxicity of Zn by sequestering excess Zn. Moreover, Zn-induced acute pancreatitis also was attenuated by treatment with aprotinin, a trypsin inhibitor. Our findings suggest that the Zn ion — either free or bound to small molecules — is involved in the development of acute pancreatitis that includes intrapancreatic trypsinogen activation.

Key words —— zinc toxicity, acute pancreatitis, metallothionein, trypsin inhibitor

*To whom correspondence should be addressed: Faculty of Nutrition, Kobe Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan. Tel.: +81-78 -974-1551; Fax: +81-78-974-5689; E-mail: min@nutr.kobegakuin.ac.jp

INTRODUCTION

It is well known that zinc (Zn) plays essential roles in almost all aspects of metabolism. Its functions include structural and catalytic roles in metalloenzymes and other metalloproteins, as well as regulatory roles in such diverse processes as synaptic signaling and gene expression.[1] As a result, Zn deficiency induces disorders of the skin and neurological, immune, and reproductive systems. However, we demonstrated that an injection of high-dose Zn (50–300 mg/kg) caused acute pancreatitis in mice.[2] In these animals, cell damage, including fibrosis and necrosis, occurred only in the pancreatic exocrine cells, not the endocrine cells, after injection of Zn.[3] Zn toxicity with pancreatic acinar necrosis also occurs in the piglet, chick and dog.[4–6] Moreover, acute pancreatitis and a high pancreatic Zn concentration followed massive ingestion of coins in a schizophrenic patient.[7] Although the Japanese Ministry of Health, Labor and Welfare has set a limit for the daily dosage of Zn in 2004, it can be easy to ingest excess supplemental Zn. Therefore, it is important to clarify the mechanism of pancreatic toxicity of Zn.

The pancreas has been suggested to play a key role in Zn homeostasis, because nearly as much Zn is released in bile-pancreatic secretions as is absorbed by the intestine under normal conditions.[8,9] The concentration of metallothionein (MT) is higher in the pancreas than in other tissues, such as the liver.[10] After treatment, most of the Zn provided binds to pancreatic MT.[11] MTs are small, cysteine-rich metal-binding proteins. The MT-I and -II genes, whose transcription is rapidly induced by Zn, are expressed in major organs, including the liver and pancreas, and MT may play a central role in Zn homeostasis.[12] Immunocytochemical study demonstrated pancreatic MT in acinar cells but not islet cells in both control

and Zn-treated mice.[13] However, it is as yet unclear how pancreatic Zn and MT are associated with the development of acute pancreatitis after injection of high-dose Zn. Pretreatment with Zn is known to produce tolerance to several of the toxic effects of Cd.[14] This tolerance has been attributed to an increase in MT levels. However, Zhou *et al*. have reported the occurrence of MT-independent Zn protection from alcoholic liver injury.[15] On the other hand, acute pancreatitis generally is believed to be triggered by activation of pancreatic trypsinogen. This activated form of trypsin initiates the activation of other digestive zymogens, leading to autodigestion of the pancreas. The activation of intrapancreatic trypsinogen is an early event of pancreatitis in experimental models of acute pancreatitis induced by an ethionine-supplemented diet, taurocholate, and the cholecystokinin analog cerulein.[16] Therefore, we investigated the effects of pretreatment with Zn, which enhances pancreatic MT synthesis, or a trypsin inhibitor on acute pancreatitis induced by Zn challenge.

MATERIALS AND METHODS

Chemicals —— Most chemicals were purchased from Nakarai Tesque, Inc. (Kyoto, Japan). Aprotinin, amylase B and lipase color tests were purchased from Wako Pure Chemicals (Osaka, Japan).

Animals —— Male mice (ddY strain) 6 weeks old and weighing approximately 30 g each were purchased from Nihon SLC (Shizuoka, Japan). Mice were maintained on a 12 : 12-hr light : dark cycle and given food and water ad libidum (Type MF, Oriental Yeast, Osaka, Japan). All animal experiments were completed under the control of the Animal Research Committee, in accordance with the Guidelines on Animal Experiments of Kobe Gakuin University and the Japanese Government Animal Protection and Management Law (No. 105).

Injection of Zn at Various doses —— Mice received a single subcutaneous injection of zinc sulfate (dose range, 10 to 100 mg Zn/kg). At the indicated times, mice (4 animals per group) were euthanized, and heparinized blood was collected from the posterior vena cava to assay for amylase and lipase activities.

Pretreatment with Aprotinin or Zn —— Mice were pretreated with 10 mg Zn/kg at 2 or 24 hr before Zn challenge (75 mg/kg). Other mice were injected twice intravenously with aprotinin (10000 kallikrein-inhibiting unit (KIU)/dose), a trypsin inhibitor, 1 hr before and 1 hr after Zn challenge. At 16 hr after challenge, mice were euthanized, and heparinized blood was collected. After digestion of whole pancreas with nitric acid, Zn concentration was analyzed by using flame atomic absorption spectrophotometry (AAS; Z-5000 Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Tokyo, Japan).[17]

Gel Filtration —— Whole pancreas (about 0.25 g) was homogenized in 2.25 ml of 50 mM Tris–HCl buffer (pH 8.0) and then centrifuged at 105000 *g* for 1 hr at 4°C. The resulting supernatant fraction (1.5 ml) was applied to a 1.5 × 48-cm Sephadex G-75 column (Amersham Biosciences, Piscataway, NJ, U.S.A.) equilibrated with Tris–HCl buffer (50 mM, pH 8.0) at 4°C; the column was eluted with the same buffer. The concentration of Zn in each fraction (3 ml) was measured using AAS.

Assay for Activity of Amylase and Lipase in Plasma —— The activities of amylase and lipase in plasma were measured using amylase B and lipase color tests (Wako Pure Chemicals), respectively.

Statistical Analysis —— Results are expressed as mean ± 1 standard deviation. Data were analyzed for significance by using Student's *t*-test and a two-tailed analysis of variance. Differences were considered significant at $p < 0.05$.

RESULTS

Chemical form of Pancreatic Zn after Injection of Zn

Figure 1 shows plasma activities of exocrine enzymes after an injection of high-dose Zn. Injection of Zn at doses higher than 50 mg/kg markedly increased both plasma amylase and lipase activities (Fig. 1A). These activities peaked after 16 to 24 hr (Fig. 1B). These data indicate that Zn caused injury to pancreatic exocrine cells, resulting in acute pancreatitis. Figure 2 shows the distribution of Zn within pancreatic cytosol, as shown by representative gel-filtration elution profiles. The concentration of Zn in the high-molecular-weight (HM) fraction did not increase with Zn doses. An injection of high-dose Zn (50–75 mg/kg), which induced acute pancreatitis, resulted in no greater increase in Zn concentration in the MT fraction and an increase in the concentration of Zn in the low-molecular-weight (LM) fraction.

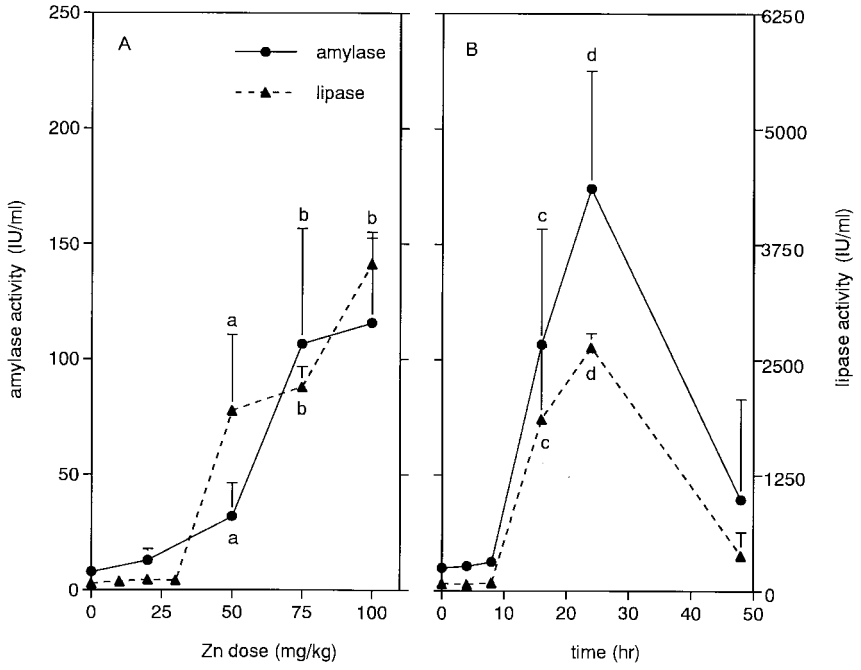

Fig. 1. Activities of Plasma Amylase and Lipase after Injection of Zn

(A) Plasma activities of endocrine enzymes after injection of Zn at various doses. (B) Time course of plasma activities of endocrine enzymes after injection of Zn at 75 mg/kg. Each mouse received a single subcutaneous injection of Zn (10 to 100 mg Zn/kg). After the times indicated, plasma was obtained and assayed for the activities of both endocrine enzymes. Data points represent the mean ± 1 standard deviation for 4 animals. a, $p < 0.05$; b, $p < 0.01$ *versus* 0 mg Zn/kg group. c, $p < 0.05$; d, $p < 0.01$ *versus* 0-hr group.

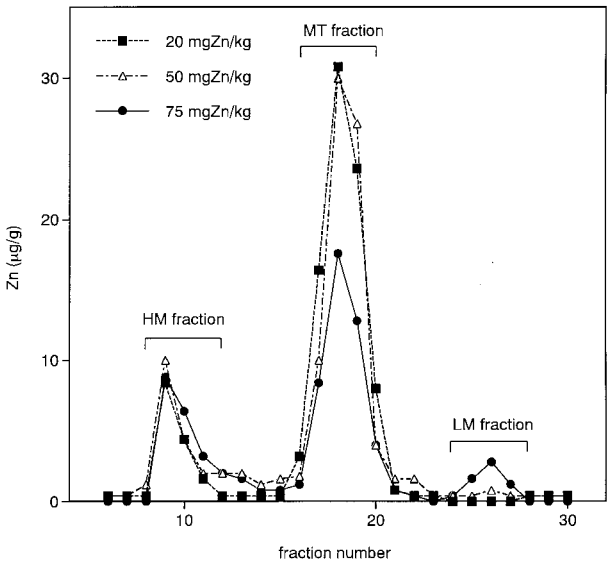

Fig. 2. Representative Gel-Filtration Elution Profiles of Zn in Pancreatic Cytosol 16 hr after Injection of Zn at Various doses

Pancreatic cytosols were prepared from mice treated with the doses shown in Fig. 1A and applied to a Sephadex G-75 column as described in MATERIALS AND METHODS. The concentration of Zn in each fraction was determined by atomic absorption spectroscopy. HM fraction, high-molecular-weight fraction; MT fraction, metallothionein-containing fraction; LM fraction, low-molecular-weight fraction.

Effects of Pretreatment with Aprotinin or Zn on Acute Pancreatitis

Table 1 shows the effects of pretreatment with aprotinin or a nontoxic dose of Zn (10 mg/kg) on acute pancreatitis induced by challenge with high-dose Zn (75 mg/kg). Pretreatment with aprotinin, trypsin inhibitor, significantly ($p < 0.05$) suppressed an increase in plasma amylase activity by Zn challenge. Pretreatment with Zn 24 hr before challenge also attenuated Zn-induced acute pancreatitis. Treatment with Zn 2 hr before challenge did not have an attenuating effect. Table 2 is a compilation of the gel-filtration data on pretreatment with Zn. Pretreatment 24 hr before challenge resulted in a marked increase in pancreatic Zn concentration compared with 2 hr before challenge, even though the total dose of Zn (pretreatment + challenge) was the same. Pretreatment with Zn 24 hr before challenge resulted in a higher concentration of Zn in the MT fraction of pancreatic cytosol and a lower concentration in the LM fraction compared with pretreatment 2 hr before. These data indicate that pancreatic MT may diminish the toxicity of Zn by sequestering excess Zn. Conversely, Zn in the non-MT fractions — excluding Zn bound to the HM fraction but including Zn ion and Zn bound to the LM fraction — partici-

Table 1. Effect of Pretreatment with Nontoxic doses of Zn and Trypsin Inhibitor on Acute Pancreatitis by Zn Challenge

Pretreatment	Zn challenge (75 mg/kg)	Plasma amylase (IU/ml)
none	none	9.08 ± 0.70
none	+	61.64 ± 10.20[a)]
Zn 2 hr before challenge	+	40.98 ± 20.52
Zn 24 hr before challenge	+	12.04 ± 4.19[b)]
aprotinin	+	11.01 ± 0.88[b)]

Mice were pretreated with 10 mg Zn/kg at 2 or 24 hr before challenge with 75 mg Zn/kg. Aprotinin (10000 KIU/dose, twice), a trypsin inhibitor, was given to mice 1 hr before and 1 hr after Zn challenge. At 16 hr after challenge, plasma was obtained and assayed for amylase activity. Data points represent the mean ± 1 standard deviation for 4 animals. *a*) $p < 0.01$ *versus* untreated control group. *b*) $p < 0.01$ *versus* nonpretreated group.

Table 2. Effects of Pretreatment with a Nontoxic dose of Zn on Intracellular Distribution of Zn after Zn Challenge

		Concentration of Zn (μg/g)			
			Cytosol		
Pretreatment (hr before challenge)	Zn challenge	Whole pancreas	HM fraction	MT fraction	LM fraction
none	none	38.04 ± 1.45	18.93 ± 0.61	13.20 ± 1.39	0.40 ± 0.40
none	+	78.29 ± 18.66[a)]	17.40 ± 3.98	34.72 ± 9.78[a)]	8.95 ± 4.65[a)]
2	+	78.73 ± 22.32	14.10 ± 1.91	41.00 ± 21.13	10.40 ± 7.13
24	+	173.93 ± 7.92[b)]	19.60 ± 5.12	94.80 ± 7.72[b)]	3.50 ± 1.44[b)]

Pancreatic cytosols were prepared from mice as shown in Table 1 and applied to a Sephadex G-75 column as described in MATERIALS AND METHODS. The concentration of Zn in each fraction was determined by AAS. Data points represent the mean ± 1 standard deviation for 4 animals. HM fraction, high-molecular-weight fraction; MT fraction, metallothionein-containing fraction; LM fraction, low-molecular-weight fraction. *a*) $p < 0.01$ *versus* untreated control. *b*) $p < 0.01$ *versus* nonpretreated Zn-challenged group.

pates in acute pancreatitis initiated by trypsinogen activation.

DISCUSSION

The pancreas is an important tissue in Zn homeostasis and contains higher concentrations of MT than does the liver.[11)] Zn is an essential metal with numerous biological functions. Our previous work found that injection of high-dose Zn causes acute pancreatitis,[2)] but the mechanism is unclear as yet. In the present study, we demonstrated clearly that pretreatment with a nontoxic dose of Zn significantly attenuated acute pancreatitis induced by high-dose Zn. This induction of tolerance to Zn pancreatic toxicity by pretreatment with Zn appears to involve the induction of pancreatic MT synthesis.

It is well known that pretreatment with Zn produces tolerance to the toxic effects of heavy metals and pro-oxidants.[14,15)] The mechanism of tolerance to the toxicities of metals such as Cd has been attributed to increases in the concentration of MT, a stress-inducible protein with a high affinity for metals. Pretreatment with Zn markedly increased the concentrations of both Zn and Zn bound to MT in the pancreas and attenuated acute pancreatitis. This suggests that MT may suppress the toxicity of Zn by sequestering excess Zn, leading to safe accumulation of Zn in the pancreas. Our findings agree with those of other investigators. For example, Kelly *et al*. demonstrated that the incidence of pancreatic acinar cell degeneration was greater in MT-null compared with control mice after treatment with a ramping dosage of Zn.[18)] Pretreatment with Zn to induce pancreatic MT synthesis also attenuated the acute pancreatitis induced by cerulein and taurocholate.[19)] In addition, De Lisle *et al*.[13)] have shown that MT is a normal component of pancreatic secretions and suggest that MT carries Zn out of the pancreas and is responsible for mediating the pancreas-to-intestine portion of this portal system. Ectopic expression of MT-III caused pancreatic acinar cell necrosis in transgenic mice.[20)] Therefore, MT is an impor-

tant component in maintaining the function and Zn homeostasis of the pancreas.

Conversely, we suggest that Zn in the LM fraction — *i.e.*, Zn ion or Zn bound to small molecules in the pancreas — participates in acute pancreatitis. This is because we found that injection of high-dose Zn (50–75 mg/kg), which induced acute pancreatitis, did not further increase the Zn concentration in the MT fraction, but there was an increase in the Zn concentration of the LM fraction. Moreover, pretreatment with Zn 2 hr before challenge — an interval that was inadequate for inducing pancreatic MT synthesis — did not attenuate Zn-induced acute pancreatitis and decrease the concentration of Zn in the LM fraction. Dineley *et al*. suggested that high intracellular free Zn promotes cell death by inhibiting cellular energy production and consequences of cellular Zn overload may include increased production of cellular reactive oxygen species, loss of mitochondrial membrane potential, and reduced cellular ATP levels.[21] Cerulein induces acute pancreatitis with depletion of glutathione and an increase in lipid peroxidation.[19] Together, these data suggest that oxidative stress is implicated in the development of both Zn- and cerulein-induced acute pancreatitis.

We showed here that pretreatment with aprotinin, a trypsin inhibitor, suppressed Zn-induced increases in plasma amylase activity. This finding suggests that the acute pancreatitis induced by Zn and cerulein are initiated by intrapancreatic activation of trypsinogen. Under physiological conditions, most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens that become activated only after they reach the duodenum. Saluja *et al*. proposed that intrapancreatic activation of trypsinogen, and presumably other zymogens, occurs within acinar cells, and that it is mediated by the lysosomal hydrolase cathepsin B.[22] This theory is supported by the evidence that genetic deletion of cathepsin B partly protects mice from pancreatitis and reduces intrapancreatic activation of trypsinogen, which occurs during pancreatitis.[23]

Our present study suggests that pancreatic MT may diminish the toxicity of Zn by sequestering excess Zn. Conversely, Zn ion or Zn in the pancreatic LM fraction may be involved in the acute pancreatitis that is triggered by activation of intrapancreatic trypsinogen. However, additional aspects of this mechanism remain unclear. Recent works suggest that oxidative stress may be involved in the development of the acute pancreatitis that follows intrapancreatic activation of trypsinogen.[24–26]

Acknowledgements This study was supported by Health Scientific Research Grant (2001) from Kobe Gakuin University.

REFERENCES

1) Cousins, R. J. (1996) Zinc. In *Present Knowledge in Nutrition*, vol. 7 (Filer, L. J. and Ziegler, E. E., Eds.), International Life Sciences Institute Nutrition Foundation, Washington, D.C., pp. 293–306.
2) Onosaka, S., Tetsuchikawahara, N., Min, K. and Kudo, K. (1998) Acute pancreatitis by Zn injection in mice. *Jpn. J. Toxicol. Environ. Health*, **44**, 305–309.
3) Minami, T., Shimane, M., Tanaka, H., Namikawa, K. and Ichida, S. (2001) Pancreatic exocrine damage induced by subcutaneous injection of a low dosage of zinc. *Biol. Trace Elem. Res*., **84**, 169–179.
4) Gabrielson, K. L., Remillard, R. L. and Huso, D. L. (1996) Zinc toxicity with pancreatic acinar necrosis in piglets receiving total parenteral nutrition. *Vet. Pathol*., **33**, 692–696.
5) Lu, J. X., Combs, G. F., Jr. and Fleet, J. C. (1990) Time-course studies of pancreatic exocrine damage induced by excess dietary zinc in the chick. *J. Nutr*., **120**, 389–397.
6) Mikszewski, J. S., Saunders, H. M. and Hess, R. S. (2003) Zinc-associated acute pancreatitis in a dog. *J. Small Anim. Pract*., **44**, 177–180.
7) Bennett, D. R., Baird, C. J., Chan, K. M., Crookes, P. F., Bremner, C. G., Gottlieb, M. M. and Naritoku, W. Y. (1997) Zinc toxicity following massive coin ingestion. *Am. J. Forensic Med. Pathol*., **18**, 148–153.
8) McClain, C. J. (1990) The pancreas and zinc homeostasis. *J. Lab. Clin. Med*., **116**, 275–276.
9) Walsh, C. T., Sandstead, H. H., Prasad, A. S., Newberne, P. M. and Fraker, P. J. (1994) Zinc: health effects and research priorities for the 1990s. *Environ. Health Perspect*., **102**, 5–46.
10) Onosaka, S., Min, K. S., Fujita, Y., Tanaka, K., Iguchi, S. and Okada, Y. (1988) High concentration of pancreatic metallothionein in normal mice. *Toxicology*, **50**, 27–35.
11) Onosaka, S., Tanaka, K. and Cherian, M. G. (1984) Effects of cadmium and zinc on tissue levels of metallothionein. *Environ. Health Perspect*., **54**, 67–72.
12) Bremner, I. (1991) Nutritional and physiologic significance of metallothionein. *Methods Enzymol*., **205**, 25–35.

13) De Lisle, R. C., Sarras, M. P., Jr., Hidalgo, J. and Andrews, G. K. (1996) Metallothionein is a component of exocrine pancreas secretion: implications for zinc homeostasis. *Am. J. Physiol*., **271**, C1103–C1110.
14) Goering, P. L. and Klaassen, C. D. (1984) Zinc-induced tolerance to cadmium hepatotoxicity. *Toxicol. Appl. Pharmacol*., **74**, 299–307.
15) Zhou, Z., Sun, X., Lambert, J. C., Saari, J. T. and Kang, Y. J. (2002) Metallothionein-independent zinc protection from alcoholic liver injury. *Am. J. Pathol*., **160**, 2267–2274.
16) Foitzik, T., Lewandrowski, K. B., Fernandez-del Castillo, C., Rattner, D. W. and Warshaw, A. L. (1994) Evidence for extraluminal trypsinogen activation in three different models of acute pancreatitis. *Surgery*, **115**, 698–702.
17) Min, K. S., Kim, H., Fujii, M., Tetsuchikawahara, N. and Onosaka, S. (2002) Glucocorticoids suppress the inflammation-mediated tolerance to acute toxicity of cadmium in mice. *Toxicol. Appl. Pharmacol*., **178**, 1–7.
18) Kelly, E. J., Quaife, C. J., Froelick, G. J. and Palmiter, R. D. (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J. Nutr*., **126**, 1782–1790.
19) Wang, Z. H., Iguchi, H., Ohshio, G., Imamura, T., Okada, N., Tanaka, T. and Imamura, M. (1996) Increased pancreatic metallothionein and glutathione levels: protecting against cerulein- and taurocholate-induced acute pancreatitis in rats. *Pancreas*, **13**, 173–183.
20) Quaife, C. J., Kelly, E. J., Masters, B. A., Brinster, R. L. and Palmiter, R. D. (1998) Ectopic expression of metallothionein-III causes pancreatic acinar cell necrosis in transgenic mice. *Toxicol. Appl. Pharmacol*., **148**, 148–157.
21) Dineley, K. E., Votyakova, T. V. and Reynolds, I. J. (2003) Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. *J. Neurochem*., **85**, 563–570.
22) Saluja, A. K., Donovan, E. A., Yamanaka, K., Yamaguchi, Y., Hofbauer, B. and Steer, M. L. (1997) Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology*, **113**, 304–310.
23) Halangk, W., Lerch, M. M., Brandt-Nedelev, B., Roth, W., Ruthenbuerger, M., Reinheckel, T., Domschke, W., Lippert, H., Peters, C. and Deussing, J. (2000) Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J. Clin. Invest*., **106**, 773–781.
24) Altavilla, D., Famulari, C., Passaniti, M., Galeano, M., Macri, A., Seminara, P., Minutoli, L., Marini, H., Calo, M., Venuti, F. S., Esposito, M. and Squadrito, F. (2003) Attenuated cerulein-induced pancreatitis in nuclear-factor-kappaB-deficient mice. *Lab. Invest*., **83**, 1723–1732.
25) Sanchez-Bernal, C., Garcia-Morales, O. H., Dominguez, C., Martin-Gallan, P., Calvo, J. J., Ferreira, L. and Perez-Gonzalez, N. (2004) Nitric oxide protects against pancreatic subcellular damage in acute pancreatitis. *Pancreas*, **28**, E9–E15.
26) Cuzzocrea, S., Genovese, T., Mazzon, E., Di Paola, R., Muia, C., Britti, D. and Salvemini, D. (2004) Reduction in the development of cerulein-induced acute pancreatitis by treatment with M40401, a new selective superoxide dismutase mimetic. *Shock*, **22**, 254–261.