The 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitor, Lovastatin (Statin) Ameliorates CCK-Induced Acute Pancreatitis in Rats

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Statin, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has an anti-inflammatory effect. The aim of this study was to investigate the effect of Lovastatin (statin) on the cholecystokinin-octapeptide (CCK)-induced acute pancreatitis in rats. In statin treated group, the pancreas weight/body weight (pw/bw) ratio in CCK-induced acute pancreatitis was significantly lower than DMSO-treated group. Statin also increased the pancreatic level of HSP 60. Additionally, the secretions of IL-1 β , TNF- α and IL-6 and the lipase levels were decreased in statin treated group. These results suggest that statin may play an important role in mitigating the progression of the inflammatory reactions during acute pancreatitis.

Key words statin; anti-inflammatory effect; acute pancreatitis

Acute pancreatitis (AP) is a clinical entity that is believed to have intracellular activation of digestive enzymes and autodigestion of the pancreas as its central pathophysiologic cause. The noninfectious destruction of pancreatic parenchyma quickly induces an inflammatory reaction at the site of injury. Histologically, AP is characterized by interstitial edema, vacuolization, inflammation and acinar cell necrosis. The diagnosis of AP is based on pancreatic edema index (pancreas weight/body weight) and pancreatic serum enzymes (pancreatic amylase, lipase, immunoreactive trypsin or elastase).^{1,2)}

The induction of the heat shock response enhances the ability of the cells to overcome the effects of the stress.³⁾ The heat shock proteins (HSPs) are involved in the synthesis, degradation, folding, transport, and translocation of proteins.^{4,5)} HSPs have been classified into six families according to their molecular mass. It is well known that the increased expression of HSPs have protective effects against caerulein-induced pancreatitis in rats or against choline-deficient ethionine-supplemented diet pancreatitis model.^{6–8)} Others reported that the pre-induction of HSP expression has a protective effect against caerulein-induced pancreatitis in mice. Many diseases result in increased levels of HSPs. But caerulein or CCK-induced pancreatitis reduces the levels of pancreatic HSPs.^{9,10)}

Cytokines are important immunoregulatory mediators. Their contribution to the pathogenesis of acute and chronic gastroenterological disorders is obvious. Increased expression of interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF)- α can be detected in inflammatory bowel disease. Pro-inflammatory cytokines such as IL-1, TNF- α , and IL-6 are elevated during AP and are considered to be involved in the pathogenesis of pancreatitis-associated multiple organ dysfunction.¹¹⁾ Elevated serum levels of these cytokines have been demonstrated clinically as well as experimentally in different animal models of AP.¹²⁾

Among the neurohormonal regulators, Cholecystokinin (CCK) is a well known gastrointestinal hormone and neural agonist to induce the release of pancreatic digestive enzymes¹³⁾. At supramaximal doses (doses greater than those

that cause maximal secretion of digestive enzymes by the pancreatic acinar cell), CCK is able to cause pancreatic responses.^{14,15)}

Statins, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are the most potent lipid-lowering agents currently available, and are being prescribed in the treatment of hyperlipidemia.^{16,17)} Statins are also effective for the reduction of vascular inflammation. Thus, we set out to investigate the effects of statin as anti-inflammatory agent on AP.

MATERIALS AND METHODS

Reagents Avidin-peroxidase and 2'-AZINO-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablets substrate and CCK-8 were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-HSP60 antibodies were purchased from Stressgen (British Columbia, Canada). Anti-rat IL-6, TNF- α and IL-1 β antibodies were purchased from R&D system (Minneapolis, MN, U.S.A.). Statin was purchased from Sigma (St. Louis, MO, U.S.A.).

Animal Models Male Wistar rats weighing 240–260 g were used. The animals were kept at a constant room temperature of 25 °C with a 12-h light–dark cycle, and were allowed free access to water and standard laboratory chow. The rats were fasted for 16 h before the induction of acute pancreatitis. In each experimental group 4–6 rats were used. The experiments performed in this study were approved by the Animal Care Committee of the university.

In Vivo Models of Pancreatitis Statin-treated group, 1 mg/kg statin was systemically administered *via* the intravenous (i.v.) catheter, followed by 75 μ g/kg CCK subcutaneously three times, after 1, 3 and 5 h. This whole procedure was repeated for 5 d. Due to the extremely poor solubility in aqueous solvents, the statin was dissolved in 100% dimethyl sulfoxide (DMSO) for systemic administration. Whereas acute pancreatitis (AP) group, 1 mg/kg 100% DMSO was systemically administered *via* the intravenous (i.v.) instead of statin, but otherwise the protocol was the same as in treat-

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ment group with statin. Finally, normal untreated group, vehicle drug 1 mg/kg DMSO was administered *via* the i.v. The animals were sacrificed by exsanguinations through the abdominal aorta 12 h after the last CCK injection. Rats were killed for HSP60 determinations. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at -70°C until use. Rats were treated in accordance with the current law and NIH Guide for Care and Use of Laboratory Animals.

Pancreas Weight/Body Weight Ratio Pancreas weight was divided by the weight of the rat.

Western Blotting Western blot analysis of pancreatic HSP60 performed from the cytosolic fraction of the pancreas homogenate. Thirty micrograms of protein were loaded per lane. Samples were electrophoresed on 10% SDS-PAGE according to the method of Laemmli.¹⁸⁾ The gels were transferred to a PVDF membrane for 3 h at 250 mA. Membranes were blocked in 5% Skim milk for 1 h and incubated with anti-HSP60 antibodies. After washing in PBS-tween 20 (PBST) several times, the blot was incubated with secondary antibody for 1 h and the antibody-specific proteins were visualized by the enhanced chemoluminescence (ECL) detection system according to the recommended procedure.

Enzyme-Linked Immunosorbent Assay (ELISA) ELISA for IL-6, TNF- α and IL-1 β were carried out in triplicate in 96-well plates (Nunc, Denmark) coated with each of 100 μl aliquots of anti-rat IL-6, TNF- α and IL-1 β monoclonal antibodies at 1 $\mu\text{g}/\text{ml}$ in PBS at pH 7.4 and was incubated overnight at 4°C . The plates were washed in PBS containing 0.05% Tween-20 (PBST) and blocked with PBST containing 1% BSA for 1 h. After additional washes, standard were added and incubated at 37°C for 2–3 h. After 2–3 h incubation at 37°C , the wells were washed and then each of 0.2 $\mu\text{g}/\text{ml}$ of biotinylated anti-rat IL-6, TNF- α and IL-1 β were added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C . Wells were again washed and ABTS substrate was added. Color development was measured at 450 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant IL-6, TNF- α and IL-1 β in serial dilutions.

Measures of Serum Amylase and Lipase Serum amylase was measured by using ADIVA 1650 (BAYER, U.S.A.). Serum lipase was measured by using a Cobas-mira (Roche, U.S.A.).

Statistical Analysis Results are expressed as means $\pm$ S.E. Differences between the experimental groups were evaluated by using analysis of variance. Values of $p < 0.05$ were accepted as significant.

RESULTS

Effect of Statin on the Pancreas Weight/Body Weight Ratio (pw/bw) Pancreatic edema is a common feature in various models of AP. The pw/bw ratio utilized to evaluate the degree of pancreatic edema. It has been shown that pw/bw is increased in CCK-induced pancreatitis rats.¹⁹⁾ As shown in Fig. 1, statin treated group, pancreas weight/body weight ratio (3.55 ± 0.286) was significantly decreased compared to that of DMSO-treated group (6.46 ± 0.66).

Pancreatic Serum Amylase and Lipase Activities

Fig. 1. Effect of Statin on the Pancreatic Weight/Body Weight Ratio (pw/bw) in CCK-Induced Acute Pancreatitis

Groups were treated as indicated in the Materials and Methods. Means $\pm$ S.E.M. for 5 animals are shown.

Fig. 2. Statin Inhibits Hyperlipidemia on CCK-Induced Pancreatitis in Rats

A and B: serum amylase and lipase levels were measured in control and CCK induced pancreatitis rats with or without statin treatment. CCK was applied as described in the Materials and Methods. Values are means $\pm$ S.E. from at least 6 animals for each group. In lipase measurement, values for animals with statin treatment were significantly lower ($p < 0.05$) than those AP without statin treatment.

Serum amylase and lipase levels are used to confirm the diagnosis of AP.^{20,21)} The serum amylase activity (18640 ± 32.52 U/I) of statin treated group was not significantly different compare to that of DMSO-treated group (17463 ± 76 U/I). However, lipase activity of statin treated group (10.5 ± 0.7 U/I) was significantly lower than that of DMSO-treated group (17 ± 0.57 U/I) (Fig. 2).

Effect of Statin on HSP 60 Expression in CCK-Induced Acute Pancreatitis Many diseases result in increased levels of HSPs. But Strowski *et al.* demonstrated that caerulein-induced pancreatitis reduces the levels of pancreatic HSPs.⁹⁾ And Rakonczay *et al.* also reported that CCK-induced pancreatitis reduces the levels of pancreatic HSPs.¹⁰⁾

Thus, we checked the effect of statin on HSP 60 expression in acute pancreatitis. As shown in Fig. 3, in statin treated group, the expression of pancreatic HSP 60 was markedly increased compare to that of DMSO-treated group.

Effect of Statin on Cytokines Secretions in CCK-Induced Acute Pancreatitis Injections of CCK increased the pancreatic TNF- α and IL-1 β levels over time.^{22–24)} So we examined whether statin has the capacity to reduce the content of several pro-inflammatory cytokines. Pretreatment of CCK-induce AP rats with statin decreased the levels of TNF- α (238 ± 25 pg/ml), IL-1 β (257.7 ± 3 pg/ml) and IL-6 productions (69 ± 2.3 pg/ml) (Fig. 4).

Fig. 3. Effect of Statin on HSP60 Expression in CCK-Induced Acute Pancreatitis.

The figure depicts representative Western blots of protein lysates (30 μ g/lane) from the pancreas of rats.

Fig. 4. Effect of Statin on TNF- α , IL-1 β and IL-6 Secretions in CCK-Induced Pancreatitis.

Rats were treated as indicated in the Materials and Methods. Means $\pm$ S.E.M. for 5 animals are shown.

DISCUSSIONS

AP is characterized by local pancreatic inflammation as well as systemic inflammatory response.^{25,26} There is a therapeutic window between onset of symptoms and the development of distant organ damage in AP, when anti-inflammatory therapy may be of use. AP is diagnosed based on pancreatic edema index (pancreas weight/body weight), pancreatic serum enzymes (e.g. pancreatic amylase, lipase, immunoreactive trypsin or elastase). Pancreatic edema formation is a common feature of AP. Although not recognized in one-fifth of AP patients, serum amylase measurement is still a standard diagnostic method for AP.

Statins are widely prescribed potent lipid-lowering agent in the treatment of hyperlipidemia. Statins have pleiotropic functions, including promotion of vasculogenesis, prevention of bone mass loss, immunomodulatory and anti-inflammatory effects.^{16,17} Statins also modulate the adhesion cascade at multiple points by targeting both the endothelium and leukocytes.^{16,27–35} Statins have been shown to affect cell adhesion by inhibiting chemokine expression (MCP-1) in activated leukocytes and endothelial cells.²⁹ Other studies indicate that statins interfere with the expression of cytokines in endothelial cells (IL-1 β and IL-6) and monocytes (IL-6 and TNF- α) and might thereby inhibit leukocyte adhesion.^{33–35}

From these reports, we set out to investigate the effect of statin on AP. In our study, no difference in serum amylase

was observed between statin-treated and non-statin-treated groups (Fig. 2). This observation is not surprising since serum amylase is not a specific diagnostic marker for AP.^{20,21} It is well known that hyperamylasemia is also observed in many extrapancreatic diseases resulting in depressed specificity. Thus, although not frequently employed for practical reasons, other serum markers such as ribonuclease, chymotrypsin, elastase, phospholipase A, pancreatic isoamylase and urinary amylase have been utilized. Pancreatic lipase is synthesized in the exocrine acinar cells. A wide variation of sensitivities and specificities has been reported for serum lipase determination in diagnosis of AP.²⁶

HSPs play a universal role in the maintenance of cellular homeostasis. They are expressed constitutively and/or at elevated levels upon the exposure of cells to a variety of stress conditions in every organ, including pancreas.³ HSPs not only help the cells to survive the stress conditions by repairing damaged proteins, but also are involved in gene regulation, the synthesis, degradation, folding, transport, and translocation of proteins.^{4,5} It has been shown that the pre-induction of HSP expression has a protective effect against cerulean induced pancreatitis in rats or choline-deficient ethionine-supplemented diet model pancreatitis in mice.^{9,10} This observation suggests that the low levels of pancreatic HSPs might be involved in the development of CCK-induced pancreatitis. Rakonczay *et al.* demonstrated that CCK-induced pancreatitis reduces the levels of pancreatic HSPs.¹⁰ Moreover, an increasing body of evidence from experimental animal studies has documented an essential role of HSPs in the prevention of AP.^{9,10} In accordance with these results, we found that supramaximal dose of CCK reduce the level of HSP 60. The decreased level of CCK, however, was ameliorated by the administration of statin (Fig. 3).

Cytokines (e.g., TNF- α and IL-1 β) are thought to mediate the systemic effects of pancreatitis such as fever, hypotension, and shock.²⁵ Serum levels of TNF- α and IL-1 β were used to evaluate the systemic cytokine response in AP. TNF- α is an important initiator of the local and systemic damage occurring in AP.³⁶ Additionally, serum levels of TNF- α correlate with severity of AP in humans.³⁶ IL-1 is a significant cytokine for the development of AP.³⁶ IL-6, which is one of the principal cytokine mediators of the acute-phase response, was suggested as a marker for predicting the severity of AP.³⁷ Our data showed that statin reduces IL-6, TNF- α and IL-1 β production on CCK-induced AP in rats, suggesting the anti-inflammatory effects of statin on AP (Fig. 4).

In conclusion, this study showed that pretreatment of statin ameliorated the severity of CCK-induced pancreatitis in rats.

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