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Captopril, an Angiotensin-Converting Enzyme Inhibitor, Attenuates the Severity of Acute Pancreatitis in Rats by Reducing Expression of Matrix Metalloproteinase 9

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CHEN, P., YUAN, Y., WANG, S., ZHAN, L. and XU, J. *Captopril, an Angiotensin-Converting Enzyme Inhibitor, Attenuates the Severity of Acute Pancreatitis in Rats by Reducing Expression of Matrix Metalloproteinase 9*. Tohoku J. Exp. Med., 2006, **209** (2), 99-107 ── It has been reported that matrix metalloproteinase 9 (MMP-9) disrupts basement membrane and increases vascular permeability. MMP-9 therefore might participate in the pathogenesis of severe acute pancreatitis (SAP). Captopril, an angiotensin-converting enzyme inhibitor, could reduce MMP-9 expression. However, the effect of captopril on the outcome of SAP is not ascertained. The aim of this study was to determine whether captopril attenuates the severity of SAP by reducing MMP-9 expression. Thirty Sprague-Dawley rats were randomly divided into 3 groups ($n = 10$ for each). Rats were given intraperitoneal injection of saline (SAP group) or captopril (4 mg/kg) (treated group), and then given retrograde infusion of 5% sodium taurocholate (1.5 ml/kg) into the pancreatic duct under laparotomy to induce SAP. One group of rats, injected with saline, underwent only sham operation (Control). Experimental samples were collected at 24 hrs after the induction of SAP or sham operation. Various markers of severity of SAP, such as serum levels of amylase and trypsinogen activation peptide and the vascular permeability, were increased in rats with SAP, but were significantly decreased in captopril-treated rats ($p < 0.01$). Likewise, the serum MMP-9 levels and expression levels of pancreatic tissue MMP-9 were significantly higher in rats with SAP than those in captopril-treated rats and control rats ($p < 0.01$ for both parameters), but showed no difference between captopril-treated and control rats. These results suggest that captopril may attenuate vascular permeability by reducing MMP-9 expression in SAP, thereby ameliorating severity of the disease. The use of captopril might become a new therapeutic agent for SAP. ──── matrix metalloproteinase-9; captopril; severe acute pancreatitis
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Severe acute pancreatitis (SAP) is an exacerbated local inflammatory reaction, and often associated with systemic inflammatory response syndrome, multiple organ failure, and sepsis (Halonen et al. 2002). Evidence in basic and clinical research suggested that various factors, which included microcirculatory disturbance (Zhou and Chen 2002), interaction of inflammatory cells and endothelial cells (Wittel et al. 2004), bacterial translocation (Samel et al. 2002), and release of inflammatory cytokines (Satol et al. 1999; Bidarkundi et al. 2002; Gomez-Cambronero et al. 2002; Matsuda et al. 2005), were involved in the development of SAP, with its mortality as high as 40% (Raraty et al. 2004). The pathophysiological changes of microcirculatory disturbance in SAP include release of acinar enzymes, increase in vascular permeability, ischaemia, leukocyte infiltration, intravascular coagulation, capillary stasis, and impaired capillary and venous drainage, consequently leading to pancreatic edema and necrosis (Hoffmann et al. 1995; Sunamura et al. 1998). Therefore, microcirculatory disturbance plays an important role in the progression of SAP.

Matrix metalloproteinase 9 (MMP-9) (molecular mass, 92 kDa) is a Zn^{2+}-containing endopeptidase that degrades a wide range of extracellular matrix (ECM) components, including collagen types IV and V, different types of gelatin, fibronectin and elastin (Visse and Nagase 2003). MMP-9 is synthesized by many types of cells, including connective tissues, endothelial, epithelial, and hematopoietic cells, and macrophages (Johnatty et al. 1997; Gibbs et al. 1999). Recent studies have shown that the increased expression of MMP-9 disrupts basement membrane causing increase of vascular permeability (Porter et al. 2004). The up-regulated MMP-9 activates neutrophil and promotes leukocyte-endothelial cell adhesion and consequently, neutrophil trafficking into inflamed tissues, and might further mediate pathological conditions (Fernandez-Patron et al. 2001). Our previous study confirmed that serum MMP-9 level was increased in patients with SAP, related with its clinical deterioration and could be used as a valuable assessment marker for severity of SAP (Chen et al. 2006). These results were consistent with previous studies in SAP, showing that MMP-9 promoted neutrophil migration and alveolar capillary leakage in pancreatitis associated with lung injury in rats, and MMP-9 inhibitor (BB-94) reduced polymorphonuclear leukocyte (PMN) transmigration and SAP development (Keck et al. 2002). So MMP-9 plays a pivotal role in the development and progression of SAP.

Captopril is usually used to treat hypertension and heart failure, as it inhibits the activity of angiotensin-converting enzyme (ACE) (Gavras and Brunner 2001; Smith and Vane 2003). Previous evidence suggested that captopril can also improve the severity of inflammation through the following pathways: First, it inhibited endothelial derived growth factor (EDGF) /nitric oxide (NO) degradation through eliminating oxygen free radicals, and reinforced EDGF/NO function (Soggard et al. 1996; Kanno et al. 2001). Secondly, it improved microcirculatory disturbance through promoting the synthesis of vascular endothelial cell and the release of prostacyclin (Pawlak et al. 2000). Thirdly, it reduced endothelin release to attenuate tissue injury (Plusczyk et al. 1999). Finally, it reduced intracellular Ca^{2+} levels by decreasing Ca^{2+} passage and inhibiting the activity of Ca^{2+} in passing through cell membrane to avoid intracellular Ca^{2+} overloading, thus attenuating cell injury (Krizanova et al. 1997). Recent studies have shown that the renin-angiotensin system (RAS) components were up-regulated in acute pancreatitis which may induce oxidative stress and pancreatic, exocrine secretion (Tsang et al. 2004). Inhibition of RAS by ACE blocker could attenuate pancreatic inflammation (Kuno et al. 2003). On the other hand, captopril could cause acute pancreatitis (Iliopoulou et al. 2001). Therefore, it is necessary to study the effect of captopril on the outcome of SAP.

Williams et al. (2005) suggested that captopril could reduce MMP-9 expression in disease. Moreover, it was reported that inhibition of MMP-9 could reduce local and distant organ injury in acute pancreatitis (Muhs et al. 2003). Therefore, the aim of this study was to demon-

strate the effect of captopril on MMP-9 expression, and its subsequent effect on the severity of SAP, and to provide a new therapeutic agent for SAP.

Materials and Methods

Animals and reagents

Thirty male Sprague-Dawley rats weighing 250-300 g were randomly divided into 3 groups: SAP group (n = 10), captopril-treated group (n = 10), and control group (n = 10). Twenty four hrs prior to the start of the experiments, the rats were deprived of food but allowed access to water. The rats were anesthetized by intraperitoneal injection of pentobarbital (30%, 0.15 ml/100 g). In rats with SAP, SAP was induced by retrograde infusion of 5% sodium taurocholate (1.5 ml/kg) into the pancreatic duct transduodenally via a 24-gauge angiocath using a constant infusion rate of 100 μl/min under laparotomy. The abdominal wounds were closed and the rats were returned to their cages with free access to water and food after surgery. In control group, rats only underwent sham operation. In treatment group, captopril was administered to rats at a dose of 4 mg/kg, dissolved in 0.9% saline (intraperitoneal injection) just before the induction of SAP. An equal volume of saline was administered to control rats and rats with SAP. All rats were sacrificed to collect material at 24 hrs after the onset of induction. The serum was separated from blood by 3,000 rpm, 10 min centrifusion for examination. The experiments were conducted according to the Guidelines of the Shanghai Animal Use and Care Committees and the National Animal Welfare Law. All reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless specifically described.

Histological score of pancreatic injury

Pancreatic tissues were excised and fixed in 4% formaldehyde for histological examination of pancreatitis. Histopathological evaluation was done under light microscopy after sectioning and staining with hematoxylin and eosin. The evaluation method of histological score of pancreatic injury was based on pancreatic tissue necrosis, vacuolization, inflammation and edema by the method previously described by Grewal et al. (1994). Two pathologists scored pancreatic tissue changes.

Pancreatic dry/wet weight ratio

Pancreatic edema was evaluated by measuring water content. A portion of the pancreas was taken immediately after sacrifice, to trim fat and weigh. Pancreatic water content was determined by calculating the wet/dry weight ratio according to the formula ([wet weight − dry weight]/dry weight*%). The initial weight of the pancreas is the wet weight and the weight after incubation at 72°C for 24 hrs is the dry weight.

Examination of vascular permeability

The vascular permeability was evaluated by ratio of Evans blue levels between peritoneal lavage fluid and serum, as well as PMN count of peritoneal lavage fluid (Patterson et al. 1992). Evans blue dye (2%, 1 ml) was administered at 1 hr before sacrifice via caudal vein. The abdominal cavity was lavaged 6 times with phosphate-buffered saline buffer (PBS, 5 ml). Five ml was reclaimed to reach 90% before sacrifice through abdominal cavity puncture site. The Evans blue levels in peritoneal lavage fluid and serum were determined by measuring the absorbance at 620 nm wave length using a spectrophotometer (Model DU530, Beckman Coulter, Inc., Fullerton, CA, USA) and the standard curve of optical density 620 nm (OD_{620}) count was made by the previously described method (Xu et al. 2001). Ratio of Evans blue levels between peritoneal lavage fluid and serum was calculated by the formula (C_S/C_U; C_S: Evans blue level of peritoneal lavage fluid; C_U: Evans blue level of serum). Determination of polymorphonuclear leukocyte (PMN) of peritoneal lavage fluid was done as follows: the peritoneal lavage cells were stained after cytocentrifugation with May-Gruenwald-Giemsa. The relative proportions of PMN were determined by differential count of 1,000 cells.

Biochemilcal assays

The serum amylase level was determined by Beckman CX7 Chemistry Analyzer (Beckman Coulter). Serum MMP-9 level (Endogen, Inc., Woburn, MA, USA) and serum trypsinogen activation peptide (TAP) level (Biotrin International Ltd., Dublin, Ireland) were evaluated by enzyme-linked immunosorbent assay. All procedures were strictly done according to the operation instruction.

Immunohistochemical examination of pancreatic tissue MMP-9

Rat ABC (avidin-biotin-peroxidase complex) staining kit (Boster Biotech, Wuhang, China) was used. 4 μm paraffin embeded pancreatic section was deparaffinized, mounted on poly-L-lysine-coated glass slides,

and rehydrated in PBS. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2/PBS for 10 min. After being immersed in goat serum blocking buffer at 37°C for 1 hrs, the specimens were incubated with mouse anti-rat MMP-9 monoclonal antibody at 4°C overnight. The specimens were incubated with biotin-conjugated goat anti-mouse secondary antibody for 2 hrs at room temperature, and then were incubated with ABC for 30 min. Finally, coloring reaction was made with DAB (3, 3-diaminobenzidine) and haematoxylin nucleus counter staining. Random observation of 5 high -power microscopic views was used to evaluate staining results in each group. Brown staining cells were defined as MMP-9 positive expression cells. Negative (−): no stained particles in cells; Weakly positive (+): rarefied stained particles in cells of single field; Moderately positive (++): rarefied stained particles in cells of 2-4 fields; Strongly positive (+++): stained particles in cells of every field. The ratio between positive expression animal numbers and total animal numbers in each group as pancreatic tissue MMP-9 positive expression ratio was calculated.

Statistical analysis

All values were expressed as mean ± S.E.M. Statistics were done by SPSS program at 11.5 version. The unpaired Student's *t*-test or one-way analysis of variance (ANOVA) was used for comparison. Categorical variables were compared using the Mann-Whitney's U-Wilcoxon Rank sum test. A p value of < 0.05 was considered as statistically significant.

Results

Histological examinations revealed that there were no remarkable pathologic changes in control rats (Fig. 1A). The broad necrosis of acinar cells and interstitial edema were seen in the pancreatic tissue of rats with SAP (Fig. 1B). The pancreatic tissue morphological changes of captopril-treated rats were characterized by slight interstitial edema, but without obvious parenchyma necrosis and

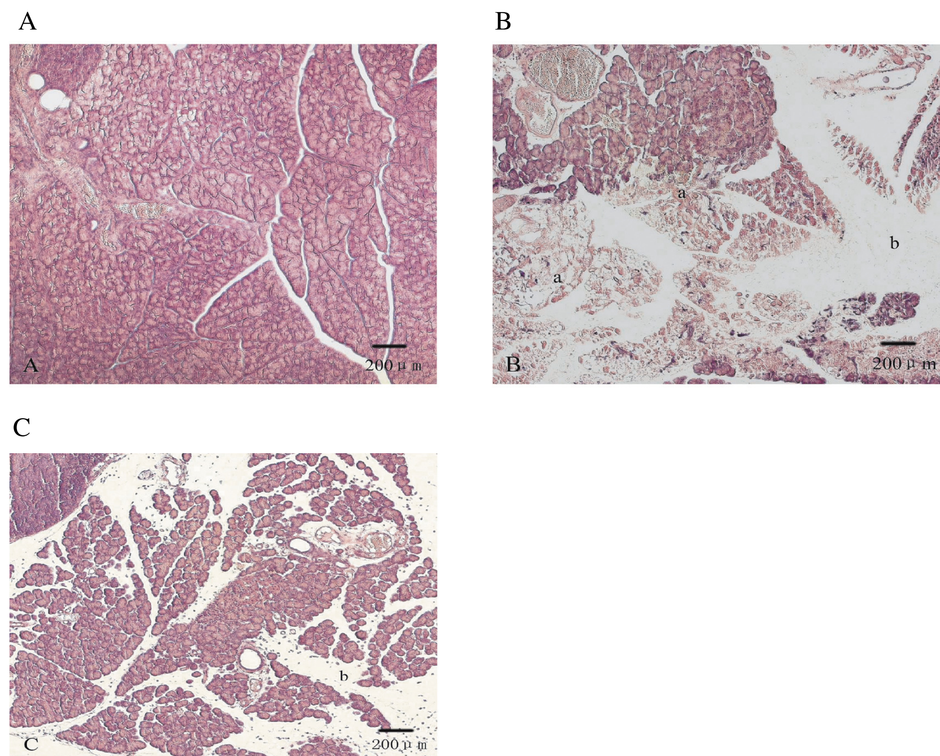

Fig. 1. Histological examination of the pancreas in each group. Shown is Hematoxylin and eosin (H-E) staining.
A: There were no remarkable pathologic changes in control rats. B: The broad necrosis of acinar cells and interstitial edema (b) in rats with SAP were recognized. C: The slight interstitial edema (b) was observed in captopril-treated rats.

TABLE 1. Histological scores of pancreatic injury, pancreatic dry/wet weight ratio, serum amylase and TAP levels (Mean ± S.E.M.).

Groups	SAP (n = 10)	Captopril (n = 10)	Control (n = 10)
Histological score of pancreatic injury	10.6 ± 1.5 [a, b]	5.2 ± 1.3 [a]	2.3 ± 0.8
Pancreatic dry/wet weight ratio (%)	3.20 ± 0.54 [a, b]	2.11 ± 0.41	1.85 ± 0.37
Serum amylase (U/liter)	2,489 ± 449 [a, b]	1,433 ± 602 [a]	685 ± 203
Serum TAP (μmol/liter)	1.712 ± 0.153 [a, b]	1.023 ± 0.113 [a]	0.623 ± 0.158

[a]$p < 0.01$, control group vs other groups; [b]$p < 0.01$, SAP vs captopril-treated rats (Captopril).
TAP, trypsinogen activation peptide.

hemorrhage (Fig. 1C).

The results of the markers for the severity of SAP in each group including pancreatic dry/wet weight ratio, histological score of pancreatic injury, serum TAP and amylase levels, were summarized in Table 1. These results showed that the reflected markers of severity of SAP were significantly decreased in captopril-treated and control animals when compared with those of rats with SAP, respectively ($p < 0.01$). In control group, histological score of pancreatic injury and serum levels of TAP and amylase were lower than those of captopril-treated rats, respectively ($p < 0.01$), but pancreatic dry/wet weight ratio showed no difference between captopril-treated and control groups ($p > 0.05$).

Fig. 2 summarized the changes of serum MMP-9 levels in each group. The serum MMP-9 level was significantly increased in SAP group (758.8 ± 181.4 ng/ml), compared to the levels in captopril-treated (324.4 ± 112.6 ng/ml) ($p < 0.01$) and control rats (227.3 ± 164.5 ng/ml) ($p < 0.01$). Importantly, there was no statistically significant difference in the serum MMP-9 level between captopril-treated and control animals.

The ratio of Evans blue levels between peritoneal lavage fluid and serum and PMN count in peritoneal lavage fluid, which are markers of vascular permeability, showed no difference between captopril-treated and control group, respectively ($p > 0.05$) (Table 2). In contrast, these parameters were significantly increased in SAP group (vs

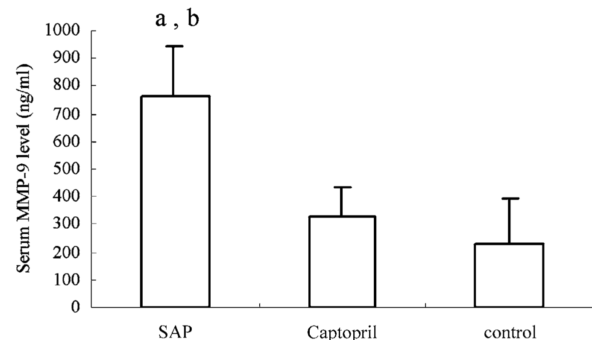

Fig. 2. Serum matrix metalloproteinase 9 (MMP-9) level in each group. (Mean ± S.E.M.) (n = 10). [a]$p < 0.01$, SAP group vs control group; [b]$p < 0.01$, SAP group vs captopril-treated group (captopril).

TABLE 2. Ratio of Evans blue levels between peritoneal lavage fluid and serum, and PMN count of peritoneal lavage fluid (Mean ± S.E.M.).

Groups	SAP (n = 10)	Captopril (n = 10)	Control (n = 10)
Ratio of Evans blue levels between peritoneal lavage fluid and serum (μg%)	22.6 ± 5.0 [a, b]	9.3 ± 4.9	5.9 ± 4.4
PMN count of peritoneal lavage fluid (× 10^6/liter)	1.245 ± 0.158 [a, b]	0.258 ± 0.124	0.158 ± 0.164

[a]$p < 0.01$, SAP vs control rats; [b]$p < 0.01$, SAP vs captopril-treated rats (Captopril).
PMN, polymorphonuclear leukocyte.

captopril-treated and control groups, $p < 0.01$, respectively).

Expression of MMP-9 was measured by immunohistochemical method in the pancreatic tissue of all animals (Fig. 3) (Table 3). The result showed that MMP-9 was stained in vascular endothelial cells of pancreatic tissue in rats with SAP (Fig. 3B), but there was no remarkable MMP-9 staining in captopril-treated (Fig. 3C) and control rats (Fig. 3A). The positive expression ratio of pancreatic tissue MMP-9 was summarized in Table 3, which showed no difference between captopril-treated (20%) and control rats (10%) ($p > 0.05$). Comparing with SAP group (100%), the difference was significant for both groups (vs captopril-treated and control groups, $p < 0.01$, respectively).

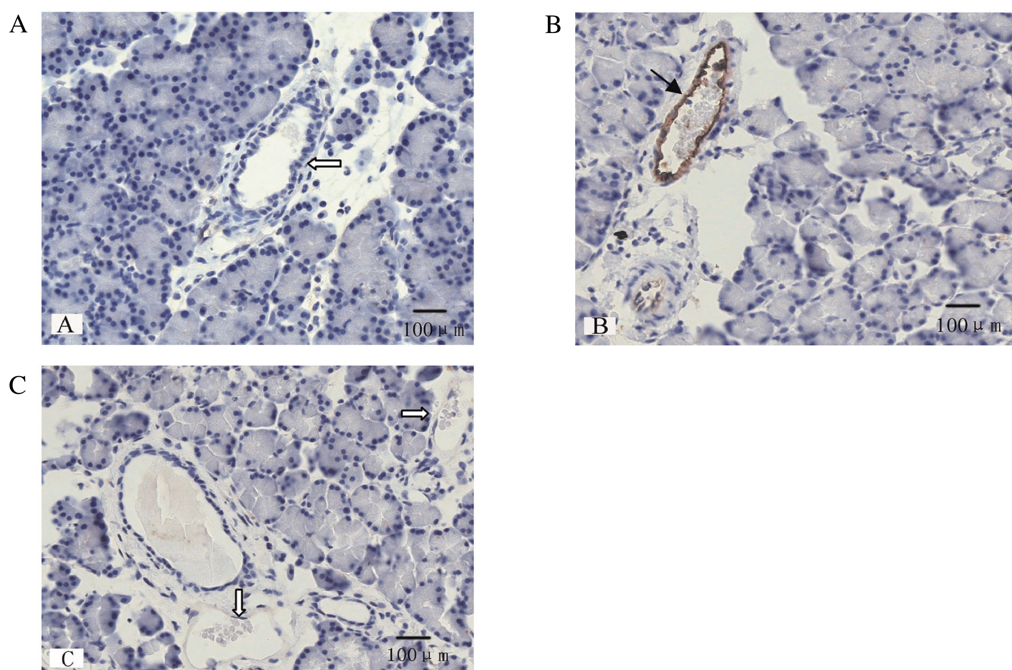

Fig. 3. Immunohistochemical localization of MMP-9 expression in pancreatic tissues.
In the pancreatic tissue of SAP rats (B), MMP-9 was strongly stained in the vascular endothelial cells in pancreatic tissue (solid arrows point). There seems to be no remarkable staining in the vascular endothelial cells in pancreatic tissue in captopril-treated (C) and control rats (A) (open arrows point).

Table 3. Immunohistochemical expression of pancreatic tissue MMP-9 ($n = 10$).

Groups	Expression of pancreatic tissue MMP-9				
	−	+	++	+++	Positive ratio (%)
SAP	0	1	4	5	100 [a, b]
Captopril	8	2	0	0	20
Control	9	1	0	0	10

[a]$p < 0.01$, SAP group vs control group; [b]$p < 0.01$, SAP group vs captopril-treated group (Captopril); p value from Mann-Whitney's U-Wilcoxon Rank sum test.
MMP-9, matrix metalloproteinase 9.

Discussion

The present study has shown that captopril decreases the serum levels of MMP-9 and pancreatic tissue MMP-9 expression in rats with SAP. Captopril, the classical ACE inhibitor, chelates Zn^{2+} in the active site of ACE to inhibit its activity (Cushman and Ondetti 1991). The activation mechanism of MMP-9 is involved in cleaving cysteine-zinc ion (Zn^{2+}) coordinate covalent bond in the interior of protein molecule. Tryggvason et al. (1990) suggested that the pathogenic factors directly or indirectly interrupted cysteine-Zn^{2+} coordinate covalent bond in the interior of protein molecule to cleave cysteine-Zn^{2+} coordinate covalent bond, thus exposing active site to activate MMP-9. Zn^{2+} chelation in active site of MMP-9 could inhibit its activity (Kontogiorgis et al. 2005). For these reasons, Zn^{2+} is absolutely required for MMP-9 activity. Therefore, given that ACE and MMP-9 share ancestory with structural homology (Matsushita et al. 1999), drugs designed to inhibit ACE may have inhibitory activity against MMP-9 (Reinhardt et al. 2002). Indeed, gelatin zymography analysis showed that the wide strap of MMP-9 gradually was narrowed following the gradual increase of captopril dosage (Volpert et al. 1996). But Nakagawa et al. (1995) demonstrated that MMP-9 inhibition by captopril could be reversed by the addition of excess Zn^{2+}. These findings suggested that MMP-9 inhibition by captopril was not cell-type-specific and was mediated by sequestration of the Zn^{2+} at the enzyme's active site. Coker et al. (2001) demonstrated that MMP-9 expression was increased when porcine cardiac myocytes were treated with 1 mM angiotensin II, suggesting that MMP-9 expression may be stimulated by angiotensin II. Thus, it was conceivable that captopril, in addition to direct inhibition of MMP-9, might reduce MMP-9 expression by blocking the stimulation of MMP-9 by angiotensin II.

A next question may arise whether the inhibition of MMP-9 expression by captopril attenuates the severity of SAP. The ratio of Evans blue levels between peritoneal lavage fluid and serum and PMN count of peritoneal lavage fluid, which indicate the extent of degradation in vascular basement membrane and elevated levels of vascular permeability, are correlated with SAP severity (Fujita et al. 2001; Mikami et al. 2002). Moreover, TAP is a small peptide (8 amino acids with a molecular mass of 900 daltons) and is cleaved from the amino-terminal end of trypsinogen during activation. Previous studies showed that the quantitation of TAP in the early phase of acute pancreatitis provides an accurate prediction for severity of the disease (Frossard 2001). Serum amylase level, histological score of pancreatic injury and pancreatic dry/wet weight ratio would reflect the severity of pancreatic damage and are increased in SAP.

The present study indicates that captopril could attenuate the severity of SAP by reducing MMP-9 expression. The inhibition of MMP-9 expression may lead to decreased PMN infiltration and reduced exacerbated inflammatory reaction in SAP, thus attenuating cellular damage and edema in injury tissue (Keck et al. 2002).

Conclusions

This is the first report to provide evidence that captopril attenuates vascular permeability by reducing the expression of MMP-9 in SAP. Captopril ameliorates necrosis of pancreas, interstitial edema, and inflammatory cell infiltration, and plays an important role in preventing the development and progression of SAP. We suggest that captopril might be a new therapeutic agent for SAP.

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