Calcineurin mediates pancreatic growth in protease inhibitor-treated mice

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Tashiro, Mitsuo, Linda C. Samuelson, Rodger A. Liddle, and John A. Williams. Calcineurin mediates pancreatic growth in protease inhibitor-treated mice. Am J Physiol Gastrointest Liver Physiol 286: G784–G790, 2004—Calcineurin (CN) (also called protein phosphatase 2B), which is uniquely activated by Ca2⁺ and calmodulin (6, 24), plays a critical role in experimental pancreatic amylase secretion, leading to the suggestion that CN may participate in regulation of pancreatic growth by CCK in mice.

Calcineurin mediates pancreatic growth in protease inhibitor-treated mice.

A VARIETY OF GASTROINTESTINAL peptides have been implicated in the regulation of proliferation and differentiation in the mature gastrointestinal tract and the pancreas (30, 56). It is well known that feeding initiates pancreatic protein synthesis and secretion and that adaptation to a high-protein diet results in pancreatic growth (17, 18). More detailed studies have shown that among gastrointestinal peptides, CCK is released in response to dietary protein leading to pancreatic growth. Injection of CCK increases pancreatic wet weight and protein and DNA content in vivo over 4–10 days (8, 37, 40, 43). Oral administration of soybean trypsin inhibitor (5, 44) or the synthetic protease inhibitor camostat (10, 15, 37, 52, 55), as well as pancreaticobiliary diversion (13, 39), increase the release of endogenous CCK into the blood and induce pancreatic growth. Moreover, CCK-A receptor antagonists that block the action of exogenous and endogenous CCK inhibit pancreatic growth (10, 13, 37, 39, 52). Therefore, most of the studies of pancreatic growth have been focused on CCK as a key regulator.

In pancreatic acinar cells, the intracellular mechanisms of enzyme secretion have been well documented. CCK activates the release of intracellular Ca²⁺ and the influx of Ca²⁺ and generates oscillations of intracellular Ca²⁺ concentration (56). Ca²⁺ then acts as an intracellular messenger and initiates the secretion of digestive enzymes. CCK has also been reported to activate a variety of other effector pathways, including protein kinase C, the MAPK cascades, phosphatidylinositol 3-kinase, tyrosine kinases, and phosphatases (50, 51, 56). At present, the intracellular mediator(s) regulating pancreatic growth stimulated by CCK is unclear.

Regulation of cellular phosphoproteins involves protein phosphatases, because the state of protein phosphorylation represents a balance between kinase and phosphatase activity. One of the serine/threonine-specific protein phosphatases is calcineurin (CN) (also called protein phosphatase 2B), which is uniquely activated by Ca²⁺ and calmodulin (6, 24). CN consists of two different subunits, A and B. CN A (a 61-kDa catalytic and calmodulin-binding subunit) is tightly bound to CN B (a 19-kDa calcium-binding subunit homologous to calmodulin). The immunosuppressants cyclosporine A (CsA) and FK506 inhibit CN activity when bound to their intracellular receptors cyclophilin A and FK506-binding protein of 12 kDa (FKBP12), respectively (29, 47). These agents, therefore, can be used as tools to investigate the role of CN in the transduction of Ca²⁺ signals. Pancreatic cytosol contains Ca²⁺-activated protein phosphatase activity and immunoreactive CN (4, 54). Groblewski et al. (20, 21) demonstrated that CCK treatment of dissociated pancreatic acini activated CN in that a specific protein substrate, calcium-regulated heat-stable protein of 24 kDa (CRHSP-24), was dephosphorylated after CCK stimulation, and this effect was blocked by CsA or FK506. These inhibitors also had a modest effect in inhibiting pancreatic amylase secretion, leading to the suggestion that CN may play a role in stimulus-secretion coupling in pancreatic acinar cells (20, 48). Recent studies in other tissues, however, have shown that CN plays a critical role in experimental models of cardiac (34, 36, 45) or skeletal muscle (11, 32, 42) hypertrophy. In the present study, we demonstrate that CN mediates the pancreatic growth induced by oral administration of the protease inhibitor camostat in mice and that endogenous CCK is required for camostat-induced pancreatic growth.

EXPERIMENTAL PROCEDURES

Induction of pancreatic growth by camostat feeding. Male ICR mice (Harlan Sprague Dawley, Indianapolis, IN), weighing ~30 g or

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female CCK-deficient mice on a 129/SV background (26), weighing ~20 g were used in all experiments. The specific pathogen-free animals were housed at 22°C on a 12:12-h light-dark cycle with free access to water and a standard rodent powdered chow (5001 rodent diet, PMI Nutrition International, St. Louis, MO). All studies were approved by the University of Michigan Committee on Use and Care of Animals, and all animals were killed by decapitation under carbon dioxide anesthesia between 8:30 and 10:00 AM.

The control diet group was fed regular powdered chow. For camostat feeding, 0.1% of camostat (provided by Ono Pharmaceutical, Osaka, Japan) was mixed with regular powdered chow. The mixture was fed ad libitum to mice beginning at 9:00 AM after conditioning by regular powdered diet. ICR or 129/SV mice were fed either regular powdered chow or camostat mixture for 1–10 days. Animals treated with CsA or FK506 were injected subcutaneously twice a day with either 15 mg/kg of CsA (Calbiochem-Novabiochem, La Jolla, CA) suspension in vehicle (5% ethanol in 0.9% saline), 3 mg/kg of FK506 (Fujisawa Healthcare, Deerfield, IL) in vehicle (8% ethanol and 2% polyoxy1 60 hydrogenated castor oil in 0.9% saline), or each vehicle alone. Injection with either CsA, FK506, or vehicle began 2 days before switching to diet containing camostat.

Biochemical determinations. After the pancreas was harvested and weighed, each pancreas was homogenized in 4 ml of buffer containing 8 mM NaCl, 0.1% Triton X-100 using a Polytron homogenizer for 20 s and then sonicated for 15 s. Protein concentrations of pancreatic homogenates were determined by protein assay reagent (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard. DNA was measured by Hoechst 33258 dye in the presence of 2.5 M NaCl using calf thymus DNA as a standard. Serum amylose activity was determined by Phadebas Amylase Test (Pharmacia and Upjohn, Uppsala, Sweden).

For determination of plasma CCK, plasma obtained by cardiac puncture was extracted by absorption and elution from C18 Sep-Paks (Waters), and CCK was quantitated by bioassay using amylase secretion from isolated rat pancreatic acini (27, 28). To obtain adequate plasma from individual mice fed camostat could be determined.

Results are means ± SE. The Student’s t-test or one-way ANOVA followed by a post hoc test for multiple group comparisons as appropriate. Differences with P < 0.05 were considered significant.

RESULTS

Effect of camostat on pancreatic growth in mice. Chronic feeding of camostat to mice for 10 days has been reported to induce pancreatic growth (37). We therefore first examined the time course of increases in pancreatic wet weight and protein (hypertrophy) and DNA (hyperplasia) in this model. Mice consumed ~4 g/day of diet resulting in an intake of ~130 mg camostat/kg body wt−1 day−1. In response to camostat, pancreatic wet weight, protein, and DNA were increased after 2 days and reached a maximum in 7–10 days (Table 1). There were no significant differences in body weight between control and camostat-fed groups during the experiment. Camostat feeding to mice did not increase the weights of other organs evaluated including liver, spleen, kidney, and heart (data not shown); oral administration of camostat also has been reported to have no effect on the weight of the intestine (14). Histolog-

Table 1. Body weight, pancreatic wet weight, pancreatic protein content, and pancreatic DNA content in camostat-fed mice

<table>
<thead>
<tr>
<th>Camostat Feeding, days</th>
<th>Body Weight, g</th>
<th>Pancreatic Wet Weight, mg</th>
<th>Wet Weight/Body Weight, mg/g</th>
<th>Protein Content, mg</th>
<th>DNA Content, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>After</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>30.9±2.0</td>
<td>31.4±1.8</td>
<td>390.0±37.5</td>
<td>12.4±0.9</td>
<td>50.9±4.9</td>
</tr>
<tr>
<td>2</td>
<td>36.1±1.0</td>
<td>34.9±0.7</td>
<td>612.6±28.7</td>
<td>17.7±0.7†</td>
<td>66.0±2.0</td>
</tr>
<tr>
<td>4</td>
<td>33.1±2.0</td>
<td>33.3±2.1</td>
<td>749.4±65.6</td>
<td>22.5±1.2†</td>
<td>69.3±6.5†</td>
</tr>
<tr>
<td>7</td>
<td>32.1±2.2</td>
<td>31.3±2.4</td>
<td>838.8±64.5†</td>
<td>27.0±1.5†</td>
<td>78.9±5.6†</td>
</tr>
<tr>
<td>10</td>
<td>32.7±1.3</td>
<td>31.7±1.6</td>
<td>874.8±52.9†</td>
<td>27.6±0.4†</td>
<td>81.0±5.7†</td>
</tr>
</tbody>
</table>

Results are means ± SE from 5 mice. Twenty-five 8-week-old male ICR mice were adjusted to powdered 5001 diet for 3 days when their average weight was 33.1 ± 0.8 g. Ten days later all mice were killed, and each pancreas was removed. Camostat was added to the diet for all except the control group for the last 2, 4, 7, or 10 days. *P < 0.05; †P < 0.01 vs. control.
ical examination of the pancreas after camostat feeding for 10 days showed intact pancreatic acini of normal size with evidence of hyperstimulation, i.e., increased basolateral basophilia, and some vacuolization compared with control acini. Pancreatic islets were of normal size although decreased in frequency per section as would be expected from distribution in a larger mass of exocrine tissue (data not shown). This selective effect on the exocrine pancreas is consistent with prior reports that total insulin content in the rat pancreas was not changed after camostat administration (16, 35).

It is believed that oral administration of protease inhibitor or dietary protein induces release of endogenous CCK, resulting in stimulation of pancreatic growth (17, 18). To confirm the role of endogenous CCK in the pancreatic growth induced by camostat, we fed camostat to CCK-deficient mice for 10 days. As observed in the first experiment, in control mice, chronic camostat feeding markedly increased the pancreatic wet weight-to-body weight ratio and protein and DNA content compared with control chow (Fig. 1). In contrast, chronic camostat feeding to CCK-deficient mice did not increase those three parameters compared with control chow. Therefore, this observation confirms that the effect of camostat is mediated by endogenous CCK in mice.

**Regulation of CN protein and its activity in the pancreas after camostat feeding.** We detected both CN A and B subunits by Western blot analysis using specific antibodies. CN A subunit ran at 61 kDa and CN B at 19 kDa in control pancreas (Fig. 2A). In response to camostat feeding, CN A and B subunits were upregulated (5.7 ± 1.5-fold and 2.8 ± 0.3-fold vs. control, n = 6) relative to total protein. In contrast to the pancreas, the amount of both CN A and B subunits were unchanged in the liver, spleen, kidney, or heart (data not shown). We also examined other proteins thought to play a role in cell growth including ERKs and p38 MAPK. In contrast to CN, these signaling proteins were unchanged in amount in mice fed camostat (Fig. 2A).

Our laboratory (21) previously identified a novel CN substrate, CRHSP-24, which is dephosphorylated by CN in response to CCK in rat pancreatic acinar cells. Thus an increase in the dephosphorylated states of CRHSP-24 indicates CN activation. From 2 to 10 days after camostat feeding, the percentage of CRHSP-24 in the dephosphorylated bands was increased, indicating CN activation in vivo (Fig. 2B). In mice fed camostat, total CRHSP-24 protein was also modestly upregulated in the pancreas (1.8 ± 0.2-fold vs. control, n = 6) when evaluated on SDS gels, although the example in the IEF gel shown in Fig. 2B shows a greater upregulation as early as 2 days.

**Both CsA and FK506 block pancreatic growth induced by camostat.** Because CsA and FK506 are CN inhibitors that depend on distinct intracellular receptors (29, 47), we injected either CsA or FK506 to camostat-fed mice to evaluate the role of CN in pancreatic growth. Neither compound affected body weight. We first used CsA, which binds to cyclophilin A, and the complex then inhibits CN. Camostat feeding for 10 days to animals injected with vehicle significantly increased the pancreatic weight-to-body weight ratio compared with normal chow with vehicle injection (Fig. 3, top). CsA injection with camostat feeding significantly reduced this increase, whereas CsA injection with normal chow had only a slight inhibitory action on the ratio (Fig. 3, top). Camostat feeding with vehicle injection also increased protein content and DNA content compared with normal chow with vehicle injection (Fig. 3, middle and bottom). CsA injection with camostat feeding blocked the increase in these parameters, whereas CsA injection with normal chow had no effect compared with vehicle injection with normal chow.

We also studied FK506, because it binds to a distinct receptor, FKBP12, with the complex inhibiting CN. In this study, camostat feeding with vehicle injection also stimulated pancreatic wet weight-to-body weight ratio, protein content, and DNA content compared with normal chow with vehicle injection (Fig. 4). FK506 injection with camostat feeding also greatly reduced the increase in the parameters, resulting in the same levels as vehicle injection with normal chow. As with CsA injection, the effect on pancreatic wet weight-to-body weight ratio was not complete, whereas the effect on protein and DNA content was complete. FK506 injection with normal chow had no effect on the parameters compared with vehicle injection with normal chow.

![Fig. 1. Requirement of CCK for camostat-induced growth of the mouse pancreas. CCK-deficient (CCK-/–) or genetically matched control 129/Sv (CCK+/+) mice were randomly assigned to two different groups and fed either regular powdered chow or camostat mixture for 10 days. Graphs show the means ± SE from 5–6 animals for each condition. *P < 0.05; **P < 0.01 vs. no camostat.](http://ajpgi.physiology.org/doi/abs/10.1152/ajpgi.00231.2003)
Histological examination showed no pancreatic damage, edema, cellular infiltration, or fibrosis induced by either CsA or FK506 in the absence or presence of camostat (data not shown). Serum amylase levels also showed no consistent difference compared with vehicle injection (data not shown). Thus the dose of CsA or FK506 used in this study had no effect to induce pancreatitis or pancreatic damage.

Neither CsA nor FK506 blocks releases of endogenous CCK by camostat. We examined the time course of plasma CCK levels during camostat feeding. In response to camostat, plasma CCK was increased after 1 day and remained elevated over the 10-day period (Fig. 5A). To confirm that the immunosuppressants do not block the release of endogenous CCK induced by camostat feeding, we pretreated with either vehicle (CsA or FK506) for 2 days and then fed camostat mixture for 2 days. Both CsA and FK506 slightly increased basal and camostat-induced CCK levels without any indication of inhibition (Fig. 5B).

DISCUSSION

In this study, we present evidence that oral administration of the protease inhibitor camostat stimulated pancreatic growth that is mediated by the gastrointestinal hormone CCK. The camostat-induced pancreatic growth requires the Ca\textsuperscript{2+}-activated protein phosphatase CN and is accompanied by selective upregulation of CN A and B subunits.
That camostat-induced pancreatic growth is mediated by CCK is not surprising given the wealth of data that CCK injection can induce pancreatic growth and the fact that most studies have shown that CCK receptor antagonists block the growth response to increasing endogenous CCK by trypsin inhibitor. However, less data exist for mice than rats, and in a recent study, a high-protein diet (containing 78.4% protein) was found to induce pancreatic growth in CCK-deficient mice similar to that seen in control mice compared with control diets (26), thus indicating the possibility of alternative mediators. In our study, camostat was added to the same control diet in powdered form but had no effect in CCK-deficient mice.

Although camostat feeding may also increase the release of endogenous secretin (49), it is clear that it is CCK that is required for camostat-induced growth. The trophic effect of CCK in rats appears primarily to be through high-affinity CCK receptors (7) located on the acinar cells (53). The effect of the high-protein diet may be mediated by another hormone or neural pathway but also may be mediated by amino acids from the dietary protein. Branched-chain amino acids, especially, are known to be effective stimulators of protein synthesis (1). Because oral administrations of amino acids are not significant stimulators of CCK release (26), it is likely that normal pancreatic growth requires both CCK and amino acids and that the amino acid composition of the normal diet was clearly sufficient.

A major finding of this study is that pancreatic growth induced by endogenous CCK can be blocked with two chemically distinct immunosuppressants whose actions are known to be mediated by inhibiting CN. Both CsA and FK506 bind to distinct intracellular proteins, cyclophilin A and FKBP-12. The ligand-binding protein then binds CN, contacting both subunits and preventing access of protein substrates to catalytic residues (23). Although a high concentration of CsA can affect mitochondrial ion permeability, FK506 does not have this action (12). The concentrations of the two agents we used are similar to those used to inhibit cardiac hypertrophy in vivo (34, 45). Moreover, minimal changes in serum amylase level or pancreatic histology were seen after administration of CsA or FK506, making a toxic effect on the pancreas unlikely. Previous reports (9, 48) suggest the potential harmful effect of these agents in rat pancreas. These differences may occur from...
species, doses, or administration route. Because we are using a pancreatic growth paradigm involving release of endogenous CCK, it was important to confirm that the immunosuppressants are not blocking endogenous CCK release. We therefore measured plasma CCK levels and showed that the increase in CCK induced by camostat was not affected by either CsA or FK506.

The mechanism by which CN mediates growth of the pancreas remains to be established. CN is activated during camostat feeding as a result of the action of CCK to increase intracellular Ca$^{2+}$ (56). That this was occurring in our study is shown by the dephosphorylation of the CN substrate CRHSP-24. However, the function of CRHSP-24 is unknown. A major known action of CN is to dephosphorylate the nuclear factor of activated T cells (NFAT) (38). NFAT is normally in the cytoplasm, but on dephosphorylation it enters the nucleus and binds to DNA along with other transcription factors and in T cells activates production of interleukin-2. Although NFAT was originally identified in T cells, multiple isoforms are now known to be broadly distributed (22, 38). One mechanism by which CN signaling initiates cardiac hypertrophy is through NFAT3 because transgenic mice expressing constitutively active CN or NFAT3 in cardiac myocytes showed cardiac hypertrophy (34). In this case, NFAT3 was believed to interact with another transcription factor, GATA4. Such a mechanism could also explain the action to induce pancreatic growth, because NFAT isoforms are expressed in the pancreas (38), although whether in acinar cells and which isoform is unknown.

A different possible mechanism could involve a role for CN in protein synthesis. CCK is known to enhance pancreatic protein synthesis in isolated pancreatic acini (25), and both exogenous and endogenous CCK activate various translational mechanisms to enhance protein synthesis (2, 3). In a brief report, Loser et al. (31) reported that CsA inhibited the induction of ornithine decarboxylase (ODC) in camostat-treated rats. Polyamines have long been known to be required for cellular transformation, and a specific serine protease inhibitor on the rat pancreas: in vitro influence of camostat on the endocrine pancreas. Scand J Gastroenterol 31: 128–135, 1995.


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