CCK-induced pancreatic growth is not limited by mitogenic capacity in mice

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Crozier SJ, Sans MD, Lang CH, D’Aleyc LG, Ernst SA, Williams JA. CCK-induced pancreatic growth is not limited by mitogenic capacity in mice. Am J Physiol Gastrointest Liver Physiol 294: G1148–G1157, 2008. First published March 20, 2008; doi:10.1152/ajpgi.00426.2007.—In mice fed trypsin inhibitor (camostat) to elevate endogenous CCK, pancreatic growth plateaus by 7 days. It is unknown whether this represents the maximum growth capacity of the pancreas. To test the ability of CCK to drive further growth, mice were fed chow containing camostat (0.1%) for 1 wk, then fed standard chow for 1 wk, and finally returned to the camostat diet for a week. Pancreatic mass increased to 245% of initial value (iv) following 1 wk of camostat feeding, decreased to 147% iv following a 1 wk return to normal chow, and increased to 257% iv with subsequent camostat feeding. Camostat feeding was associated with significant increases in circulating CCK and changes in pancreatic mass were paralleled by changes in protein and DNA content. Moreover, regression of the pancreas following camostat feeding was associated with changes in the expression of the autophagosome marker LC3. Pancreatic protein synthetic rates were 130% of control after 2 days on camostat but were equivalent to control after 7 days. Changes in the phosphorylation of 4E-BP1 and S6, downstream effectors of mammalian target of rapamycin (mTOR), paralleled changes in protein synthetic rates. Cellular content of Akt, an upstream activating kinase of mTOR, decreased after 7 days of camostat feeding whereas expression of the E3 ubiquitin-ligases and the cell cycle inhibitor p21 increased after 2 days. These results indicate that CCK-stimulated growth of the pancreas is not limited by acinar cell mitogenic capacity but is due, at least in part, to inhibition of promitogenic Akt signaling.

pancreatic acinar cell; protein metabolism; cholecystokinin

GASTROINTESTINAL HORMONES can regulate cell cycle progression (mitogenesis) and cellular hypertrophy of exocrine acinar cells of the pancreas. In particular, the hormone cholecystokinin (CCK) has been implicated in stimulating acinar cell growth (22). Exogenous CCK administration stimulates pancreatic growth in rodents (9, 30) and cell division in acinar cell cultures (23). In addition, feeding an oral trypsin inhibitor is associated with pancreatic growth (6, 26, 28, 42) concomitant with increased concentrations of circulating CCK (15, 21). Furthermore, oral trypsin inhibitor-induced pancreatic growth is abated by coadministration of CCK antagonists (46) and is absent in CCK-deficient (42) or CCK-A receptor-deficient mice (36).

In the exocrine pancreas, CCK binds to its receptor, stimulates intracellular Ca2+ release (45), and thereby activates the protein phosphatase calcineurin (4, 17). Calcineurin activation is required for CCK-induced stimulation of protein synthetic rates in rat pancreatic acini (34) and oral trypsin inhibitor-induced pancreatic growth in mice (42). CCK also stimulates the protein kinase mTOR (mammalian target of rapamycin) in the exocrine pancreas via the PI3K signaling pathway (44). Similar to calcineurin, mTOR activation is required for CCK-induced stimulation of protein synthetic rates in rat pancreatic acini (3). In our laboratory’s recent study (8), we also demonstrated that activation of the mTOR signaling pathway is necessary for the stimulation of pancreatic protein synthesis, cell division, and growth following feeding of a synthetic trypsin inhibitor (camostat) in mice.

Tashiro et al. (42) have demonstrated that whereas pancreatic growth plateaus after 7 days of camostat feeding, circulating CCK levels and pancreatic calcineurin activity remain significantly elevated even after 10 days on this diet. In contrast, phosphorylation of a downstream target of mTOR, the ribosomal protein S6, declined after only 2 days of camostat feeding (41). Interestingly, 5-bromo-2-deoxyuridine incorporation, an indicator of cell division, also peaks after 2 days of camostat feeding and then declines (8). Whether growth of the exocrine pancreas following camostat feeding is limited by the capacity of acinar cells to divide or the ability of the mTOR pathway to remain activated and thus drive cell division is currently unknown. The objective of this study was to establish whether multiple rounds of camostat-induced growth occur in the exocrine pancreas of the mouse and to determine the mechanism whereby exocrine pancreas growth plateaus following chronic CCK-stimulation.

METHODS

Materials. Camostat (FOY-305) was generously provided by Ono Pharmaceutical (Osaka, Japan). CCK radioimmunoassay kits were from Alpco Diagnostics (Salem, NH). Betanechol (carbamyl-β-methylcholine chloride) was from Sigma (St. Louis, MO). Protease inhibitors were from Roche (Indianapolis, IN), L-[2,3,4,5,6-3H]phenylalanine (3H-Phe) was from Amersham, and amino acid derivatization reagents were from Waters (Milford, MA). Bovine anti-goat horseradish peroxidase-conjugated IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); sheep anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated IgG from Amersham Pharmacia Biotech (Piscataway, NJ); enhanced chemiluminescence reagents from Amersham and Pierce (Rockford, IL); and precast electrophoresis gels, SDS-PAGE molecular weight markers, nitrocellulose membranes, and protein assay reagent from Bio-Rad (Hercules, CA). 4E binding protein-1 (4E-BP1) antibody was obtained from Calbiochem; pan-S6 and p21 antibodies from Santa

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Cruz, cyclophilin-A antibody from Upstate (Lake Placid, NY); amylase antibody from Sigma; chymotrypsin, elastase, and ribonuclease antibodies from Cortex (San Leandro, CA); and all other antibodies from Cell Signaling (Beverly, MA). RNAlater was from Ambion (Austin, TX), TRIZol reagent was from Invitrogen (Carlsbad, CA), and RNeasy was from Qiagen (Valencia, CA).

Animal care. Male ICR mice age 6–8 wk from Harlan (Indianapolis, IN) weighing ~30 g were maintained on a 12-h light-dark cycle with free access to water and chow (5001 Rodent Diet, PMI Nutrition International, St. Louis, MO). All animals were killed by exsanguination under carbon dioxide anesthesia. The University of Michigan Committee on Use and Care of Animals approved the animal facilities and the experimental protocol used in these studies.

Experimental design. In all experiments, animals were acclimated to chow in powdered form for 3 days prior to further experimental manipulation. Animals were then divided into groups provided with either standard powdered chow or powdered chow containing 0.1% camostat for 2–7 days. After 7 days on the camostat-containing chow, animals were provided standard powdered chow for up to 14 days. After 7 days on standard powdered chow, some mice were again provided camostat-containing chow for an additional 7 days. Blood was collected via cardiac puncture under carbon dioxide anesthesia. Plasma was separated, rapidly frozen, and later assayed for CCK-8 equivalents by radioimmunoassay using a version of the manufacturer’s protocol scaled down for the small sample volumes attained. The pancreas was quickly excised and weighed. Portions of the pancreas were either processed immediately, stored in RNAlater, or frozen in liquid nitrogen for later analysis.

Quantitation of pancreatic growth. Following determination of total pancreatic wet weight, a frozen portion was weighed and homogenized in a solution (2 ml/100 mg pancreas) containing 0.1% Triton X-100 (vol/vol) and 5 mM MgCl₂ and subsequently sonicated. The homogenate was collected via cardiac puncture under carbon dioxide anesthesia. Plasmas were separated, rapidly frozen, and later assayed for CCK-8 equivalents by radioimmunoassay using a version of the manufacturer’s protocol scaled down for the small sample volumes attained. The pancreas was quickly excised and weighed. Portions of the pancreas were either processed immediately, stored in RNAlater, or frozen in liquid nitrogen for later analysis.

Histology. Small blocks of pancreas were fixed for 2 h with a mixture of 2% glutaraldehyde and 2% formaldehyde (prepared fresh from paraformaldehyde) in PBS, postfixed for 45 min with 2% OsO₄, and then dehydrated and embedded in Epon. Semithin plastic sections (1 μm thick) were stained with 1% toluidine blue in 1% sodium borate and examined with a light microscope equipped with a digital camera. Morphological analysis was then performed in a blinded manner.

Terminal dUTP nick-end labeling (TUNEL) staining was performed on paraffin-embedded pancreas samples with the ApopTag Red in situ apoptosis detection kit (Chemicon International, Temecula, CA). Slides were mounted with long gold antifade reagent containing DAPI (Molecular Probes; Carlsbad, CA) and examined with a fluorescence microscope (Olympus Optical; Melville, NY).

Administration of metabolic tracer and measurement of protein synthesis. Fractional rates of protein synthesis in the pancreas were determined by the flooding dose method (12) as modified for mice by Lundholm et al. (24) and for pancreas by Sans et al. (33). Briefly, mice were given an intraperitoneal injection of [³H]-Phe (0.4 μCi/g body wt) and unlabeled l-Phe (1.5 μmol/g body wt). Ten minutes later, the pancreas was removed and frozen in liquid nitrogen. A portion of frozen pancreas was later homogenized in 0.6 N perchloric acid (PCA) (1 ml/100 mg) and processed as described previously (19). l-Phe was measured by HPLC, and protein synthesis was calculated from the rate of [³H]-Phe incorporation into pancreatic protein by using the specific radioactivity of pancreatic PCA-soluble l-Phe as the precursor pool and normalized per milligram of pancreatic protein.

Immunoblot analysis. Pancreas samples were prepared for immunoblot analysis exactly as described previously (8). Equal amounts of protein were subjected to SDS-PAGE, after which the protein was transferred to nitrocellulose membrane and Western blotting was performed as described previously (13). Proteins were visualized by enhanced chemiluminescence using an AlphaEase FC8900 imaging system (Alpha Innotech; San Leandro, CA). Changes in the expression of the autophagosomal marker LC3 were determined by normalizing the amount of lipidated protein (17-kDa isoform) to the total amount of LC3. Changes in the phosphorylation state of S6 were determined by normalizing the amount of phosphorylated protein to the total amount of S6 prior to data transformation. When subjected to SDS-PAGE, 4E-BP1 resolves into multiple electrophoretic forms whereby the most highly phosphorylated γ-form exhibits the slowest mobility, thus allowing for an assessment of both protein content and phosphorylation status. Changes in 4E-BP1 phosphorylation were assessed by calculating the proportion of 4E-BP1 in the γ-form. Changes in digestive enzyme and p21 content, as well as Akt content and phosphorylation, were not normalized; however, cyclophilin-A was used as a loading control.

RNA extraction, RNase protection assay (RPA), and quantitative real-time RT-PCR. Total RNA was isolated from pancreatic tissue stored in RNAlater and isolated using TRIZol and an RNeasy kit. Primer sequences for RPA template were determined with the aid of Genefisher software (14) and were as follows:

MABfx/atrogen-1: forward (5'-GCA GAA TTC CAT ACC CCT ATG CAC ACT GGT GCA-3'), reverse (5'-GCA GGT ACC AGT AGC TGT TCC TCT CTC TGC TCA GAG A-3'); MuRF1: forward (5'-GCA GAA TTC AGA AGG ACA CCT CTT CCT ACC ACC AA-3'); E3avl: forward (5'-GCA GAA TTC TCC TAA CCC AGC ACA GAG GGA A-3'), reverse (5'-GCA GGT ACC ACT TGC AGA GCG GCC GTA AGT G-3'); E3aIf: forward (5'-GCA GAA TTC CGT AAT GTC ATG CAG GGA ATG GA-3'), reverse (5'-GCA GGT ACC CCA CCG AGT GAC GTA AAA TAC TGA-3'); and L32: forward (5'-GCA GAA TTC CGG CCT CTG GTG AAG CCC AA-3'), reverse (5'-GCA GGT ACC CCT TCT CCG CAC CCT GTC TGT A-3').

mRNA expression was determined by RPA as described previously (11). Total RNAs were reversed transcribed with a Superscript First Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA), and quantitative PCR was performed by using Quantitect SYBRgreen PCR kits (Qiagen) exactly as described previously (29) with previously published primers for mouse E3 ligases (11). Primer sequences for RPA template were determined with the aid of Genefisher software (14) and were as follows:

Statistical analysis. Data are expressed as means ± SE. Data were analyzed via the GraphPad Prism statistical software package (GraphPad Software, San Diego, CA). Statistical significance was assessed by a one-way ANOVA and a Newman-Keuls posttest. P values < 0.05 were considered significant.

RESULTS

The pancreas undergoes multiple rounds of camostat-induced growth. To address whether growth of the exocrine pancreas following camostat feeding is limited by the capacity of acinar cells to divide, mice were fed camostat-enriched chow for 1 wk, then fed control chow the subsequent week, and finally fed camostat-enriched chow for an additional week. As has been described previously (42), 1 wk of camostat feeding was not associated with any change in body weight (data not
shown), but there was a significant increase in pancreatic weight (Fig. 1A) after only 2 days, which increased to 245% of its initial value after 7 days. Growth of the pancreas was primarily hyperplastic, with a significant increase in total DNA content (Fig. 1B) after only 2 days and a significant increase in total protein content (Fig. 1C) by 7 days. When camostat was removed from the diet, the weight of the pancreas (Fig. 1A) decreased, although it remained significantly larger, at 147% of its initial value, following 1 wk of camostat removal. The decrease in the size of the pancreas was paralleled, with one notable exception that shall be presented in the following section, by reductions in DNA (Fig. 1B) and protein content (Fig. 1C). The observation that both DNA and protein decreased after removal of camostat indicates that the atrophy of the pancreas is due to cell loss and not simply decreased cell size. Growth of the pancreas during the second round of camostat feeding was analogous to that observed during the initial feeding. After 7 days, the weight of the pancreas (Fig. 1A) increased to 257% of its initial value. This growth was again associated with cellular hyperplasia, as demonstrated by the significant increases in both DNA (Fig. 1B) and protein content (Fig. 1C). The observed changes in pancreatic size, DNA, and protein content were associated with concomitant changes in plasma CCK levels (Fig. 1D). Similar to what has been reported previously by Tashiro et al. using a bioassay (41), CCK levels increased to ~10 pmol/l within a day of camostat feeding and remained elevated throughout the first week of camostat feeding. Furthermore, CCK concentrations returned to basal levels within 1 day of camostat removal and were again elevated during the subsequent week of camostat feeding.

**Acinar cell digestive enzyme content transiently increases following removal of camostat.** Changes in the weight of the pancreas in response to the addition or removal of camostat from the diet were mirrored by changes in DNA and protein content with one major exception. In mice fed control chow for 1 day following week-long camostat feeding, the total pancreatic protein content (Fig. 1C) was increased above that observed in week-long camostat-fed mice. Although this result was unexpected, histological examination of pancreas samples (Fig. 2, A–D) indicated that, in contrast to the modest increase in pancreatic zymogen granule content after week-long camostat feeding (Fig. 2B), there was a dramatic increase in zymogen granules 1 day following camostat removal (Fig. 2C). To confirm this result, Western blot analysis was performed, and, regardless of changes in respective digestive enzyme content during camostat feeding (data not shown), there were indeed significant increases in the amount of four different digestive enzymes per unit of protein (Fig. 2E) in the pancreas of mice fed control chow for 1 day following week-long camostat feeding compared with those from mice fed camostat for 1 wk.

**Pancreatic protein synthetic rates are normal following 7 days of camostat feeding.** Increased rates of protein synthesis are required for the accretion of protein observed during pancreatic growth. We have demonstrated previously (8) that camostat feeding is associated with an acute increase in pancreatic protein synthetic rates; however, it is not known whether protein synthesis remains elevated during chronic camostat feeding. As demonstrated in Fig. 3, pancreatic protein synthetic rates were significantly higher in mice fed camostat for 2 days compared with chow-fed controls. In contrast, after 7 days there was no difference between camostat and chow-fed controls.

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**Fig. 1.** Effect of feeding trypsin inhibitor on pancreatic growth in mice. Pancreatic weight (A), DNA content (B), protein content (C), and plasma CCK levels (D) during periods of trypsin inhibitor (TI) feeding and withdrawal. On TI, camostat added to the diet; off TI, camostat removed from the diet. Values represent means ± SE; n = 5–11. *Significantly different from initial control value; †significantly different from 7-day camostat-fed value, P < 0.05.
mice per unit pancreas; total pancreatic protein synthesis, however, is increased in parallel to the increase in pancreatic mass. Moreover, and in accordance with the pancreatic regression observed following removal of camostat from the diet, synthetic rates fell to only 58% of control values in mice fed control chow for 1 day following week-long camostat feeding. The mTOR pathway is stimulated acutely but returns to control levels following 7 days of camostat feeding. The protein kinase mTOR integrates diverse hormonal and nutrient signals to regulate pancreatic protein synthesis (35), and in our laboratory’s recent study (8) we demonstrated that mTOR activation was required for camostat-induced growth of the pancreas. To establish whether the loss of stimulation of pancreatic protein synthesis following 7 days of camostat feeding corresponds to diminished mTOR signaling, Western blot analysis was performed to assess the phosphorylation status of two major downstream targets of mTOR, the ribosomal protein S6 and the eukaryotic initiation factor 4E-BP1. The phosphorylation of S6 (Fig. 4A) and 4E-BP1 (Fig. 4B) was significantly increased in the pancreas of mice fed camostat for 2 days compared with chow-fed mice. In contrast, after 7 days of camostat feeding phosphorylation of 4E-BP1 was not statistically different from control levels and phosphorylation of S6 was 83% below the values observed after 2 days of camostat feeding.

Total Akt is diminished following 7 days of camostat feeding. Binding of CCK to its receptor on rodent acinar cells results in activation of the PI3K/Akt pathway, which lies upstream of mTOR (3, 35). To elucidate whether the diminished mTOR response to camostat after 7 days of feeding was due to altered Akt signaling, we first used Western blot analysis to assess the phosphorylation status of Akt at Ser473, a residue whose phosphorylation is often indicative of Akt kinase activity (1). We have shown previously that the phosphorylation of Akt is significantly elevated in the pancreas of mice fed camostat for 2 h (8); however, there was no significant increase in pancreatic Akt phosphorylation after 2 or 7 days of camostat feeding when the data were normalized to total amounts of Akt (data not shown). In contrast, there was a significant decrease in Akt phosphorylation after 7 days of camostat feeding when phos-
Phosphorylation was assessed without normalization (Fig. 5A). This discrepancy led us to evaluate total Akt content (Fig. 5B), and, interestingly, the decrease in Akt phosphorylation after 7 days of camostat feeding was paralleled by a decrease in the total amount of Akt per unit of pancreatic protein (Fig. 5B). This effect was not observed in mice fed camostat for 2 days. Moreover, the observation that there were no changes in total cyclophilin-A content (Fig. 5B), and, interestingly, the decrease in Akt phosphorylation after 7 days of camostat feeding was paralleled by a decrease in the total amount of Akt per unit of pancreatic protein (Fig. 5B). This effect was not observed in mice fed camostat for 2 days. Moreover, the observation that there were no changes in total cyclophilin-A content (Fig. 5B, inset) indicated that this is not a generalized effect.

Ubiquitin-ligase and p21 expression is enhanced during camostat feeding. Activated Akt suppresses the forkhead box, subgroup O (FOXO) transcription factors and thereby inhibits the expression of the E3 ubiquitin ligases (39) that regulate ubiquitin-mediated protein degradation. To determine whether the changes in Akt phosphorylation and content observed with camostat feeding were associated with a loss of FOXO suppression, the mRNA abundance of E3α1 and E3α2, as well as “muscle-specific” atrogin-1 and MuRF1, were quantitated. Although constitutive expression of all four E3 ligases was detectable by RPA on whole pancreas (Fig. 6A), the predominant mRNA transcript was that of E3α2. Moreover, E3α1 and E3α2 mRNA levels were greater than control values after 2 days of camostat feeding and further elevated following 7 days of camostat feeding based on RPA. Qualitative real-time PCR confirmed that the expression of E3α1 (Fig. 6B) and E3α2 mRNA (Fig. 6C) is significantly increased after 2 days of camostat feeding compared with control levels. In addition, the expression of E3α2, but not E3α1, mRNA is further increased after 7 days. Suppression of FOXO transcription factors by activated Akt is also associated with downregulation of the cell cycle inhibitor p21 (37). To further evaluate whether the observed changes in Akt were associated with a loss of FOXO suppression, p21 expression was evaluated by Western blotting. The expression of p21 (Fig. 7) was significantly increased above control levels after 2 days of camostat feeding and remained elevated after 7 days of camostat feeding.

Cell loss increases following removal of camostat. Although the observed decrease in pancreatic protein synthetic rates following removal of camostat from the diet would preclude pancreatic growth, it does not adequately explain why cell loss occurs following camostat removal. To elucidate the potential mechanisms driving pancreatic regression, TUNEL staining was utilized to evaluate whether camostat withdrawal results in increased rates of apoptosis. TUNEL-positive cells were not observed in pancreas samples from mice fed chow (data not shown) or camostat for 1 wk (Fig. 8A). In contrast, there was a modest visible increase in TUNEL-positive cells in some (Fig. 8C), but not all (Fig. 8B), pancreas samples from mice fed control chow for 3 days following week-long camostat feeding. To evaluate whether a mechanism other than apoptosis could also contribute to the observed pancreatic regression, LC3 expression was evaluated by Western blotting. LC3 is a recently discovered protein that associates with autophagosomes when lipidated (18, 40). Increases in the lipidated form of LC3 are therefore indicative of autophagy and, as demonstrated in Fig. 9, the proportion of the more quickly migrating lipidated

![Graph](image)
DISCUSSION

This study demonstrates that the exocrine pancreas of the mouse has the capacity to undergo multiple rounds of CCK-stimulated growth. Moreover, the results show that the growth arrest that occurs following chronic camostat feeding, when the pancreas reaches ~2.5 times its original weight, is not due to an inherent limit on acinar cell division. Otherwise, after having diminished in size following camostat removal, a second round of camostat feeding would either not have stimulated pancreas growth or would have only stimulated hypertrophic growth. Our observation that pancreatic DNA content increased during both rounds of camostat-induced growth shows this not to be the case.

The growth plateau that occurred following 7 days of camostat feeding was associated with a loss of CCK-induced stimulation of pancreatic protein synthesis. A potential explanation for the observed decrease in protein synthesis following 7 days of camostat feeding is a decrease in CCK concentrations or CCK activity. However, previous studies have reported that circulating CCK levels remain elevated during prolonged camostat feeding (42) and the results from the present study clearly demonstrate

Fig. 5. Effect of feeding T1 on Akt phosphorylation and content in the pancreas. The phosphorylation (A) and total content (B) of Akt were assessed by Western blotting. Cyclophilin-A served as a loading control. Insets: representative immunoblots. Akt(P), Akt phosphorylated on Ser473; Akt(T), total Akt content; Cyc, total cyclophilin-A content. Values represent means ± SE; n = 14–16. *Significantly different from chow-fed control value; †significantly different from 2-day camostat-fed value, P < 0.05.

Fig. 6. Effect of feeding T1 on ubiquitin-ligase expression in the pancreas. Representative RNase protection assay of ubiquitin-ligase expression in the pancreas (A). Relative mRNA levels of total E3α1 (B) and E3α2 (C) as determined by quantitative PCR. L32 served as a loading control. Values represent means ± SE; n = 4. *Significantly different from chow-fed control value; †significantly different from 2-day camostat-fed value, P < 0.05.
that CCK levels remain elevated until camostat is withdrawn from the diet. Moreover, CCK receptor activation is unlikely to be affected by chronic camostat feeding, as evidenced by the significantly greater calcineurin activity observed after 10 days of camostat feeding, compared with control values (42). Downstream of the CCK receptor, however, there is a divergence between the signaling events required for CCK-induced calcineurin and mTOR activation. Consistent with results published previously by Tashiro et al. (41), the results from this study indicate that stimulation of the mTOR signaling pathway diminishes after prolonged camostat feeding. Thus the loss of camostat-induced protein synthesis is likely to be mediated, at least in part, by altered mTOR signaling.

Unlike calcineurin, which is regulated by Ca\(^{2+}\), mTOR receives input from the PI3K/Akt pathway (3, 35). Activation of Akt is demarcated by increased phosphorylation at Ser473 (1), but despite the observed increase in mTOR signaling in the pancreas after 2 days of camostat feeding, there was not a significant increase in Akt phosphorylation at this time. Therefore, it is possible that camostat feeding may stimulate the mTOR pathway through Akt-independent mechanisms. An additional possibility, however, is that camostat-induced activation, as well as phosphorylation, of Akt transiently peaks during the first 2 days of camostat feeding and then quickly returns to control levels. This would be consistent with our prior observation that camostat-induced DNA synthesis peaks by 2 days of camostat feeding (8) as well as the observations by Tashiro et al. (41) indicating peak mTOR activation within the first day of camostat feeding.

Akt Ser\(^{473}\) phosphorylation was significantly decreased after 7 days of camostat feeding compared with chow-fed controls, which is indicative of decreased Akt activity. Further evaluation demonstrated that this decreased Akt phosphorylation was not due solely to alterations in upstream kinase and/or phosphatase activity but, at least in part, to a decrease in total Akt content. Although the underlying cause(s) of the decreased Akt content have not been identified, mechanisms affecting cellular Akt levels have been described previously. Increased Akt activity is often associated with translocation of Akt to nuclear and membrane-associated components of the cell (5, 47). It is possible that there would be bias for loss of membrane-associated and nuclear Akt during the preparation of whole cell

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**Fig. 7.** Effect of feeding TI on p21 content in the pancreas. The total content of p21 was assessed by Western blotting. Cyclophilin-A served as a loading control. Note that p21 formed a doublet during Western blotting and values are representative of both bands. Insets: representative immunoblots. Values represent means ± SE; n = 6. *Significantly different from chow-fed control value, P < 0.05.

**Fig. 8.** Effect of feeding TI on a marker of apoptosis in the pancreas. Terminal dUTP nick-end labeling (TUNEL) staining of pancreatic acinar cell nuclei costained with DAPI (A–C). Mice were fed camostat for 7 days (A) or control chow for 3 days following 7 days of camostat feeding (B and C). DAPI-positive nuclei are stained blue and TUNEL-positive nuclei are stained red.

**Fig. 9.** Effect of feeding TI on a marker of autophagy in the pancreas. The expression of lipidated (17 kDa) and nonlipidated (19 kDa) LC3 was assessed by Western blotting. Inset: representative immunoblot. Values represent means ± SE; n = 6. *Significantly different from chow-fed control value.
lysates used for Western blotting in this study. However, decreased Akt content was associated with decreased Akt pathway activation in the present study, suggesting that Akt translocation is unlikely to account for our findings. Changes in cellular levels of Akt could also be explained by alterations in Akt synthesis. Preliminary studies did not indicate any decrease in the levels of Akt1 or Akt2 mRNA from the pancreas of mice fed camostat for 7 days compared with chow-fed controls (S. J. Crozier and J. A. Williams, unpublished data). Therefore altered rates of Akt transcription seem an unlikely explanation. In contrast, the recent observation that translation of Akt mRNA is attenuated under conditions of cell stress (48) could contribute to the observed changes in Akt content.

Changes in Akt content could also be explained by increased rates of Akt degradation following chronic camostat feeding. Although ubiquitin-mediated protein degradation is well studied in some tissues, such as skeletal muscle, there are few published data on ligase expression in pancreas. Hence we determined the mRNA content of two E3 ligases that have a published data on ligase expression in pancreas. Although our data show the constitutive expression of all four ligases in the pancreas, the predominance of the E3α enzymes, particularly E3α2, was clear. The mRNA content of this ligase was increased after 2 and 7 days of camostat feeding. Medina et al. (27) have shown that decreased Akt signaling is associated with increased ubiquitination of Akt in adipocytes. Therefore, the elevated level of E3α mRNA may, in part, explain why pancreatic Akt levels are diminished following chronic camostat feeding. Moreover, the increased capacity for ubiquitin-mediated protein degradation, as demarcated by increased E3 ligase content, is also likely to contribute to the pancreatic growth arrest that occurs following 7 days of camostat feeding. Regardless of whether or not the loss of Akt content was due to E3 ligase-mediated ubiquitination, this loss was associated with increased expression of E3 ligases and the cell cycle inhibitor p21. Akt suppresses FOXO transcription factors that regulate the expression of these proteins; therefore increased expression of E3 ligases and p21 following prolonged camostat feeding is indicative of diminished Akt activity. Thus it appears that camostat-induced pancreatic growth plateaus when mitogenic signaling pathways, such as the Akt pathway, are countered by inhibitory signaling pathways that thereby prevent further protein accretion.

In the present study, removal of camostat from the diet was associated with decreased protein synthesis and a progressive loss of pancreatic mass, protein, and DNA content. Importantly, the loss of DNA indicates that regression of the pancreas is due to cell death and not simply a decrease in cell size. A similar response has been reported in the rat pancreas after prolonged feeding and subsequent removal of raw soya flour, which contains a natural trypsin inhibitor, from the diet (7). However, in rat compared with mouse, regression of the pancreas occurs at a much faster rate, with DNA content returning to control values within 2 days and protein content and pancreatic weight returning within 7 days (32). Although these species-specific differences cannot be explained here, it is noteworthy that histological examination of the regressing rat pancreas revealed the formation of apoptotic bodies 1, 2, and 7 days after removal of trypsin inhibitor from the diet (32). In contrast, TUNEL staining indicated that there was only a small increase in apoptosis following the removal of camostat in the present study. These data suggest that whereas apoptosis does occur in the mouse pancreas, it occurs at a much slower rate than in the rat pancreas. This would be in agreement with the relatively slow regression of the mouse pancreas following camostat removal. Cell loss in the mouse pancreas may also occur through autophagy following camostat removal. This possibility is particularly attractive given that whereas activated mTOR inhibits autophagy (2, 31), the decrease in protein synthetic rates observed 1 day following camostat removal suggests that growth-promoting signaling pathways, such as the mTOR pathway, are inhibited during pancreatic regression. Autophagosome formation was not observed in our histology samples. However, the slow regression of the pancreas observed in the present study would likely mean that relatively few autophagic vacuoles are present at any one time, thereby making their visualization difficult. In contrast, there was a significant increase in lipidated LC3 following camostat removal. This result is highly supportive of increased autophagy and elucidating the role of autophagy in pancreatic regression following camostat feeding will be an important avenue of future research.

Removing camostat from the diet for a single day after 1 wk of feeding resulted in a significant increase in pancreas protein content as well as the number ofzymogen granules despite a concomitant decrease in pancreatic protein synthesis. Interestingly, Green et al. (16) have reported a similar increase in pancreatic digestive enzyme content 1 day after administration of a CCK receptor antagonist to rats fed a high-protein diet for 1 wk. The authors hypothesized that digestive enzymes accumulated within the pancreas because the antagonist blocked the known stimulatory effects of CCK on enzyme secretion (25, 28) but did not affect the availability of amino acids to serve as substrate for digestive enzyme synthesis (16). The situation appears to be similar in the present study since there would be no limitation on amino acid availability (on the contrary, intracellular acinar cell amino acid levels might increase because of the breakdown of pancreatic structural protein during regression) and CCK receptor activity would decline as CCK levels fell following the removal of camostat from the diet (21, 43). If the secretory response to diminished CCK is greater and/or faster than the protein synthetic response, an accumulation of digestive enzyme within the pancreas would be predicted. This concept of differential sensitivity to the effects of CCK seems highly plausible, given that zymogen granule content appears to normalize within 3 days of camostat removal based on pancreatic protein content measurements and histological examination.

In conclusion, the results presented herein demonstrate for the first time that camostat-induced growth of the pancreas is not limited by a capacity for acinar cell division. The results indicate that whereas pancreatic protein synthesis is stimulated following 2 days of camostat feeding, no such stimulation exists after 7 days. Moreover, ubiquitin-ligase and p21 expression appears to increase during chronic camostat feeding. Thus the size to which the pancreas grows in response to elevated CCK levels appears to be limited by the duration of time that mitogenic stimulation outweighs that of cell cycle repression and protein degradation. After 7 days of camostat feeding, the
cellular content of Akt was diminished and there was a corresponding decrease in signaling through the growth-promoting mTOR pathway, as well as an apparent corresponding increase in FOXO-mediated transcription. Thus it appears as though the ability to sustain Akt activity limits casomalt-induced pancreatic growth. Elucidating the mechanisms whereby Akt content is diminished following chronic casomalt feeding will be important for furthering our understanding of the regulation of pancreatic growth in health and disease.

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