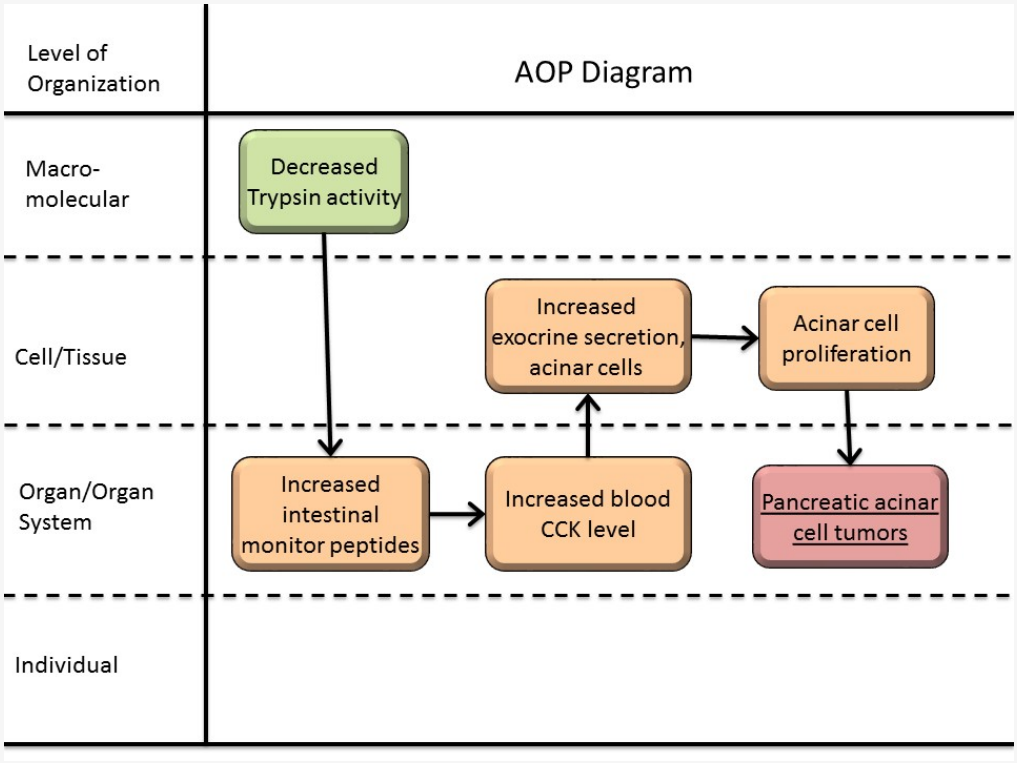
AOP ID and Title:

AOP 316: Trypsin inhibition leading to pancreatic acinar cell tumors
Short Title: TI-induced AC tumors

Graphical Representation



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Status

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Abstract

Pancreatic exocrine secretion is controlled mainly by the gastrointestinal hormone cholecystokinin (CCK), which is secreted by CCK-producing I cells located in the mucosa of the small intestine. Once the contents in the stomach is transported to the small intestine, I cells are stimulated to release CCK into the bloodstream. Several mechanisms to stimulate CCK release are involved.

In rats, pancreatic acinar cells secrete monitor peptide (MP) into the intestinal lumen as a pancreatic soluble trypsin inhibitor (TI). MP stimulates I cells to release CCK into the bloodstream through their surface MP receptors. Then, the increased blood concentration of CCK induces pancreatic exocrine secretion. When fasting, trypsin–MP complexes are formed to decrease the level of free MP in the small intestinal lumen; thereafter, CCK release is suppressed. Meanwhile, upon feeding, partially ingested proteins in the diet consume trypsin to increase the luminal concentration of free MP followed by stimulation of CCK release.

When soybean powder (raw soya flour) containing trypsin inhibitory molecules or TIs such as camostat are given to rats, the intestinal concentration of free MP is increased due to trypsin–TI complex formation. Then, intestinal I cells are stimulated to release CCK. The resulting increased blood level of CCK stimulates pancreatic exocrine secretion of MP, which induces further CCK release via a positive feedback loop. A sustained increase in the CCK level might induce pancreatic hypertrophy and hyperplasia and ultimately result in acinar cell tumor formation.

This increased blood CCK level induced by trypsin inhibition may also occur in humans and other mammalian species

including rats. Luminal CCK-releasing factors (LCRFs) are trypsin-sensitive peptides secreted from small intestinal mucosa that stimulate CCK release by intestinal I cells. Luminal levels of LCRFs are increased after TI ingestion; however, the resultant increase in CCK levels does not stimulate further release of LCRFs, in contrast to MP.

Species differences in CCK-mediated stimulation of pancreatic enzyme secretion have been described in rats and humans. In rats, CCK stimulates pancreatic exocrine secretion and/or proliferation directly via CCK1 receptors expressed on acinar cell surfaces or indirectly via vagal afferent nerves expressing CCK1 receptors, especially at physiological blood CCK concentrations. In contrast to rats, the secretory function of human pancreatic acinar cells is indirectly innervated by vagal afferent nerves expressing CCK1 receptors; however, CCK receptors (mainly CCK2 receptors) expressed on human acinar cell surfaces are not involved in both exocrine secretion and proliferation. These findings suggest that, in humans, innervation of acinar cells in response to elevated CCK blood levels affects mainly secretory functions, with less of an effect on cell proliferation, although the effects of vagal stimulation on acinar cell proliferation are still unclear.

In conclusion, long-term administration of TIs induces pancreatic acinar cell tumors in rats. The main factor contributing to carcinogenesis is a sustained increase in plasma CCK levels mediated by an increased luminal concentration of trypsin-sensitive MP. The risk of trypsin inhibition-induced pancreatic tumors in humans seems to be low or equivocal because of the following reasons:

1. MP, a pancreatic soluble TI that protects against auto-injury induced by trypsin, stimulates CCK release and thereby pancreatic exocrine secretions containing MP, via a positive feedback loop, in rats only.
2. An increased CCK level directly stimulates pancreatic acinar cells to proliferate via surface CCK1 receptors in rats but not in humans. It is still unclear whether vagal stimulation of acinar cells promotes proliferation of acinar cells.

Background

Raw soy flour and purified trypsin inhibitors (TI) cause pancreatic hypertrophy and hyperplasia in some mammalian species, and prolonged treatment with high levels of TI contained in raw soy induced pancreatic nodular hyperplasia and acinar cell adenoma [Rackis JJ, 1965; McGuinness EE et al, 1984; McGuinness EE et al, 1980; McGuinness EE et al, 1985; McGuinness EE and Wormsley KG, 1986; Gumbmann MR et al, 1986]. TI also promoted nodular hyperplasia and tumor formation in rats treated with low levels of pancreatic carcinogens such as azaserine [McGuinness EE et al, 1984; McGuinness EE et al, 1987; Lhoste EF et al, 1988]. These findings question the safety of TI-containing plant foods, and many different studies and reviews have been published to date. The important factors for TI-induced pancreatic acinar cell tumors seem to be a high level of CCK release and CCK-stimulated acinar cell proliferation. In the present AOP, the pathway progressing from trypsin inhibition to pancreatic acinar cell tumor formation is considered from the viewpoints of such key factors.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1720	Trypsin inhibition	Inhibition, trypsin
	KE	1721	Increased intestinal monitor peptide level	Increased monitor peptide
	KE	1722	Increased blood CCK level	Increased blood CCK level
	KE	1723	Increased exocrine secretion from pancreatic acinar cells	Increased acinar cell exocrine secretion
	KE	1724	Acinar cell proliferation	Acinar cell proliferation
	AO	1725	Pancreatic acinar cell tumors	Acinar cell tumors

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Trypsin inhibition	adjacent	Increased intestinal monitor peptide level	Moderate	Low

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased intestinal monitor peptide level	adjacent	Increased blood CCK level	High	Moderate
Increased blood CCK level	adjacent	Increased exocrine secretion from pancreatic acinar cells	High	High
Increased exocrine secretion from pancreatic acinar cells	adjacent	Acinar cell proliferation	High	Moderate
Acinar cell proliferation	adjacent	Pancreatic acinar cell tumors	High	High

Overall Assessment of the AOP

Long-term treatment with trypsin inhibitors (TIs) such as raw soya flour (RSF) in rats increases the incidence of pancreatic acinar cell tumors [McGuinness EE et al, 1984; Gumbmann MR et al, 1986; McGuinness EE et al, 1987; Woutersen RA et al, 1991]. The causative factors for tumorigenesis are a TI-induced increase in cholecystokinin (CCK) release from small intestinal I cells into the blood and direct stimulation of acinar cell proliferation via surface CCK1 receptors [Watanapa P and Williamson RC, 1993].

Differences in these tumor risk factors between rodents and humans are described below.

In rats, trypsin-sensitive monitor peptide (MP), a pancreatic soluble TI (PSTI) found in pancreatic juice that protects against the auto-injury induced by trypsin [Iwai K et al, 1987; Iwai K et al, 1988; Tsuzuki S et al, 1991; Tsuzuki S et al, 1992], plays a major role in stimulating pancreatic exocrine secretion via CCK release [Miyasaka K et al, 1989; Fushiki T et al, 1989; Miyasaka K and Funakoshi A, 1998]. TIs increase the luminal concentration of MP to stimulate CCK release, which in turn increases the MP level as well as pancreatic enzyme secretion via positive regulation. Moreover, repeated injection of CCK into rats increased the level of MP mRNA in the pancreas [Tsuzuki S et al, 1992]. Therefore, the TI-induced increase in CCK release seems to be robust in rodents compared with other species.

On the other hand, in humans, PSTIs do not directly stimulate CCK release [Miyasaka K et al, 1989]. Furthermore, other trypsin-sensitive CCK-releasing peptides (luminal CCK-releasing factors, LCRFs) secreted by intestinal mucosal cells are found in multiple species including rodents and humans [Spannagel AW et al, 1996; Herzig KH et al, 1996; Tarasova N et al, 1997; Li Y et al, 2000; Owyang C, 1999; Wang Y et al, 2002]. TIs increase luminal concentrations of LCRFs, which stimulate CCK release; however, the increase might be mild compared with that induced by MP, because LCRF release does not increase in response to increased CCK levels.

Regarding mitotic activity, high plasma levels of CCK directly stimulate proliferation of rodent pancreatic acinar cells via their surface CCK1 receptors [Povoski SP et al, 1994; Myer JR et al, 2014]. In humans, surface CCK receptors (mainly CCK2 receptors) are not involved in stimulating pancreatic functions; the secretory functions of human acinar cells are innervated mainly by vagal afferent nerves expressing CCK1 receptors [Dufresne M et al, 2006]. However, the vagal contribution to acinar cell proliferation is controversial. Oral ingestion of raw soya flour containing TIs has been reported to stimulate CCK release in humans [Calam J et al, 1987]. In addition, some epidemiological surveys suggest that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978]. On the other hand, a strong relationship between pancreatic cancer and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported.

Therefore, the present AOP supports a pathway from trypsin inhibition to tumor formation originating from pancreatic acinar cells in rodents. The relevance of these findings to humans seems low, although some evidence of a TI-induced increase in blood CCK levels suggests the need for case-by-case risk assessment of pancreatic cancer in humans.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI
Macaca fascicularis	Macaca fascicularis	Low	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

Trypsin

Trypsin is a pancreatic digestive enzyme that has been identified in many animals, including insects, fish, and mammals. The natural substrate of trypsin is generally any peptide that contains Lys or Arg. The active site of trypsin, which is composed of a catalytic triad, is fully conserved, with a similar three-dimensional structure among species, although there are species differences in the amino acid sequence of the enzyme [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017]. TIs such as soybean flours and camostat suppress the activity of trypsin in animal species, including rats and humans [Savage GP and Morrison SC, 2003].

Monitor peptide and related peptides with trypsin inhibitory activity

Pancreatic soluble trypsin inhibitors (PSTIs) are found in the pancreatic juice of multiple mammalian species, including rodents and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced self-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: monitor peptide (MP, also known as PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal I cells via their surface MP receptors, whereas the latter does not [Yamanishi R et al, 1993]. Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989].

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the blood steam from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007].

In rats, however, different from other mammalian species, MP secreted by pancreatic acinar cells plays a major role in protein-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of trypsin inhibitors increases the intestinal level of MP, especially in the intestines during fasting, causing a subsequent increase in the blood level of CCK. Increased levels of CCK stimulate pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these isoforms of CCK have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Species differences in pancreatic exocrine secretion

In rats, physiological plasma level of CCK stimulates pancreatic exocrine secretion and acinar cell growth directly via CCK1 receptors expressed on the cell surface, and exocrine secretion is also innervated by vagal afferent nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014]. Higher plasma levels of CCK may stimulate acinar cell proliferation only via surface CCK receptors but not by vagal nerve innervation [Yamamoto M et al, 2003].

On the other hand, human pancreatic acinar cells express CCK2 receptors, which are not involved in secretion nor proliferation, and exocrine secretion is regulated exclusively by innervation of vagal nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], although there is some evidence of direct stimulation of exocrine secretion of human pancreatic acinar cells [Murphy JA et al, 2008].

Species differences in CCK receptors

Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, and all CCK isoforms function as ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

In rats, CCK1 receptors are expressed in pancreatic acinar cells and sensory vagal afferent nerves, whereas in humans, CCK1 receptors are expressed in vagal afferent nerves but not pancreatic acinar cells. Acinar cells instead express CCK2 receptors; however, these CCK2 receptors are not involved in pancreatic exocrine secretion [Ji B et al, 2001; Dufresne M et al, 2006].

Risk of TI-induced tumor formation from pancreatic acinar cells in humans

The mode of action of TI-induced tumor formation from pancreatic acinar cells in rats is based on a persistent increase in the blood level of CCK, which is induced by an increased intestinal level of MP, resulting from positive regulation of pancreatic exocrine secretion and TI activity.

It was reported that raw soya flour increases CCK release in humans [Calam J et al, 1987]. In addition, the plasma CCK concentration was found to increase after oral administration of fat in patients after subtotal gastrectomy [Hopman WP et al, 1984], and a strong association between pancreatic cancer and a history of subtotal gastrectomy was demonstrated in these patients [Hopman WP et al, 1984].

Therefore, based on the findings from animal studies of persistently increased blood CCK levels accompanied by histopathologic changes in acinar cell proliferation, the tumor risk should be evaluated carefully in humans, despite the lower risk compared with rodents.

Essentiality of the Key Events

MP:

Atropine-treated rats with diversion of pancreatic juice were infused with a mixture consisting of MP, purified trypsin, and various food proteins into the small intestine after intraluminal lavage, followed by examination of pancreatic exocrine secretion. Exocrine secretion was fully reconstructed by the constituent, suppressed in the absence of MP, and the treatment with an anti-MP antibody decreased this exocrine secretion [Fushiki T et al, 1989]. These results suggest that MP is an essential factor for regulating pancreatic exocrine secretion.

CCK:

CCK-deficient mice generated by gene targeting in embryonic stem cells showed no abnormalities in body weight or pancreatic weight or histopathology, but they showed protein-induced increases in pancreatic growth and proteolytic enzyme secretion, suggesting that other regulatory pathways are modified to compensate for the CCK deficiency [Lacourse KA et al, 1999]. The TI camostat increased pancreatic wet weight and protein and DNA levels in a time-dependent manner over a 10-day period in normal mice, but not in CCK-deficient mice [Tashiro M et al, 2004]. These results suggest that CCK is needed for TI-induced pancreatic hyperplasia.

CCK receptors:

In an experiment in which CCK1 receptor-deficient rats were fed a diet containing 0.1% TI (camostat, ONO-3403, or soybean TI) for 7 days, the CCK mRNA level increased without any change in the protein level in pancreatic juice in each TI treatment group. These results suggest that TI treatment enhances the release of CCK, and that CCK-induced secretion of pancreatic digestive enzymes is mediated by CCK1 receptors [Kawanami T et al, 1999].

Experiments using CCK1-receptor-deficient Otsuka Long-Evans Tokushima Fatty rats showed the following:

1. The CCK1 receptor plays a role in the increased cell size associated with normal growth of the pancreas [Miyasaka K et al, 1996].
2. The CCK1 receptor is not an absolute requirement for normal growth of the pancreas but is important for pancreatic regeneration [Miyasaka K et al, 1997].
3. Absence of the CCK1 receptor did not affect the acute phase of pancreatitis but significantly retarded regeneration of pancreatic tissue [Miyasaka K, Ohta M et al, 1998].

Weight of Evidence Summary

Biological Plausibility

KER	KE_{up}-KE_{down}	Plausibility	Rationale supported by the literature

KER1	Trypsin inhibition increases the luminal concentration of MP	Middle	In rodents, a certain level of monitor peptide (MP) is secreted from pancreatic acinar cells, even between meals or under fasting conditions. However, intestinal MP level is maintained at a low level because of its rapid degradation by trypsin and other proteases (or because of MP–trypsin complex formation, which decreases the level of luminal free MP) [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998]. After ingestion of trypsin inhibitor (TIs), the intestinal content of MP increases rapidly especially in the fasting state [Iwai K et al, 1988; Liddle RA, 1995]. In other species, trypsin-sensitive CCK-releasing peptide (luminal CCK-releasing factor, LCRF) is released from small intestinal mucosal cells [Owyang C, 1999]. TIs increase the luminal concentration of LCRF; however, the increase in LCRF is not as high compared with MP [Liddle RA, 1995].
KER2	The increased luminal concentration of MP increases the blood CCK level	Strong	In rats, CCK release from I cells in the small intestinal mucosa is regulated by trypsin-sensitive MP [Miyasaka K et al, 1989; Cuber JC et al, 1990; Guan D et al, 1990]. In the empty intestine after dietary protein is digested, secreted MP forms complex with trypsin to be degraded, and luminal level of free MP is kept at low levels, during which CCK release is suppressed Once TIs are ingested, the intestinal concentration of free MP is increased due to trypsin–TI interactions [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].Then, the increased MP directly stimulates I cells via their surface MP receptors to release CCK into the blood, leading to increased plasma CCK levels [Bouras EP, 1992; Cuber JC, 1990; Guan D, 1990]. The luminal MP level is further increased along with increased pancreatic exocrine secretion due to the increased plasma level of CCK via a positive feedback loop and trypsin inhibition [Liddle RA, 1995]. In other species including rats, TI increases the luminal level of trypsin-sensitive LCRF to stimulate CCK release, but the increase is transient due to the lack of the positive feedback loop between CCK and LCRF [Liddle RA, 1995].

KER3	The increased blood CCK level stimulates exocrine secretion by pancreatic acinar cells	Strong	Pancreatic exocrine secretion is regulated by CCK released from CCK-producing I cells located in the small intestinal mucosa. CCK stimulates exocrine secretion from pancreatic acinar cells directly via surface CCK receptors and indirectly via vagal afferent nerves expressing CCK receptors in rats. However, in humans, pancreatic secretion is innervated by vagal afferent nerves [Pandiri AR, 2014]. Of the two types of CCK receptors (CCK1 and CCK2 receptors), the former show high affinity to CCK and the latter high affinity to both CCK and gastrin [Dufresne M et al, 2006]. In rats, CCK1 receptors are expressed on pancreatic acinar cells and vagal afferent nerves. On the other hand, in humans, CCK1 receptors are expressed on vagal afferent nerves but not on pancreatic acinar cells, on which CCK2 receptors are expressed instead. CCK2 receptors are not involved in acinar cell functions [Pandiri AR, 2014].
KER4	The exocrine secretion induced by pancreatic acinar cells increases proliferation of pancreatic acinar cells	Strong	An increased plasma level of CCK directly induces proliferation of pancreatic acinar cells via surface CCK1 receptors, as well as exocrine secretion, in rats [Yanatori Y and Fujita T, 1976; Folsch UR et al, 1978; Longnecker DS, 1987; Povoski SP et al, 1994; Tashiro M et al, 2004]. However, the involvement of vagal afferent innervation in acinar cell proliferation under an increased blood level of CCK might be low in humans, but this is unclear [Chandra R and Liddle RA, 2009].
KER5	The increased proliferation of pancreatic acinar cells leads to pancreatic acinar cell tumor formation	Strong	A sustained increase in acinar cell proliferation promotes tumor formation [McGuinness EE et al, 1985]. An increased blood CCK level is the main factor involved in sustained acinar cell proliferation, which promotes acinar cell tumor formation [Douglas BR et al, 1989].

Empirical Support

KER	Empirical support for KERs

MIE=>KE1 Trypsin inhibition increases the luminal concentration of MP	Empirical support for the MIE => KE1 is strong. Rationale No study has demonstrated a direct relationship between trypsin inhibition and an increased luminal concentration of monitor peptide (MP). However, several studies have reported a relationship between trypsin inhibitor (TI) treatment and an increased plasma CCK level. Considering that MP directly stimulates CCK release from I cells in the small intestine in rodents, increased plasma CCK levels induced by TIs suggest increased luminal MP levels. The plasma CCK8 level in rats after 18-hour fasting was 0.31 ± 0.05 pM (mean ± SE) and increased to 6.2 ± 1.8 pM 7.5 minutes after feeding and increased to 10.3 ± 1.8 pM 15 minutes after intragastric instillation of a soybean trypsin inhibitor [Liddle RA et al, 1984]. Immediately after oral feeding of camostat at 400 mg/kg in rats, the plasma CCK level increased 10-fold above that in controls, reached a maximum after 90 min, remained elevated for more than 6 h, and then returned to control levels 24 h after administration of camostat [Goke B et al, 1986].
KE1 =>KE2: An increase in the luminal concentration of MP increases the blood CCK level	Empirical support for the KE1 => KE2 AO is strong. Rationale MP at concentrations ranging from 3×10^{-12} to 3×10^{-8} M stimulated CCK release from isolated mucosal cells of the rat duodenum in a dose-dependent manner with highest level at 15 minutes after stimulation [Bouras EP et al, 1992]. MP at a concentration range of 2–12 µg/mL induced within a few minutes a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodenum/jejunum [Cuber JC et al, 1990]. In rats with biliary and pancreatic fistulas, duodenal infusion of MP at 0.9 µg/rat increased pancreatic secretion and the plasma CCK level [Miyasaka K et al, 1989]. Sorted CCK-positive rat intestinal mucosal cells stimulated with 30 nM MP increased the secretion of CCK in a time-dependent manner as soon as 5 min after the start of stimulation [Liddle RA et al, 1992].

KE2 =>KE3: An increase in the blood CCK level induces exocrine secretion by pancreatic acinar cells	Empirical support of the KE2 => KE3 is strong. Rationale In rats, diversion of bile pancreatic juice induced more than ten-times increase in plasma concentration of CCK at the end of two hours and caused rapid and sustained increase in pancreatic protein secretion with more than two folds at 60 minutes of diversion compared with the basal levels [Li Y and Owyang C, 1994]. Repeated injections of cholecystokinin (CCK) at 130 IU s.c. for 3 weeks significantly increased the pancreatic content and secretion of amylase and trypsin during stimulation with 60 IU/kg-hour of CCK. Peak secretion rates of the enzymes were obtained 45 minutes after the start of the stimulation [Folsch UR et al, 1978]. CCK-mediated feedback control of pancreatic enzyme secretion is also observed in humans. Intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Simultaneous intraduodenal perfusion of trypsin with phenylalanine lowered plasma CCK level at 24% and pancreatic output of chymotrypsin at 63% compared with the perfusion of phenylalanine alone. Moreover, intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].
KE3 =>KE4: Induction of exocrine secretion by pancreatic acinar cells increases proliferation of pancreatic acinar cells	Empirical support for the KE2 => KE3 is strong. Rationale KE3/KE4: In rats fed 20, 40, and 100% RSF-containing diet for up to 36 weeks, pancreatic hypertrophy was found in all RSF-fed groups, and hyperplasia was found only in the 40 and 100% RSF-fed groups [Crass RA and Morgan RG, 1982]. KE3: Intraduodenal administration of 30 mg RSF increased the total amount of 1-hour pancreatic protein output at 2.2 ± 1.1 mg/h (mean ± SE) in rats in which bile and pancreatic juice were returned to the duodenum [Jordinson M et al, 1996]. KE4: In rats, administration of TIs in drinking water (“Trypsin soybean inhibitor” (Miles), 400mg/100mL) or injection of CCK (CCK-PZ or CCK-33,400 Ivy Dog unit) for 7 days increased acinar cell proliferation as well as acinar cell hypertrophy [Yanatori Y and Fujita T, 1976], and RSF feeding at libitum increased acinar cell proliferation from 7 to 28 days of treatment leading to hypertrophy and hyperplasia [Oates PS and Morgan RG, 1984]. These results show that trypsin inhibition-induced acinar cell proliferation (hyperplasia) developed at higher doses of RSF compared with those of pancreatic hypertrophy caused by increased secretion, or that pancreatic exocrine secretion and increased acinar cell proliferation were detected after 1 h and 7 days, respectively, after the start of TI or CCK treatment.

KE4 =>AO: Increased proliferation of pancreatic acinar cells induces pancreatic acinar cell tumors	Empirical support for the KE4 => AO is strong. Rationale Rats were fed a diet containing 100 or 200 mg TI concentrates prepared from RSF or potato juice. KE4: After 28 days of feeding, both sources of TI induced pancreatic hypertrophy. AO: After 95 weeks of feeding, both TIs induced dose-related pancreatic changes in terms of nodular hyperplasia and acinar adenoma [Gumbmann MR et al, 1989]. Rats continuously fed a diet containing 5% or more RSF developed pancreatic micro/macroscopic nodules and stimulated azaserine-induced nodular hyperplasia and tumorigenesis, and those fed a diet containing 25, 50 and 100% RSF 2 days per week developed pancreatic macro/microscopic nodules, and 100% RSF-fed rats developed pancreatic cancer [McGuinness EE and Wormsley KG, 1986]. Rats fed a diet containing as little as 0.02% camostat 3 days per week developed pancreatic hypertrophy and hyperplasia [Lhoste EF et al, 1988]. F344 rats injected s.c. twice with azaserine at 30 mg/kg BW and treated with camostat at 200 mg/kg BW by gavage 5 days a week for 18 weeks developed azaserine-induced pancreatic preneoplastic lesions. In azaserie-treated Lewis rats, treatment with camostat in diet at 0.5 g/kg diet for 4 weeks and then 0.2 mg/kg diet 3 consecutive days per week for 8 or 16 weeks also promoted the growth of azaserine-induced neoplastic lesions [Lhoste EF et al, 1988].

Quantitative Consideration

KER1:

No study has shown a dose–response relationship between trypsin inhibition and the luminal concentration of MP in rodents. (further research is needed).

KER2:

MP at concentrations ranging from 3×10^{-12} to 3×10^{-8} M stimulated CCK release within 5 minutes from isolated mucosal cells from the rat duodenum in a dose-dependent manner [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced a dose-dependent transient (within several minutes) increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodeojejunum. MP at 36 µg/mL showed lower CCK release [Cuber JC et al, 1990].

KER3:

The effect of CCK on the stimulation of pancreatic secretion is dose dependent.

Intravenous infusion of CCK-8 at 20 and 40 pM/kg/hour or high affinity CCKR agonist CCK-JMV-189 at 22, 44 and 88 µg/kg/hour in rats induced dose-dependent increases in pancreatic protein secretion from 15 minutes of infusion [Li Y et al, 1997].

Physiological plasma CCK doses (up to ~10 pM) stimulate the vagal afferent pathway, whereas supraphysiological CCK doses stimulate intrapancreatic neurons and pancreatic acini to secret pancreatic protein [Owyang C, 1996].

KER4:

In rats injected subcutaneously with CCK at 7.5 or 30 Ivy dog units (IU) twice daily for 20 days, pancreatic wet weight and DNA content / 100g BW increased with a same manner compared with saline-treated rats, however, pancreatic output of amylase and trypsin in response to submaximal intravenous stimulation with CCK at 15 IU/kg/hour increased with dose-dependent manner [Folsch UR et al, 1978].

Rats were fed diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%) and were then sacrificed at 3-month intervals starting at 6 months [Rackis JJ et al, 1985]. Trypsin and chymotrypsin activities per 100g BW, RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI and protein-

fed animals after 6-month dosing, although pancreatic nodules were increased in number at 15 months of dosing or later at 215 mg TI/100 g diet or higher [Liener IE et al, 1985].

KER5:

Rats were fed diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%) and were then sacrificed at 3-month intervals starting at 6 months [Rackis JJ et al, 1985]. RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI- and protein-fed animals after 6-month dosing. Pancreatic nodules were increased in number at 15 months of dosing or later and at 215 mg TI/100 g diet or higher[Liener IE et al, 1985].

Considerations for Potential Applications of the AOP (optional)

TBD

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Appendix 1

List of MIEs in this AOP

Event: 1720: Trypsin inhibition

Short Name: Inhibition, trypsin

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Term	Scientific Term	Evidence	Links
Macaca fascicularis	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Trypsin is a digestive enzyme expressed in many vertebrates, and its molecular weight and isoforms vary among animal species, for example, human cationic and anionic trypsins (trypsins 1 and 2) and mesotrypsin, bovine cationic and anionic trypsins, and rat anionic trypsin and P23 [Chen JM and Claude Férec C, 2013; Fukuoka S and Nyaruhucha CM, 2002]. However, their three-dimensional structures are highly conserved among species [Baird Jr TT, 2013].

The natural substrate for trypsin is generally any peptide that contains Lys or Arg. The active site of trypsin has a specific catalytic triad structure composed of serine, histidine, and aspartate, and the flanking amino acid sequences are entirely conserved [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017].

Therefore, TIs show comparable enzymatic inhibition of trypsin molecules among animal species including humans and rats [Savage GP and Morrison SC, 2003].

Key Event Description

Trypsin is a digestive enzyme secreted by pancreatic acinar cells that cleaves peptide bonds at the carboxyl end of basic amino acids (lysine and arginine). Acinar cells secrete trypsinogen, the inactive form of trypsin, into the lumen of the duodenum; in turn, trypsinogen is auto-hydrolyzed by enterokinase into β-trypsin, composed of an uncleaved single chain, and α-trypsin, composed of two cleaved chains bound by a disulfide bridge [Santos AMC et al, 2008]. Trypsin is required for the partial hydrolysis of chymotrypsinogen to chymotrypsin, and most pancreatic digestive enzyme precursors are activated by trypsin in the same manner as chymotrypsin in the intestinal lumen.

As part of the defense against trypsin-induced self injury in the pancreas, internal TIs such as the serine protease inhibitor Kazal type 1 (SPINK1 or human pancreatic trypsin inhibitor) and bovine pancreatic TI in the pancreatic juice and α1-antitrypsin in the serum bind tightly to active trypsin [Voet D and Voet JG, 1995].

Secretion of pancreatic digestive enzymes including trypsin is regulated mainly by CCK released from enteroendocrine I cells in the duodenal mucosa of the small intestine [Wang BJ and Cui ZJ, 2007], and CCK release is controlled by multiple mechanisms [Caron J et al, 2017]. One such mechanism is trypsin-mediated negative feedback regulation, in which increased trypsin secretion leads to decreased levels of trypsin-sensitive luminal CCK-releasing factors (LCRFs) in several mammalian species and MP in rodents [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].

Therefore, ingestion of RSF containing trypsin inhibitory action or protease inhibitors such as camostat inhibits trypsin activity in the intestinal lumen, which leads to increased luminal levels of the abovementioned trypsin-sensitive peptides and thereby stimulation of CCK release [Green GM and Miyasaka K, 1983; Cuber JC et al, 1990; Miyasaka K et al, 1989; Cuber JC et al, 1990; Komarnytsky S et al, 2011].

How it is Measured or Detected

Activity of trypsin inhibitors is measured colorimetrically using mixture of multiple dilutions of samples (TIs), trypsin and its substrate. Standard procedures for measuring TI activities in soy bean products are released as AACCI Method 22-40.01 [AACCI, 2009] and AOCS Method Ba 12-75 [AOCC, 2017]. ISO standard for measuring TI activities is also established as Standard 14902:2001 [ISO, 2012]. The two methods of modified AACC 20-40.01 and ISO 14902 were compared to show that the values obtained by these two methods are not directly comparable [Sueiro S et al, 2015]. Modified standard method is proposed reconsidering the levels of dilutions and volumes, reaction sequence and other factors [Liu K, 2019].

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List of Key Events in the AOP

Event: 1721: Increased intestinal monitor peptide level

Short Name: Increased monitor peptide

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	KeyEvent

Biological Context

Level of Biological Organization

Level of Biological Organization

Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI
Macaca fascicularis	Macaca fascicularis	Low	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Feedback regulation of pancreatic enzyme secretion mediated by trypsin-sensitive intestinal peptides other than MP has been reported in mammals. Such peptides include luminal CCK-releasing factors (LCRFs) secreted by duodenal mucosal cells in response to intestinal diet in some mammalian species including rats, pigs (diazepam-binding inhibitor) and humans [Miyasaka K and Funakoshi A, 1998; Wang Y, 2002; Wang BJ and Cui ZJ, 2007]. In humans, different from rodents, LCRF is not secreted spontaneously in the intestine, however luminal amino acids and fatty acids were reported to induce CCK release [Liddle RA, 1997].

MP is one of pancreatic soluble TIs (PSTIs), which are found in the pancreatic juice of many mammalian species including pigs, dogs, and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced auto-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: MP (or PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal CCK I cells via their surface MP receptors [Yamanishi R et al, 1993], whereas the latter does not [Guan D et al, 1990].

Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989], and no PSTI except MP has been reported to stimulate CCK release.

Key Event Description

Trypsin-mediated feedback regulation of pancreatic exocrine secretion is commonly found among vertebrate species.

In rats, trypsin-sensitive monitor peptide (MP), a pancreatic soluble trypsin inhibitor (TI) found in pancreatic juice that protects against trypsin-induced auto-injury [Iwai K et al, 1987; Iwai K et al, 1988; Tsuzuki S et al, 1991; Tsuzuki S et al, 1992], plays a major role in stimulating pancreatic exocrine secretion via cholecystokinin (CCK) release [Miyasaka K et al, 1989; Fushiki T et al, 1989; Miyasaka K and Funakoshi A, 1998].

MP is a peptide consisting of 61 amino acids with a molecular weight of approximately 6000 and is secreted from pancreatic acinar cells along with other pancreatic enzymes [Iwai K et al, 1987].MP is reported to have trypsin inhibitory activity [Lin YZ et al, 1990], and it forms complexes with trypsin in the empty intestine, similar to other pancreatic soluble TIs [Voet D and Voet JG, 1995], which keeps the intestinal level of free MP low. However, once the gastric contents are transported to the small intestine, secretion of the pancreatic proteases with MP are induced, where trypsin is used for protein hydrolysis, and the level of free MP is subsequently increased [Iwai K et al, 1988; Graf R, 2006]. The increased MP level stimulates CCK release from I cells lining the small intestinal mucosa via MP receptors [Liddle RA et al, 1992; Yamanishi R et al, 1993; Yamanishi R et al, 1993; Liddle RA et al, 1992], and the resulting increase in CCK stimulates exocrine secretion from the pancreas. MP secretion is simultaneously increased to stimulate CCK release further. Therefore, MP-mediated regulation of trypsin and related proteases appears to act via a positive feedback loop as long as duodenal contents remain to consume trypsin for proteolysis.

In accordance with the increased secretion of pancreatic enzymes, proteolysis of the intestinal contents lowers protein levels in the intestinal lumen, which once again lowers the intestinal level of free MP due to the excess of trypsin. CCK release is decreased in accordance with the decreased intestinal MP level, followed by a decrease in pancreatic exocrine secretion [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998].

After ingestion of raw soya flour, which contains trypsin inhibitory activity, or TIs such as camostat, TI–trypsin complexes are formed, and the intestinal level of free MP is increased to stimulate CCK release [Yamanishi R et al, 1993], increasing the blood CCK level. Increased CCK further stimulates MP as well as other pancreatic enzymes via positive feedback regulation [Liddle RA, 1995].

How it is Measured or Detected

No literatures that describe the methods of measuring intestinal concentration of MP are found although some authors reported the isolation and measurement of MP from synthesis reaction solution, pancreas or pancreatic juice.

Synthesized crude peptides were eluted through gel filtration chromatography. PSTI-I-specific peak was confirmed by mas spectrometric measurement and analytical HPLC was performed [Graf R et al, 2003].

In rats fed control and high protein diets, zymogen granules were isolated and concentrations of MP and PSTI-II in zymogen granules can be determined by HPLC [Tsuzuki S et al, 1991].

Rat anionic trypsinogen and a pancreatic secretory trypsin inhibitor were purified from the pure pancreatic juice of rats by reverse-phase HPLC [Iwai K et al, 1987].

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Event: 1722: Increased blood CCK level

Short Name: Increased blood CCK level

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	KeyEvent

Biological Context

Level of Biological Organization

Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Macaca fascicularis	Macaca fascicularis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

There are species differences in the regulation of CCK release.

Fats, fatty acids, proteins, and amino acids stimulate CCK release in humans, and fatty acids and amino acids are the key factors regulating CCK release in dogs. These factors stimulate intestinal I cells to release CCK either directly via cell surface receptors such as Ca-sensing receptors and the G protein-coupled receptor GPR93 or indirectly via LCRFs [Caron J et al, 2017]. Amino acids directly stimulate LCRF release from small intestinal mucosal cells in humans [Wang BJ and Cui ZJ, 2007].

On the other hand, in rodents, trypsin-mediated negative and positive feedback regulation loops involved in CCK release have been identified; the former is mediated by LCRF secreted from intestinal mucosal cells and the latter via MP secreted from pancreatic acinar cells [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Miyasaka K and Funakoshi A, 1998]. This mechanism of CCK release regulation is plausible in rodents, because of their diet of wild legumes and cereal grains, which contain trypsin inhibitors, and the short digestion time in the stomach.

Multiple isoforms of CCKs (e.g., CCK-83, -58, -39, -33, -22, -8, and others) have been identified, and their expression differs among species (humans express CCK-33, -22, and -58; dogs express CCK-58; cats express CCK-8, -33, and -58; pigs express CCK-22, -58, -3, and -8; rabbits express CCK-22 and -8; and rats express CCK-58). All CCK isoforms contain a highly conserved region of amino acids and serve as ligands for CCK1 receptors [Wang BJ and Cui ZJ, 2007; Rehfeld JF, 2017].

Key Event Description

Pancreatic exocrine secretion is controlled by multiple mechanisms [Caron J et al, 2017; Wang BJ and Cui ZJ, 2007; Wang Y et al, 2011], many of which are mediated by CCK secreted by CCK-producing I cells lining the mucosa of the small intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. CCK is also synthesized in cerebral neurons and expressed in several endocrine and certain other cells, and they are involved in many functions other than pancreatic exocrine secretion, including gall bladder contraction, gut motility, and satiety [Rehfeld JF, 2017].

CCK is initially synthesized as a peptide prohormone comprising 150 amino acids, which is processed into active CCK by prohormone convertases specific to the cell type and species [Rehfeld JF et al, 2003; Wang BJ and Cui ZJ, 2007]. CCKs exist as several isoforms that differ due to post-translational modifications, although the C-terminal amino acid sequences are conserved among these isoforms [Rehfeld JF et al, 2001; Rehfeld JF, 2017].

CCK release is stimulated mainly by gastric contents containing fatty acids and amino acids transported into the small intestine. The factors in gastric chyme that stimulate CCK release differ among species, with fats, fatty acids, proteins, and amino acids being the key players in humans, fatty acids and amino acids in canines, and digested/undigested proteins in rats [Wang BJ and Cui ZJ, 2007; Caron J et al, 2017]. These factors stimulate intestinal mucosal I cells to release CCK into the blood either directly via specific receptors such as calcium-sensing receptors and the G protein-coupled receptor GPR93 or indirectly via luminal CCK-releasing factors (LCRFs) [Caron J et al, 2017]. LCRFs are released from intestinal mucosal cells in response to amino acids and fatty acids in humans [Liddle RA, 1997; Liddle RA, 2000] ; however, the peptides mediate negative feedback regulation of CCK release via CCK degradation by pancreatic proteases [Wang BJ and Cui ZJ, 2007].

In addition to the negative feedback regulation of CCK release in rodents, CCK release is stimulated by monitor peptide (MP), a pancreatic soluble trypsin inhibitor (PSTI) secreted into the upper intestine from pancreatic acinar cells [Wang BJ and Cui ZJ, 2007]. MP, which is trypsin-sensitive, stimulates intestinal I cells to release CCK via positive feedback regulation, in that the resulting increased CCK level stimulates the secretion of MP together with other pancreatic enzymes [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Miyasaka K and Funakoshi A, 1998]

When trypsin is inhibited in rodents, trypsin-sensitive MP-induced CCK release is overstimulated due to positive feedback regulation of CCK release by trypsin.

How it is Measured or Detected

Plasma was first extracted on octadecylsilyl silica columns, and the CCK concentration was measured in the resulting extracts, based on the ability of the extracts to stimulate amylase release from isolated rat pancreatic acini [Liddle RA et al, 1984].

The STC-1 cell line, which is derived from murine enteroendocrine tumor cells, secretes several enteroendocrine hormones including CCK, GLP-1, and GLP-2 in response to many different stimulants such as monosaccharides, aromatic amino acids, peptidomimetic compounds, and bitter tastants [Wang BJ and Cui ZJ, 2007].

CCK release from STC-1 cells or intestinal cell preparation were measured by sensitive and specific radioimmunoassay, which recognizes biologically active forms of CCK [Wang Y et al, 2002; Wang Y et al, 2011].

In order to assess the effects of protein hydrolysates on CCK release from enteroendocrine cells, each of protein hydrolysates and STC-1 cells were incubated and CCK release is measured by ELISA [Foltz M et al, 2008].

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Event: 1723: Increased exocrine secretion from pancreatic acinar cells

Short Name: Increased acinar cell exocrine secretion

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Macaca fascicularis	Macaca fascicularis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

CCK1 and CCK2 receptors are expressed in various organs and tissues including the digestive and nervous systems, and there are species differences in the expression localization and levels of these receptors.

In rats, it has been reported that pancreatic acinar cells express mainly CCK1 receptors but not CCK2 receptors [Bourassa J et al, 1999]. CCK1 receptors are also expressed in vagal afferent nerve fibers of the gastroduodenal tract; stimulation of the vagus nerve via CCK1 receptors as well as via physical stimulation of stomach wall distention by ingested food also promotes pancreatic exocrine secretion [Dufresne M et al, 2006].

Meanwhile, in humans, CCK2 receptors are dominantly expressed in pancreatic acinar cells, with low expression of CCK1 receptors [Nishimori I et al, 1999]. Ji reported the following using human pancreatic acini: 1) the mRNA level of the CCK2 receptor is higher than that of the CCK1 receptor, 2) an in situ hybridization experiment showed no expression of either receptor type, and 3) human pancreatic cells did not show any response to the CCK1 receptor agonist CCK8 or the CCK2 receptor agonist gastrin in vitro [Ji B et al, 2001]. Therefore, human pancreatic acinar cells respond to CCK more weakly compared with rodents, because the CCK receptor subtypes expressed in the pancreas are different between humans and rodents, and CCK receptor expression levels are lower in humans than rodents.

In addition, exocrine secretion from the human pancreas is regulated mainly by innervation of vagal afferent nerves via CCK1 receptors and less so by direct stimulation of acinar cells via CCK receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014]. Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, with 98% homology between rats and mice, 90% between rats and humans, 98% between cynomolgus monkeys and humans, and 89% between dogs and humans [Wang BJ and Cui ZJ, 2007].

Multiple molecular forms of CCKs, including CCK-83, -58, -39, -33, -22, -8 among others, have been identified; all of these isoforms have a highly conserved region of 5 amino acid sequences at the C-terminal, and all are ligands for CCK1 receptors [Wank SA, 1995].

Key Event Description

The major function of pancreatic exocrine secretion is secretion of digestive enzymes, fluid, and bicarbonate in response to food intake. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes, such as trypsinogen, chymotrypsinogen, proesterase, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted into the small intestine, where trypsinogen is converted to trypsin by enteropeptidase, and the newly generated trypsin activates more trypsinogen molecules and other proenzymes [Berg JM et al, 2002].

Pancreatic exocrine secretion is regulated mainly by CCK released from CCK-producing I cells located within the mucosa of the small intestine. CCK stimulates exocrine secretion either directly via CCK receptors expressed on acinar cells or indirectly by the vagovagal reflex via CCK receptors. There are species differences in these CCK regulatory mechanisms [Singer MV and Niebergall-Roth E, 2009; Chandra R and Liddle RA, 2009].

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin) receptors. The CCK1 receptor exhibits high affinity to all CCK isoforms, whereas the CCK2 receptor exhibits affinity to both CCK and gastrin, in which the last five amino acid sequences at the C-terminus end are identical [Dufresne M et al, 2006; Rehfeld JF, 2017].

In rats, pancreatic acinar cells express mainly CCK1 receptors, and blood CCK directly stimulates exocrine secretion and acinar cell proliferation [Dufresne M et al, 2006]. Moreover, the vagal afferent nerves also stimulate pancreatic exocrine secretion; CCK stimulates CCK1 receptors expressed on the vagal afferent nerve fibers of the vago–vagal reflex loop, and the acetylcholine generated acts on M3 muscarinic cholinergic receptors to promote pancreatic exocrine secretion [Bourassa J et al, 1999; Adler G, 1997; Ji B et al, 2001; Li Y et al, 1997; Owyang C, 1996]. Moreover, when the gastric wall is distended with ingested food, the vagus nerve is stimulated to promote pancreatic exocrine secretion [Dufresne M et al, 2006].

In humans, the density of CCK receptors expressed on acinar cells is lower than that in rodents, whereas CCK2 receptors are dominantly expressed. Therefore, the responses of acinar cells to CCK seem to be weaker compared with rodents, and pancreatic exocrine secretion in humans is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014].

How it is Measured or Detected

Ex vivo procedure for measuring secretion from pancreatic acini is reported [Geron E, 2014], where ex vivo culture of pancreatic acini isolated from mice is used for amylase secretion assay as a global measure and direct imaging of

pancreatic secretion with subcellular resolution.

The release of amylase was measured in dispersed acini from human pancreas [Miyasaka K et al, 2002].

Pancreatic exocrine secretion was measured in rats with chronic pancreatitis and pancreatic and biliary fistulas [Green GM and Miyasaka K, 1983]. Pancreatic juice was collected from the jejunum and the amounts of protein and pancreatic enzymes were measured.

Pancreatic enzyme activities in pancreatic outlet were measured after CCA injection [Folsch UR et al, 1978]. After repeated subcutaneous injections of CCK in rats, pancreatic enzymes were collected by perfusing duodenum. trypsin in the perfusate was activated with enterokinase and its activity was measured photometrically using benzoyl arginine as substrate. Amylase activity in the perfusate was measured using Zulkovsky starch as substrate. The concentration of protein per weight of DNA, the total level of pancreatic DNA, and the pancreatic levels of amylase and trypsin were also measured in rats after repeated CCK administration.

In the rats fed the TI camostat, pancreatic weight and protein, DNA, and enzyme contents and trypsinogen, chymotrypsinogen, and amylase levels in the pancreatic homogenates prepared 24 hours after the last administration were measured [Goke B et al, 1986]. Levels of trypsinogen and chymotrypsinogen were measured as trypsin and chymotrypsin after activation.

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19. Wank SA: Cholecystokinin receptors. Am J Physiol 269:G628-646,1995

Event: 1724: Acinar cell proliferation

Short Name: Acinar cell proliferation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
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Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

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List of Adverse Outcomes in this AOP

Event: 1725: Pancreatic acinar cell tumors

Short Name: Acinar cell tumors

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:316 - Trypsin inhibition leading to pancreatic acinar cell tumors	AdverseOutcome

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

In monkeys receiving repeated dosing of the CCK1 receptor agonist GI181771X for up to 52 weeks, no hypertrophy or histopathological changes of the pancreas were observed, but these results differed from those of rats [Myer JR et al, 2014]. In humans, obese patients treated with GI181771X for 24 weeks showed no abnormal changes in the pancreas by ultrasonography or MRI [Jordan J et al, 2008]. On the other hand, oral ingestion of raw soya flour, which contains trypsin inhibitors, increased the release of CCK in humans [Calam J et al, 1987]. In addition, some epidemiological studies reported that the incidence of pancreatic tumors in humans administered high levels of protease inhibitors was decreased [Messina M and Messina V, 1991; Miller RV, 1978]. These results suggest no relevance between pancreatic growth/tumor development and CCK-agonist treatment in humans or non-human primates.

As indicated above, the effects of CCK on acinar cell proliferation differ between rodents and humans. In rodents, proliferation of pancreatic acinar cells is regulated directly via CCK1 receptors expressed on their surfaces. However, in humans, CCK1 receptor density on the surface of pancreatic acinar cells is low, and exocrine secretion is innervated by vagal afferent nerves, with little effect on acinar cell proliferation.

Key Event Description

Several reports have shown that increased blood CCK levels directly stimulate acinar cell proliferation via CCK1 receptors in rats as follows:

In rats with a sustained increase in the CCK level due to treatment with a CCK1 receptor agonist (CCK-8), acinar cell proliferation and pancreatic hypertrophy were induced [Folsch UR et al, 1978; Povoski SP et al, 1994]. Endogenous and exogenous increases in blood CCK levels induced pancreatic hypertrophy due to the direct action of CCK on acinar cells [Yamamoto M et al, 2003]. Repeated administration of the CCK1 receptor agonist GI181771X to rats and mice resulted in pancreatic injury, hypertrophy and diffuse/focal hyperplasia of acinar cells, and zymogen degranulation depending on the dose and dosing period [Myer JR et al, 2014].

Administration of the trypsin inhibitor A8947 to rats increased pancreatic weight; however, infusion of the selective CCK1 receptor antagonist MK-329 using an osmotic minipump completely diminished this effect of A8947 on pancreatic weight [Obourn JD et al, 1997].

These results indicate that CCK directly stimulates pancreatic acinar cell proliferation via CCK1 receptors, and trypsin inhibition enhances acinar cell proliferation due to an increased plasma level of CCK.

How it is Measured or Detected

Pancreatic acinar cell proliferation is evaluated based on measurements of pancreatic weight and DNA and RNA levels [Folsch UR et al, 1978; Povoski SP et al, 1994; Tashiro M et al, 2004], as well as histopathological examination [Povoski SP et al, 1994]. In these experiment, pancreatic weight, protein content, RNA content, DNA content, protein-DNA ratio, RNA-DNA ratio, pancreatic area per nucleus, and number of mitoses per 10,000 acinar cells could be determined. Among such parameters, Increased DNA content and number of mitoses per 10,000 acinar cells are indicative of acinar cell hyperplasia, and the others suggest pancreatic or acinar cell hypertrophy and increased pancreatic protein synthesis.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2028: Inhibition, trypsin leads to Increased monitor peptide

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI
Macaca fascicularis	Macaca fascicularis	Low	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Isoforms of trypsin are found in many species, for example, cationic and anionic trypsins (trypsins 1 and 2) and mesotrypsin in humans, cationic and anionic trypsins in cows, and anionic trypsin and P23 in rats [Chen JM and Claude Férec C, 2013; Fukuoka S and Nyaruhucha CM, 2002] . Despite differences among species, the three-dimensional structures of the isoforms are highly conserved among species, and the natural substrates for the enzymes are generally any peptide that contains Lys or Arg [Baird Jr TT, 2017]. The active site of trypsin has a specific catalytic triad structure composed of serine, histidine, and aspartate, and the flanking amino acid sequences are entirely conserved [Baird Jr TT and Craik CS, 2013; Baird Jr TT, 2017]. Therefore, trypsin inhibitors have comparable effects on the enzymatic activity of trypsin isoforms among animal species including humans and rats [Savage GP and Morrison SC, 2003].

MP secreted from rat pancreatic acinar cells into the small intestine stimulates I cells of the small intestinal mucosa to release CCK.

MP-like peptides are also found in rats and other mammalian species [Eddeland A and Ohlsson K, 1976]. Rat soluble trypsin inhibitor [Tsuzuki S et al, 1992; Tsuzuki S et al, 1991], human soluble trypsin inhibitor [Pubols MH et al, 1974; Kikuchi N et al, 1985], and bovine soluble trypsin inhibitor [Greene LJ and Giordano JS Jr, 1969; Guy O et al, 1971] are homologous peptides, all of which show trypsin inhibitory activity but no CCK-stimulatory activity [Miyasaka K et al, 1989a; Miyasaka K et al, 1989b; Marchbank T et al, 1998; Voet D and Voet JG, 1995].

Key Event Relationship Description

Pancreatic acinar cells secrete digestive enzymes including trypsin into the small intestine.

In rats, one of the pancreatic soluble trypsin inhibitors (TIs), monitor peptide (MP), is simultaneously secreted in the pancreatic juice. MP forms complexes with trypsin in the empty intestine, which keeps the intestinal level of free MP low. Once the gastric contents are transported to the small intestine, secretion of the pancreatic proteases including trypsin and MP is induced, where trypsin is used for protein hydrolysis, and the level of free MP is subsequently increased. The increased MP level stimulates CCK release from I cells lining the small intestinal mucosa via MP receptors, and the resulting increase in CCK stimulates exocrine secretion including MP from the pancreas. Increased MP further stimulates CCK secretion via a positive feedback loop as long as duodenal contents remain to consume trypsin for proteolysis.

After trypsin inhibitors are ingested, the intestinal content of free MP increases rapidly, especially in an empty intestine, via positive feedback regulation.

Evidence Supporting this KER

TBD

Biological Plausibility

Trypsin is a digestive enzyme secreted by pancreatic acinar cells that cleaves peptide bonds at the carboxyl end of basic amino acids (lysine and arginine). Secretion of pancreatic digestive enzymes including trypsin is regulated mainly by cholecystokinin (CCK) released from enteroendocrine I cells located in the duodenal mucosa of the small intestine [Wang BJ and Cui ZJ, 2007], and CCK release is controlled by multiple mechanisms [Caron J et al, 2017]. These mechanisms involve feedback regulation of trypsin-sensitive CCK-releasing peptides, one being positive feedback regulation of MP and the other negative feedback regulation of luminal CCK-releasing factor (LCRF) [Miyasaka K and Funakoshi A, 1998; Wang BJ and Cui ZJ, 2007; Guan D et al, 1990].

MP is one of the PSTIs in rats, which stimulates CCK release from duodenal enteroendocrine I cells as well as inhibition of trypsin activity. MP consists of 61 amino acids and has a molecular weight of approximately 6000. MP was first purified from rat pancreatic juice, and its amino acid sequence was subsequently determined [Iwai K et al, 1987; Lin YZ et al, 1990].

MP is bound to trypsin in the empty intestine.Once gastric contents are transported into the small intestine, secretion of the pancreatic proteases with MP is increased, where trypsin instead hydrolyzes these proteins, leading to an increase in the free MP level [Iwai K et al, 1988; Liddle RA, 1995; Graf R, 2006]. The increased level of MP stimulates CCK release from I cells, and then pancreatic exocrine secretion is stimulated [Liddle RA et al, 1992; Guan D et al, 1990; Cuber JC et al, 1990]. It was shown that MP binds to the surface of CCK-immunoreactive mucosal cells of the small intestine [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b].

Following the increased secretion of pancreatic enzymes, proteolysis decreases intestinal protein contents, which once again decreases the intestinal level of free MP due to the excess of trypsin and in turn CCK release [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006].

When raw soya flour (RSF), which contains trypsin inhibitory activity, or TIs such as camostat are ingested, trypsin activity is inhibited to increase the intestinal level of free MP especially in the empty intestine, followed by an increase in the blood level of CCK [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998]. TI ingestion-induced increases in blood levels of CCK leads to further CCK release due to increased pancreatic secretion of proteins including MP in a positive feedback manner. On the other hand, TIs may elevate the luminal concentration of LCRF to stimulate CCK release; however, this increase might not be as exaggerated as that of MP, because increased blood level of CCK does not induce further secretion of LCRF.

Empirical Evidence

No study has shown a relationship between the trypsin inhibitor dose or degree of trypsin activity and the luminal concentration of MP. Trypsin inhibitor dosing and CCK levels are presented here. Considering that MP directly stimulates CCK release from I cells in the small intestine in rodents, increased plasma CCK levels induced by TIs seems to be appropriate as a surrogate of increased luminal MP levels.

The plasma CCK8 level in rats after 18-hour fasting was 0.31 ± 0.05 pM (mean ± SE) and increased to 6.2 ± 1.8 pM 7.5 minutes after feeding and increased to 10.3 ± 1.8 pM 15 minutes after intragastric instillation of a soybean trypsin inhibitor [Liddle RA et al, 1984].

Immediately after oral feeding of camostat at 400 mg/kg in rats, the plasma CCK level increased 10-fold above that in controls, reached a maximum after 90 min, remained elevated for more than 6 h, and then returned to control levels

24 h after administration of camostat [Goke B et al, 1986].

Plasma concentrations of CCK were measured after administration of a single dose of 200 mg/kg camostat by gavage or 2.5 μg/kg CCK8 by subcutaneous administration to rats. The maximum CCK level, 9.6 ± 2.7 pM, was reached at 30 min after administration of CCK8, and that of 4.9 ± 1.2 pM over the time period of 15–240 min per animal with basal CCK concentration of about 2.5 pM [Douglas BR et al, 1989].

In isolated vascularly perfused rat duodenum/jejunum 30-min of infusion of trypsin with ovalbumin hydrolysate reduced CCK release by approximately 60% of that induced by the peptone alone. This effect was reversed by co-infusion of soybean trypsin inhibitor with the trypsin–peptone mixture [Cuber JC et al, 1990].

Eleven healthy volunteers consumed one of two meals: one containing raw soya flour and the other heat-treated soya flour. The two flours contained 34 and 3 mg trypsin/g flour, respectively. The peak CCK response was 16.8 ± 8.1 (mean ± SE) pmol/l for the raw soya flour diet versus 4.9 ± 2.8 pmol/l for the heat-treated soya flour diet ($P < 0.05$) [Calam J et al, 1987].

Uncertainties and Inconsistencies

In normal rats, positive regulation of CCK release by MP seems to require some level of pancreatic secretion before to be effective. In the presence of nutritional protein in the duodenum, trypsin is used for digestion of protein and increased levels of MP stimulates CCK release. On the other hand, after most of the protein is digested, increased free MP might be inactivated with excess of trypsin or other proteases, as follows [Foltz M, 2008]:

1) MP is degraded by trypsin and other proteases.

2) MP forms a complex with trypsin as other PSTIs.

3) MP forms a complex with trypsin, thereafter degraded by proteases.

Quantitative Understanding of the Linkage

TBD

Response-response relationship

No study has shown a direct quantitative relationship between MIE and KE1.

Time-scale

No study has reported the time from trypsin inhibition to alteration of intestinal MP content. However, as mentioned above, treatment with trypsin inhibitors or MP increased the plasma concentration of CCK within 30 min in rats.

Known modulating factors

Raw soya flour and trypsin inhibitors such as camostat inhibit trypsin activity, leading to an increase in CCK release from the upper intestine into the bloodstream, where the increased CCK released seems to be mediated by increased luminal concentration of MP due to trypsin inhibition [Green GM and Miyasaka K, 1983; Liddle RA et al, 1984; Goke B et al, 1986; Douglas BR et al, 1989; Cuber JC et al, 1990; Playford RJ et al, 1993; Obourn JD et al, 1997; Tashiro M et al, 2004; Komarnytsky S et al, 2011; Calam J et al, 1987] .

Known Feedforward/Feedback loops influencing this KER

MP stimulates CCK release from intestinal I cells, and the increased CCK level in turn promotes pancreatic acinar cells to secrete pancreatic enzymes including CCK-stimulating MP. Therefore, MP-mediated CCK release is under positive feedback regulation [Liddle RA, 1995; Wang BJ and Cui ZJ, 2007; Chey WY and Chang T, 2001] and the effects of trypsin inhibitors seem robust. As discussed previously, trypsin-sensitive LCRF released from intestinal mucosal cells also stimulate duodenal I cells to release CCK with negative feedback loop.

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Relationship: 2029: Increased monitor peptide leads to Increased blood CCK level

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Low	NCBI
Macaca fascicularis	Macaca fascicularis	Low	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Mixed	High

Monitor peptide and related peptides with trypsin inhibitory activity

Pancreatic secretory trypsin inhibitors (PSTIs) are found in the pancreatic juice of multiple mammalian species, including rodents and humans [Greene LJ et al, 1968; Pubols MH et al, 1974; Eddeland A and Ohlsson K, 1976; Kikuchi N et al, 1985]. Secreted PSTIs bind tightly to trypsin to protect against trypsin-induced auto-injury in the pancreas and intestinal tracts [Voet D and Voet JG, 1995].

In rats, two types of PSTIs have been isolated: monitor peptide (MP, also known as PSTI-I) and PSTI-II [Tsuzuki S et al, 1991; Tsuzuki S et al, 1992]. Both are similar in amino acid sequence; however, the former directly stimulates CCK release from intestinal I cells via their surface MP receptors, whereas the latter does not [Miyasaka K et al, 1989b; Yamanishi R et al, 1993a]. Human PSTIs do not directly stimulate CCK release from intestinal mucosal cells [Miyasaka K et al, 1989a]. PSTIs from other mammalian species including dogs and pigs might neither directly stimulate CCK release although no related reports are found.

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the bloodstream from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007].

In rats, in contrast to other mammalian species, MP secreted by pancreatic acinar cells plays a major role in protein-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of TIs increases the intestinal level of MP, especially after all nutrient protein is digested in the intestines, causing a subsequent increase in the blood level of CCK. Increased levels of CCK stimulate pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these CCK isoforms have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Key Event Relationship Description

Pancreatic exocrine secretion is regulated mainly by cholecystokinin (CCK) via multiple mechanisms. In the digestive system, CCK is released by I cells located in the duodenal mucosa of the small intestine. CCK release is at least in part under negative or positive feedback regulation mediated by trypsin-sensitive CCK-releasing peptides.

In rats, CCK release from I cells is regulated actively by monitor peptide (MP) secreted from pancreatic acinar cells in the presence of nutritional protein in the duodenum [Graf R, 2006].

In the empty intestine, secreted MP binds to trypsin and thus maintained at low intestinal levels; in this situation, CCK release is suppressed. Once the gastric contents are transported to the small intestine, secretion of pancreatic juice including trypsin and MP is stimulated, where trypsin is used for digestion, and the level of free MP is subsequently increased. The increased free MP level stimulates CCK release from I cells via MP receptors, and the resulting increase in CCK stimulates pancreatic exocrine secretion including MP. The resulting increased level of MP directly stimulates I cells to release CCK further; this positive feedback regulation might be continued as long as duodenal contents remain to consume trypsin for proteolysis.

Meanwhile, soon after nutritional protein is digested, free MP and excessive trypsin binds together to be subsequently degraded followed by decreases in blood level of CCK and pancreatic secretion. However, after ingestion of trypsin inhibitors, the intestinal concentration of MP is increased continuously with positive feedback manner due to inhibition of its degradation by trypsin.

On the other hand, in mammalian species including rodents, negative feedback regulation of trypsin secretion is mediated by trypsin-sensitive luminal CCK-releasing peptide (LCRF) secreted from the mucosa of the upper intestine into the intestinal lumen in response to dietary components such as amino acids and peptides. LCRF directly stimulates I cells to secrete CCK, with a resulting increase in trypsin secretion from pancreatic acinar cells, and trypsin then degrades LCRF, indicating negative feedback regulation of trypsin-mediated CCK release.

Evidence Supporting this KER

TBD

Biological Plausibility

Regulation of pancreatic secretion

Pancreatic exocrine secretion is controlled mainly by the gastrointestinal hormone cholecystokinin (CCK), which is secreted by CCK-producing I cells located in the mucosa of the small intestine. Multiple mechanisms are involved in the stimulation of CCK release [Wang BJ and Cui ZJ, 2007; Caron J et al, 2017].

Regulation of CCK release mediated by monitor peptide(MP) in rats

In rats, CCK release from I cells in the duodenal mucosa of the small intestine is regulated actively by MP [Miyasaka K et al, 1989a; Fushiki T et al, 1989; Iwai K et al, 1988; Miyasaka K and Funakoshi A, 1998], which consists of 61 amino acids with a molecular weight of approximately 6000. It was first purified from rat pancreatic juice, and its amino acid sequence was subsequently determined [Iwai K et al, 1987].

In the empty intestine, secreted MP is bound to trypsin and thus free MP is maintained at a low level in the intestine; in this situation, CCK release is suppressed. However, after the gastric contents are transported to the small intestine, proteases are postulated to be used for protein hydrolysis, allowing the amount of free MP to increase [Iwai K et al, 1988; Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006]. The increased MP stimulates mucosal I cells to release CCK via their surface MP receptors, stimulating pancreatic exocrine secretion [Liddle RA et al, 1992; Guan D et al, 1990; Cuber JC et al, 1990]. MP binds to the surface of CCK-immunoreactive mucosal cells in the small intestine [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b]. After proteolysis of the intestinal contents, the luminal level of free trypsin is increased, which causes the luminal MP level to return to a low level, followed by a decrease in CCK release [Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Graf R, 2006].

Another role of MP as a pancreatic secretory trypsin inhibitor (TI)

Similar to other pancreatic soluble TIs, MP forms complexes with trypsin in the empty intestine to prevent auto-injury by trypsin [Lin YZ et al, 1990; Voet D and Voet JG, 1995]. Once TI is ingested, TI–trypsin complexes are formed, and the intestinal level of free MP is increased to stimulate CCK release [Yamanishi R et al, 1993b], increasing the blood CCK level even on an empty intestine. TIs other than MP show no effect on CCK release [Miyasaka K, 1989a; Tsuzuki S, 1991].

Effects of TIs on MP-mediated CCK release

In contrast, once TIs are ingested, the intestinal concentration of MP is increased due to inhibition of its binding with trypsin and degradation, and the increased MP directly stimulates I cells to release CCK into the blood. In turn, the increased CCK stimulates pancreatic acinar cells to secrete MP as well as pancreatic enzymes, and the secretion of MP further upregulates CCK release via a positive feedback mechanism, especially under trypsin inhibition [Wang BJ and Cui ZJ, 2007; Liddle RA, 1995; Miyasaka K and Funakoshi A, 1998; Liddle RA, 1995].

Some studies have reported that intraduodenal injection of MP stimulates CCK release in rats with external biliary and pancreatic fistulae [Miyasaka K et al, 1989a; Longnecker DS, 1987].

Raw soya flour containing TIs and protease inhibitors such as camostat directly inhibit trypsin activity, and rats treated with these agents showed an increased blood level of CCK [Liddle RA et al, 1984; Goke B et al, 1986; Calam J et al, 1987; Douglas BR et al, 1989; Cuber JC et al, 1990; Playford RJ et al, 1993; Obourn JD et al, 1997; Tashiro M et al, 2004; Komarnytsky S et al, 2011] . The mechanism underlying the increase in CCK release by TIs is thought to involve an increase in the intestinal MP level resulting from trypsin inhibition [Iwai K et al, 1988; Cuber JC et al, 1990; Miyasaka K et al, 1989a].

CCK

CCK is a peptide hormone secreted by I cells located in the mucosa of the small intestine, and it regulates pancreatic exocrine secretion. CCK is secreted as peptide prohormone consisting of 150 amino acids. Several CCK isoforms exist, composed of different numbers of amino acids due to post-transcriptional modifications, although the amino acid sequence of the C-terminal end is common among these isoforms [Rehfeld JF, 2017; Wang BJ and Cui ZJ, 2007].

In addition, MP receptors are thought to be expressed on I cells, based on the findings that MP binds to CCK-positive cells in the mucosa of the small intestine, and this binding is inhibited by TIs [Yamanishi R et al, 1993a; Yamanishi R et al, 1993b].

Empirical Evidence

MP at concentrations ranging from 3×10^{-12} to 3×10^{-8} M stimulated CCK release from isolated mucosal cells from the rat duodenum in a dose-dependent manner with highest level at 15 minutes after stimulation [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced within a few minutes a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodenum/ jejunum [Cuber JC et al, 1990].

In rats with biliary and pancreatic fistula, duodenal infusion of MP at 0.9 µg/rat increased pancreatic secretion and the plasma CCK level [Miyasaka K et al, 1989a].

Sorted CCK-positive rat intestinal mucosal cells stimulated with 30nM MP increased the secretion of CCK in a time-dependent manner starting at 5 min after the start of MP incubation [Liddle RA et al, 1992].

Uncertainties and Inconsistencies

TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship

MP at concentrations ranging from 3×10^{-12} to 3×10^{-8} M stimulated mucosal cells isolated from the rat duodenum to release CCK in a dose-dependent manner [Bouras EP et al, 1992].

MP at a concentration range of 2–12 µg/mL induced a dose-dependent transient increase in portal CCK-like immunoreactivity in isolated vascularly perfused rat duodeojejunum MP at 36 µg/mL showed lower CCK release [Cuber JC et al, 1990].

Time-scale

MP stimulated CCK release from isolated mucosal cells from the rat duodenum, sorted CCK-positive rat intestinal mucosal cells, or isolated vascularly perfused rat duodenum/jejunum after or within several minutes from the incubation [Liddle RA et al, 1992; Bouras EP et al, 1992; Cuber JC et al, 1990].

Known modulating factors

In addition to by MP in rats, CCK release from duodenal I cells is stimulated by gastric contents containing fatty acids and amino acids, either directly by specific receptors such as Ca-sensing receptors and the G protein-coupled receptor GPR93 or indirectly by luminal CCK-releasing factors (LCRF) in rats and humans[Caron J et al, 2017]. In humans, LCRF is released from intestinal mucosal cells in response to amino acids and fatty acids, and the LCRF mediate negative feedback regulation of CCK release via LCRF degradation by trypsin [Wang BJ and Cui ZJ, 2007].

Known Feedforward/Feedback loops influencing this KER

In rodents, monitor peptide, a pancreatic secretory trypsin inhibitor, is secreted by pancreatic acinar cells along with trypsin and other digestive enzymes stimulated by CCK [Iwai K et al, 1988; Tsuzuki S et al, 1991]. Because MP binds tightly to trypsin [Voet D and Voet JG, 1995], trypsin inhibition increases the intraluminal concentration of MP in a positive feedback manner [Liddle RA et al, 1984; Wang BJ and Cui ZJ, 2007].

Meanwhile, in mammalian species including rodents, TIs might stimulate CCK release into the bloodstream via an increased luminal concentration of trypsin-sensitive CCK-releasing peptides secreted by duodenal mucosal cells [Miyasaka K et al, 1989c; Lu L et al, 1989; Guan D et al, 1990; Owyang C, 1994; Liddle RA, 1995; Spannagel AW et al, 1996; Herzig KH et al, 1996; Miyasaka K and Funakoshi A, 1998; Marchbank T et al, 1998; Li Y et al, 2000; Owyang C, 1999; Wang Y et al, 2002] . Increased blood level of CCK does not stimulate further secretion of LCRF different from the positive feedback regulation of CCK release by MP.

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Relationship: 2030: Increased blood CCK level leads to Increased acinar cell exocrine secretion

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Macaca fascicularis	Macaca fascicularis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Species differences in the mechanism of CCK release

Pancreatic exocrine secretion is controlled mainly by CCK released into the blood steam from intestinal mucosal I cells of the small intestine in response to the gastric contents transported to the intestine [Singer MV and Niebergall-Roth E, 2009; Rehfeld JF, 2017]. Peptides released from gastrointestinal digestion, along with fatty acids, are the main stimuli of CCK release involving several direct and indirect pathways [Caron J et al, 2017].

In humans and canines, amino acids and fatty acids in the gastric contents transported to the small intestine play a major role in stimulating CCK release, which regulates pancreatic exocrine secretion, but MP is not involved in exocrine regulation [Wang BJ and Cui ZJ, 2007]. CCK-stimulated pancreatic exocrine secretion seems to be regulated with negative feedback manner via LCRF.

In rats, however, different from other mammalian species, nutrient protein and protein hydrolysate stimulate CCK release and MP secreted by pancreatic acinar cells plays an active role in protein/protein hydrolysate-stimulated CCK release [Iwai K et al, 1988; Fushiki T et al, 1989]. Ingestion of trypsin inhibitors increases the intestinal level of MP, especially after all nutrient protein is digested in the intestines, causing a subsequent increase in the blood level of CCK. The increased CCK level stimulates pancreatic exocrine secretion of proteins including MP, which in turn further increases the release of CCK. This positive feedback response associated with MP secretion might lead to continuously elevated plasma levels of CCK [Liddle RA, 1995].

Species differences in CCKs

Several isoforms of CCK, including CCK-83, -58, -39, -33, -22, and -8, have been identified, and there are species differences in CCK isoforms (e.g., CCK-33, -22 and -58 are expressed in humans, CCK-58 in dogs, CCK-8, -33 and -58 in cats, CCK-22, -58, -3 and -8 in pigs, CCK-22 and -8 in rabbits, and CCK-58 in rats). All of these CCK isoforms have a highly conserved region of amino acids, and all are ligands of CCK1 receptors [Wang BJ and Cui ZJ, 2007].

Species differences in pancreatic exocrine secretion

In rats, CCK stimulates pancreatic exocrine secretion and acinar cell growth directly via CCK1 receptors expressed on the cell surface, and exocrine secretion is also innervated by vagal afferent nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014; Yamamoto M et al, 2003].

On the other hand, human pancreatic acinar cells do not express CCK1 receptors, and CCK-mediated exocrine secretion is regulated exclusively by innervation of vagal nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], although there is some evidence of direct stimulation of exocrine secretion of human pancreatic acinar cells [Murphy JA et al, 2008].

Species differences in CCK receptors

CCK1 and CCK2 receptors are expressed in various organs and tissues including digestive and nervous systems, and there are species differences in distribution and expression levels of the receptors.

In rats, pancreatic acinar cells express mainly CCK1 receptors and no CCK2 receptors [Bourassa J et al, 1999]. CCK1 receptors are also expressed in vagal afferent nerve fibers of the gastroduodenal tract. Stimulation of the vagal nerve via CCK1 receptors as well as via physical stimulation by stomach wall distention from ingested food also promotes pancreatic exocrine secretion [Dufresne M et al, 2006].

In humans, on the other hand, CCK2 receptors are dominantly expressed in pancreatic acinar cells, with low expression of CCK1 receptors [Nishimori I et al, 1999]. Ji reported the following: 1) the mRNA level of the CCK2 receptor is higher than that of the CCK1 receptor in the human pancreas; 2) an in situ hybridization experiment showed no expression of either receptor type in the human pancreas, and 3) human pancreatic cells did not show any response to the CCK1 receptor agonist CCK8 or the CCK2 receptor agonist gastrin in vitro [Ji B et al, 2001]. Therefore, human pancreatic acinar cells respond to CCK more weakly compared with the response in rodents.

Although the distribution of CCK receptors is different between humans and rodents, the structures of CCK1 receptors are highly conserved among mammalian species, with 98% homology between rats and mice, 90% between rats and humans, 98% between cynomolgus monkeys and humans, and 89% between dogs and humans [Wang BJ and Cui ZJ, 2007].

Key Event Relationship Description

Pancreatic exocrine secretion is regulated mainly by cholecystokinin (CCK) released by CCK-producing I cells located in the mucosa of the upper small intestine. CCK stimulates exocrine secretion directly via CCK receptors expressed on

acinar cell surfaces or indirectly via vagal afferent nerves expressing CCK receptors.

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin) receptors. The former shows high affinity to CCK and the latter to both CCK and gastrin [Wang BJ and Cui ZJ, 2007; Dufresne M et al, 2006].

There are species differences in CCK-mediated pancreatic exocrine secretion. In rats, exocrine secretion from pancreatic acinar cells is regulated directly by CCK1 receptors expressed on the surface of acinar cells and indirectly by vagal afferent nerves expressing CCK1 receptors. Meanwhile, in humans, pancreatic exocrine secretion is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007].

The major function of pancreatic exocrine secretion is the production and secretion of digestive enzymes. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes such as trypsinogen, chymotrypsinogen, proesterases, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted by acinar cells into the small intestine, where they are activated by pepsins and peptidases [Berg JM et al, 2002].

Evidence Supporting this KER

TBD

Biological Plausibility

Pancreatic exocrine secretion

The major function of pancreatic exocrine secretion is the release of digestive enzymes, fluid, and bicarbonate in response to food intake. Zymogen granules located at the apical site of pancreatic acinar cells contain the precursors of multiple digestive enzymes, such as trypsinogen, chymotrypsinogen, proesterase, procarboxypeptidase A and B, as well as pancreatic lipase and amylase α. These precursors are secreted into the small intestine, where trypsinogen is converted to trypsin by enteropeptidase, and the newly generated trypsin activates more trypsinogen molecules and other proenzymes [Berg JM et al, 2002].

Regulation of pancreatic exocrine secretion via CCK and CCK receptors

Pancreatic exocrine secretion is regulated mainly by CCK released from CCK-producing I cells located within the mucosa of the small intestine. CCK stimulates exocrine secretion either directly via CCK receptors expressed on acinar cells or indirectly by the vagovagal reflex via CCK receptors. There are species differences in these CCK regulatory mechanisms [Singer MV and Niebergall-Roth E, 2009; Chandra R and Liddle RA, 2009].

CCK receptor subtypes

There are two types of CCK receptors: CCK1 (CCK-A) and CCK2 (CCK-B or gastrin receptor) receptors. The CCK1 receptor exhibits high affinity to all CCK isoforms, whereas the CCK2 receptor exhibits affinity to both CCKs and gastrin [Dufresne M et al, 2006; Rehfeld JF, 2017].

Direct and indirect innervation-mediated regulation of exocrine secretion from acinar cells via CCK receptors

In rats, pancreatic acinar cells express mainly CCK1 receptors, and blood CCK directly stimulates exocrine secretion and acinar cell proliferation [Dufresne M et al, 2006]. Moreover, the vagal afferent nerves also stimulate pancreatic exocrine secretion; CCK stimulates CCK1 receptors expressed on the vagal afferent nerve fibers of the vago–vagal loop, and the acetylcholine generated acts on M3 muscarinic cholinergic receptors to promote pancreatic exocrine secretion [Bourassa J et al, 1999; Adler G, 1997; Ji B et al, 2001; Li Y et al, 1997; Owyang C, 1996].

In humans, the density of CCK receptors expressed on acinar cells is lower than that in rodents, whereas CCK2 receptors are dominantly expressed. Therefore, the responses of acinar cells to CCK seem to be weaker compared with rodents, and pancreatic exocrine secretion in humans is regulated mainly by vagal afferent nerves expressing CCK1 receptors [Wang BJ and Cui ZJ, 2007; Owyang C, 1996; Pandiri AR, 2014].

Empirical Evidence

In rats, diversion of bile pancreatic juice induced more than ten-times increase in plasma concentration of CCK at the end of two hours diversion and caused rapid and sustained increase in pancreatic protein secretion with more than two folds at 60 minutes of diversion compared with the basal levels [Li Y and Owyang C, 1994].

Repeated injections of CCK at 1390 IU s.c. for 3 weeks significantly increased the pancreatic levels and secretion of amylase and trypsin during stimulation with 60 IU/kg-hour of CCK. Peak secretion rates of the enzymes were obtained 45 minutes after the start of the stimulation [Folsch UR et al, 1978].

Trypsin-mediated feedback control of pancreatic enzyme secretion has also been observed in humans.

Intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Simultaneous intraduodenal perfusion of trypsin with phenylalanine lowered plasma CCK level at 24% and pancreatic output of chymotrypsin at 63% compared with the perfusion of phenylalanine alone. Moreover, intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].

Uncertainties and Inconsistencies

TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship

CCK action on the stimulation of pancreatic secretion is dose dependent. Doses of CCK that induce physiological concentrations of plasma CCK (up to ~10 pM) stimulate the vagal afferent pathway, whereas doses that produce supraphysiological CCK levels act to stimulate intrapancreatic neurons and pancreatic acini. The brief summaries are as follows:

Intravenous infusion of CCK-8 at 20 and 40 pM/kg/hour or high affinity CCKR agonist CCK-JMV-189 at 22, 44 and 88 μg/kg/hour in rats induced dose-dependent increases in pancreatic protein secretion from 15 minutes of infusion, which was blocked by the CCK1 receptor antagonist L-364,718 [Li Y et al, 1997].

Physiological level of plasma CCK (up to ~10 pM) result in stimulation of the vagal afferent pathway originating from the gastroduodenal mucosa, whereas doses that induce supraphysiological CCK levels result in stimulation of intrapancreatic neurons and pancreatic acini [Owyang C, 1996].

Time-scale

In rats in which bile and pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF stimulated a 1-h integrated increase in pancreatic protein output of 2.2 ± 1.1 mg/h (mean ± SE) [Jordinson M et al, 1996].

Bile-pancreatic juice diversion in rats increases pancreatic protein secretion with more than two fold 60 minutes after the start of diversion with elevated blood level of CCK [Li Y and Owyang C, 1994].

Intravenous infusion of CCK at 60 IU/kg/hour induces the pancreatic secretion of amylase and trypsin with peak level at 45 minutes after the start of the stimulation [Folsch UR et al, 1978].

In human intraduodenal perfusion of phenylalanine at 10mM, 5mL/min induced a several times increase in the plasma level of CCK within 15 minutes and a four-times increase in one-hour pancreatic outputs of trypsin and chymotrypsin. Intravenous infusion of CCK-8 at 20 and 40 ng/kg/h for 60 minutes showed a dose-dependent increase in pancreatic output of chymotrypsin [Owyang C et al, 1986].

These results suggest that CCK-induced pancreatic exocrine secretion occur within a short time after CCK infusion or stimulation of CCK release.

Known modulating factors

Disruption of the CCK1 receptor in rats also affects pancreatic exocrine secretion [Miyasaka K et al, 1998].

Capsaicin and atropine inhibit cholinergic vagus nerve reflexes to reduce CCK-mediated pancreatic enzyme secretion [Li Y et al, 1997; Yamamoto M et al, 2003; Li Y and Owyang C, 1994; Soudah HC et al, 1992; Owyang C et al, 1986].

Known Feedforward/Feedback loops influencing this KER

TBD

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Relationship: 2031: Increased acinar cell exocrine secretion leads to Acinar cell proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effect of CCK on acinar cell proliferation differs between rodents and humans.

In rats, CCK stimulates pancreatic exocrine secretion directly via CCK1 receptors expressed on the cell surface and also via innervation of afferent vagal nerves expressing CCK1 receptors [Singer MV and Niebergall-Roth E, 2009; Pandiri AR, 2014]. Higher plasma levels of CCK might also directly stimulate acinar cell proliferation via surface CCK receptors [Yamamoto M et al, 2003].

In contrast to rats, monkeys receiving repeated doses of the CCK1 receptor agonist GI181771X for up to 52 weeks showed no hypertrophy or histopathological changes in the pancreas [Myer JR et al, 2014]. Regarding humans, obese patients treated with GI181771X for 24 weeks showed no abnormal changes in the pancreas by ultrasonography or MRI [Jordan J et al, 2008]. Moreover, some epidemiological surveys suggested that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978], although oral ingestion of raw soya flour containing TIs was reported to stimulate CCK release in humans [Calam J et al, 1987].

These findings suggest that exocrine secretion in humans and primates is regulated exclusively by innervation of vagal afferent nerves expressing CCK1 receptors [Soudah HC et al, 1992; Beglinger C et al, 1992; Singer MV and Niebergall-Roth E, 2009], with little effect on acinar cell proliferation, although the possibility of direct stimulation of exocrine secretion from human pancreatic acinar cells has been suggested [Murphy JA et al, 2008].

Meanwhile, a strong relationship between pancreatic cancers and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported. Therefore, the effect of CCK on acinar cell proliferation in humans is controversial.

Key Event Relationship Description

In rats, an increased blood level of CCK stimulates pancreatic acinar cells to secrete digestive enzymes directly via surface CCK1 receptors and indirectly via innervation of vagal afferent nerves expressing CCK1 receptors. A persistent increase in the blood CCK level stimulates pancreatic acinar cell proliferation directly via surface CCK1 receptors. On the other hand, human pancreatic acinar cells express CCK2 receptors, which do not respond to CCK in terms of secretion and proliferation. Pancreatic enzyme secretion in humans is innervated by afferent vagal nerves expressing CCK1 receptors; however, its involvement in acinar cell proliferation is unclear.

Evidence Supporting this KER

TBD

Biological Plausibility

CCK-induced pancreatic acinar cell proliferation

An increased plasma level of CCK directly induces proliferation of pancreatic acinar cells via surface CCK1 receptors as well as exocrine secretion in rodents. Consuming raw soya flour for 30 days, administration of trypsin inhibitor in drinking water for 7 days, or repeated injection of cholecystokinin for 7 days induced pancreatic hypertrophy and hyperplasia [Yanatori Y and Fujita T, 1976]. Repeated administration of CCK for 21 days [Folsch UR et al, 1978] and treatment with the CCK8 and CCK1 receptor agonist A-71623 for 3 weeks [Povoski SP et al, 1994] also induced pancreatic hyperplastic changes in mice [Tashiro M et al, 2004]. Addition of 0.1% camostat in the diet for 10 days increased pancreatic weight and protein and DNA levels in a time-dependent manner in mice [Tashiro M et al, 2004].

The CCK1 receptor agonist GI181771X induced pancreatitis due to abnormal basolateral secretion of Zymogen

granules at the high dose and acinar cell hypertrophy at the middle and low doses in ratsan. The author mentioned JAK1/2–STAT1/3 activation leading to p38MAPK activation as a mechanism underlying acinar cell proliferation.

Direct effect of CCK on acinar cell proliferationvia CCK receptors

In rats, the trypsin inhibitor FOY-305 increased pancreatic weight and induced acinar cell hypertrophy, and denervation of vagal nerves had little effect on these hypertrophic changes [Aki T et al, 1989]. Administration of CCK-8 at physiological doses induced exocrine secretion, and atropine and vagal nerve denervation suppressed this exocrine secretion but not that induced by non-physiological doses of CCK-8 [Li Y and Owyang C, 1993]. These results suggest that the involvement of vagal nerve innervation in acinar cell proliferation under an increased blood CCK level might be low, and this may also be the case in humans, but the evidence is unclear [Chandra R and Liddle RA, 2009].

Empirical Evidence

KE3/KE4

In rats fed 20, 40, or 100% RSF-containing diet for up to 36 weeks, pancreatic hypertrophy was found in all RSF-fed groups, whereas hyperplasia was found only in the 40 and 100% RSF-fed groups [Crass RA and Morgan RG, 1982].

KE3

In rats in which bile/pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF increased the total amount of 1-h pancreatic protein output at 2.2 ± 1.1 mg/h (mean ± SE) with highest CCK levels at 30 or 40 minutes after RSF administration [Jordinson M et al, 1996].

KE4

In rats, administration of TIs in drinking water (“Trypsin soybean inhibitor” (Miles), 400mg/100mL) or injection of CCK (CCK-PZ or CCK-33,400 Ivy Dog unit) for 7 days increased acinar cell proliferation as well as acinar cell hypertrophy [Yanatori Y and Fujita T, 1976], and RSF feeding at libitum increased acinar cell proliferation from 7 to 28 days of treatment leading to hypertrophy and hyperplasia [Oates PS and Morgan RG, 1984].

These results showed that trypsin inhibition-induced acinar cell proliferation (hyperplasia) developed at higher TI doses compared with the development of pancreatic hypertrophy caused by increased secretion, and that pancreatic exocrine secretion and increased acinar cell proliferation were found at 1 h and 7 days, respectively, after the start of TI or CCK treatment.

Uncertainties and Inconsistencies

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in acinar cell proliferation after 7 days, followed by a decrease to control levels by 28 days [Obourn JD et al, 1997]. The reason why the TI-induced increase in acinar cell proliferation is transient is unclear.

In humans, the involvement of innervation of vagal nerves in acinar cell proliferation under an increased blood level of CCK might be low, but this is unclear [Chandra R and Liddle RA, 2009].

Quantitative Understanding of the Linkage

TBD

Response-response relationship

KE3 and KE4 in rats injected with CCK

In rats repeatedly injected subcutaneously with CCK at 7.5 or 30 Ivy dog units (IU) twice daily for 20 days, pancreatic wet weight and DNA content / 100g BW increased with a same manner compared with saline-treated rats, however, pancreatic output of amylase and trypsin in response to submaximal intravenous stimulation with CCK at 15 IU/kg/hour increased with dose-dependent manner. [Folsch UR et al, 1978].

KE3 and KE4 in rats treated with TIs

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm, or 56 days, at 0 and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in pancreatic weight, acinar cell proliferation, diffuse acinar cell hypertrophy, and the plasma CCK level after 7 days. The increases in pancreatic weight and the CCK level were maximum at day 14 and then maintained throughout the study, whereas acinar cell proliferation peaked at day 7 but then decreased to control levels by day 28 [Obourn JD et al, 1997]. MK-329, a specific CCKA receptor antagonist, completely abolished the increase in pancreatic weight induced by 30,000 ppm A8947 after 7 days [Obourn JD et al, 1997].

Weanling male Wistar rats were fed 15 diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%), as well as raw and heat-treated soy flour containing 10% protein. Rats were sacrificed at 3-month intervals, starting at 6 months, over a period of 22 months [Rackis JJ et al, 1985]. Trypsin and chymotrypsin activities per 100g BW, RNA and DNA contents of pancreas indicative of pancreatic hypertrophy and hyperplasia, respectively, were already increased in all of the TI and protein-

fed animals after 6-month dosing, although pancreatic nodules were increased in number at 15 months of dosing or later at 215 mg TI/100 g diet or higher [Liener IE et al, 1985].

Time-scale

In rats in which bile and pancreatic juice had been returned to the duodenum, intraduodenal administration of 30 mg RSF stimulated a 1-h integrated increase in pancreatic protein output of 2.2 ± 1.1 mg/h (mean ± SE) [Jordinson M et al, 1996].

Pancreatic hypertrophy was observed in rats fed an RSF-containing diet within 9 days [Rackis JJ, 1965; Watanapa P and Williamson RC, 1993].

Rats fed RSF showed a biphasic increase in acinar and duct cell proliferation, as determined by [3H]-thymidine incorporation into pancreatic DNA, on days 2–4 and again on days 7–28 after the start of RSF feeding. The first peak in DNA synthesis may represent a regenerative response to tissue damage. The second more delayed peak appears to represent the development of hyperplasia in response to a trophic stimulus [Oates PS and Morgan RG, 1984].

Rats administered TIs in drinking water for 7 days or repeatedly injected with CCK for 7 days [Yanatori Y and Fujita T, 1976] exhibited mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Oates PS and Morgan RG, 1984].

A8947, a broadleaf herbicide with trypsin inhibitory action, was fed to male rats for up to 28 days, at doses of 0, 300, 10,000, and 30,000 ppm. A8947 at 10,000 and 30,000 ppm induced significant increases in acinar cell proliferation after 7 days, followed by a decrease to control levels by 28 days [Obourn JD et al, 1997].

In the abovementioned studies [Rackis JJ et al, 1985; Liener IE et al, 1985], the increases in exocrine activity and acinar cell hyperplasia and hypertrophy were found at the earliest sacrifice (6 months). The exocrine activities and hypertrophic changes remained unchanged thereafter, whereas the hyperplastic changes became more pronounced until the final sacrifice (22 months).

These findings show that pancreatic exocrine secretion and increased acinar cell proliferation were found at 1 h and 7 days, respectively, after the start of TI or CCK treatment.

CCK was released within 1 h after intraduodenal administration of RSF, and acinar cell proliferation was elevated approximately 7 days after the start of RSF feeding, although some TIs induced transient acinar cell proliferation within 7 days as a regenerative change to acute pancreatic injury.

Known modulating factors

TIs including RSFs are reported to induce pancreatic acinar cell proliferation as well as acinar cell hypertrophy due to increased pancreatic protein secretion in rats. Administration of CCK receptor agonist and CCK also induce acinar cell hyperplasia and hypertrophy as follows.

Acinar cell changes induced by a CCK receptor agonist

A novel CCK1 receptor agonist, GI181771X, was administered to mice and/or rats at doses of 0.25–250 mg/kg/day from 7 days to 26 weeks, and pancreatic acinar cell responses were examined. The treated animals showed a wide range of morphological changes in the pancreas that were dose and time dependent, including necrotizing pancreatitis, acinar cell hypertrophy/atrophy, zymogen degranulation, focal acinar cell hyperplasia, and interstitial inflammation [Myer JR et al, 2014].

Acinar cell proliferation in rats injected with CCK

Rats 1) fed raw soybeans for 30 days, 2) administered TIs in drinking water for 7 days, or 3) repeatedly injected with CCK for 7 days exhibited increased mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Myer JR et al, 2014].

Known Feedforward/Feedback loops influencing this KER

TBD

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Relationship: 2032: Acinar cell proliferation leads to Acinar cell tumors

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Trypsin inhibition leading to pancreatic acinar cell tumors	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Rats fed a diet supplemented with soy and potato TI concentrates for 28 days developed pancreatic hypertrophy, and after long-term feeding (95 weeks), the rats developed nodular hyperplasia and acinar adenoma in a dose-dependent manner. Although mice responded similarly to rats to soy TIs in short-term (28 days) feeding experiments, they did not form these pathologies (hyperplasia or acinar adenoma) following long-term feeding. This considerable species difference suggests that the propensity to develop preneoplastic and neoplastic lesions in the pancreas is not predicted by short-term pancreatic hypertrophic and hyperplastic responses to TIs [Gumbmann MR et al, 1989].

The effects of TI-containing diets were evaluated in rats, mice, and hamsters for 30 weeks. In rats and mice, pancreatic weight and DNA, RNA, and protein levels increased in response to a diet consisting of RSF (which contains TIs). Only rats fed RSF developed reversible micro- and macro-nodules after 6 months of treatment, and longer treatment with RSF resulted in further growth in the pancreas and, ultimately, development of adenomas and carcinomas from pancreatic acinar cells [McGuinness EE et al, 1985].

The reasons for the abovementioned species differences in tumor outcome based on hyperplastic changes in acinar cells are unclear, even in rodents.

Meanwhile, a strong relationship between pancreatic cancer and a history of subtotal gastrectomy [Mack TM et al, 1986], which induced a higher plasma CCK level in response to fat [Hopman WP et al, 1984], was reported. On the other hand, some epidemiological surveys suggested that long-term ingestion of TI-containing foods does not increase the risk of pancreatic cancer [Miller RV, 1978], although oral ingestion of raw soya flour containing TIs was reported to stimulate CCK release in humans [Calam J et al, 1987]. Therefore, the effect of CCK on acinar cell proliferation in humans is controversial.

In cases where acinar cell proliferation is enhanced due to a certain treatment, the risk of acinar cell tumor formation may be high in humans as well as rodents.

Key Event Relationship Description

An increased blood level of CCK is the main factor responsible for a sustained increase in acinar cell proliferation and subsequent tumor formation.

Evidence Supporting this KER

TBD

Biological Plausibility

Trypsin inhibitor-induced pancreatic tumor formation

Ingestion of raw soya flour, which contains trypsin inhibitory activity, by rats for 2 years induced pancreatic hypertrophy due to acinar cell hyperplasia and acinar cell tumors [Rackis JJ et al, 1985; Woutersen RA et al, 1991]. Rats given raw soya flour or the trypsin inhibitor camostat exhibited pancreatic hypertrophy and acinar cell hyperplasia, and rats administered the pancreatic carcinogen azaserine followed by camostat exhibited acinar cell tumor formation [Gumbmann MR et al, 1986; Lhoste EF et al, 1988; Bell RH Jr et al, 1992].

Promotion of pancreatic acinar cell tumors via CCK

In addition, the suggestion that trypsin inhibition-induced pancreatic acinar cell tumor formation is promoted by increased acinar cell proliferation via CCK receptors is supported by the following study.
After initiating treatment with 30 mg/kg azaserine at 19 days of age, rats were treated with camostat, CCK8, or gelatin control, in combination with or without the CCK receptor antagonist CR-1409 (once daily, 3 days/week for 16 weeks). After 16 weeks, both camostat and CCK8 stimulated pancreatic growth and the development of azaserine-induced acidophilic putative preneoplastic foci. CR-1409 almost completely abolished the effect of CCK8 and significant attenuated the effect of camostat [Douglas BR et al, 1

Soybean trypsin inhibitor

KE4/AO:

Soy and potato trypsin inhibitor (TI) concentrates were prepared from defatted raw soy flour and potato juice. Rats and mice were fed a diet supplemented with each concentrate to provide 100 and 200 mg of trypsin inhibitor activity per 100 g of diet. In short-term (28 d) experiments in rats, both sources of TI induced pancreatic hypertrophy (KE4). After long-term feeding (95 weeks) in rats, soy and potato TI induced dose-related increases in pancreatic nodular hyperplasia and acinar adenoma (AO) [Gumbmann MR et al, 1989].

Rats were continuously fed diets containing lower amounts of raw soya flour (RSF, 5%, 25% and 50%) with weekly intraperitoneal injection of either azaserine at 5mg/kg BW or saline for up to 85 weeks or were fed RSF intermittently (2 days per week).After a maximum of 2 years of study, continuous feeding of as little as 5% RSF developed pancreatic micro/macroscopic nodules and stimulated the development of azaserine-initiated nodular hyperplasia and tumorigenesis. Intermittent feeding of 25 , 50 and 100% RSF also induced nodular hyperplasia. In addition, consuming a 100% RSF diet for 2 days per week resulted in the development of pancreatic cancer in some of the rats [McGuinness EE and Wormsley KG, 1986].

Protease inhibitor camostat:

KE4:

Adult Fischer 344 (F344) and Lewis rats fed camostat mixed in the diet to define a level that induced pancreatic hypertrophy and hyperplasia. As little as 0.02% fed 3 days per week was effective [Lhoste EF et al, 1988].

AO:

F344 rats were injected s.c. twice with azaserine at 30 mg/kg BW and thereafter were given camostat at 200 mg/kg BW by gavage 5 days a week until autopsy 18 weeks later. In addition, azaserine-treated Lewis rats were fed camostat in the diet at 0.5 g/kg diet for 4 weeks and then 0.2 g/kg diet 3 consecutive days a week for 8 or 16 weeks until autopsy. In these experiments the number and size of atypical acinar cell foci and nodules (AACN) were increased in comparison with the control groups. The data suggest a promoting effect of dietary camostat on the growth of azaserine-induced preneoplastic lesions in the pancreas of both rat strains [Lhoste EF et al, 1988].

989].

Sustained pancreatic growth (acinar cell proliferation) leading to acinar cell tumor formation

Rats fed a diet containing raw soya flour developed micro- and macro-nodules. Longer treatment with raw soya flour resulted in further growths in the pancreas and, ultimately, development of adenomas and carcinomas in the acinar pancreas. The pancreatic changes were reversible up to 6 months of consuming the raw soya flour diet but became irreversible thereafter [McGuinness EE et al, 1985].

Empirical Evidence

Soybean trypsin inhibitor

KE4/AO:

Soy and potato trypsin inhibitor (TI) concentrates were prepared from defatted raw soy flour and potato juice. Rats and mice were fed a diet supplemented with each concentrate to provide 100 and 200 mg of trypsin inhibitor activity per 100 g of diet. In short-term (28 d) experiments in rats, both sources of TI induced pancreatic hypertrophy (KE4). After long-term feeding (95 weeks) in rats, soy and potato TI induced dose-related increases in pancreatic nodular hyperplasia and acinar adenoma (AO) [Gumbmann MR et al, 1989].

Rats were continuously fed diets containing lower amounts of raw soya flour (RSF, 5%, 25% and 50%) with weekly intraperitoneal injection of either azaserine at 5mg/kg BW or saline for up to 85 weeks or were fed RSF intermittently (2 days per week).After a maximum of 2 years of study, continuous feeding of as little as 5% RSF developed

pancreatic micro/macroscopic nodules and stimulated the development of azaserine-initiated nodular hyperplasia and tumorigenesis. Intermittent feeding of 25 , 50 and 100% RSF also induced nodular hyperplasia. In addition, consuming a 100% RSF diet for 2 days per week resulted in the development of pancreatic cancer in some of the rats [McGuinness EE and Wormsley KG, 1986].

Protease inhibitor camostat:

KE4:

Adult Fischer 344 (F344) and Lewis rats fed camostat mixed in the diet to define a level that induced pancreatic hypertrophy and hyperplasia. As little as 0.02% fed 3 days per week was effective [Lhoste EF et al, 1988].

AO:

F344 rats were injected s.c. twice with azaserine at 30 mg/kg BW and thereafter were given camostat at 200 mg/kg BW by gavage 5 days a week until autopsy 18 weeks later. In addition, azaserine-treated Lewis rats were fed camostat in the diet at 0.5 g/kg diet for 4 weeks and then 0.2 g/kg diet 3 consecutive days a week for 8 or 16 weeks until autopsy. In these experiments the number and size of atypical acinar cell foci and nodules (AACN) were increased in comparison with the control groups. The data suggest a promoting effect of dietary camostat on the growth of azaserine-induced preneoplastic lesions in the pancreas of both rat strains [Lhoste EF et al, 1988].

Uncertainties and Inconsistencies

TBD

Quantitative Understanding of the Linkage

TBD

Response-response relationship

Hypertrophy/hyperplasia of acinar cells and tumor development in rats fed TI-containing diet were examined in the same rat study reported as follows:

Weanling male Wistar rats were fed 15 diets consisting of four concentrations of purified soybean TIs (93, 215, 337, and 577 mg/100 g diet) and three protein concentrations (10%, 20%, and 30%), as well as raw and heat-treated soy flour containing 10% protein. Rats were first sacrificed at 6 months and at 3-month intervals thereafter over a period of 22 months [Rackis JJ et al, 1985]. In this study, the following dose responses for KE4 and AO were obtained.

KE4:
Hypertrophy and hyperplasia of the pancreas determined by pancreas weight and RNA and DNA content developed at 6 months and were likewise positively correlated with the levels of TI and protein. Although the hypertrophic response remained unchanged, hyperplasia became more pronounced as the period of exposure to TI was prolonged [Liener IE et al, 1985].

AO:
Nodular hyperplasia of acinar cells was observed in the first sacrifice group at 6 months. Incidence of the lesion was positively related to both time of exposure and level of dietary TI. Acinar cell adenoma was first observed at 18 months and was most prevalent in rats fed the highest concentration of TI [Spangler WL et al, 1985].

Time-scale

KE4:

Several studies have suggested that acinar cell proliferation is induced approximately 7 days after treatment with TIs or CCK. Rats fed RSF showed a biphasic increase in the proliferation of acinar and duct cells on days 2–4 and again on days 7–28 after the start of RSF feeding. The first peak may represent a regenerative response to tissue damage. The second more delayed peak appears to represent the development of hyperplasia in response to a trophic stimulus [Oates PS and Morgan RG, 1984]. Rats administered TIs in drinking water for 7 days or repeatedly injected with CCK for 7 days exhibited increased mitotic figures in the acinar, centroacinar, and intercalated portions of the pancreas and in excretory duct cells, as well as marked pancreatic hypertrophy [Yanatori Y and Fujita T, 1976].

AO:

Increased CCK-mediated acinar cell proliferation might lead to acinar cell tumor formation, as shown by the following findings:
In rats fed soybean TIs, acinar cell hyperplasia was observed at the first sacrifice time point (6 months) and became more pronounced with prolonged TI exposure. Nodular hyperplasia of acinar cells was also found at 6 months and increased at later dosing periods. Acinar cell adenomas were first observed at 18 months of TI exposure [Liener IE et al, 1985; Spangler WL et al, 1985].

Morgan et al. reported that rats fed an RSF diet for 24 weeks developed pancreatic hypertrophy and hyperplasia, as determined by DNA, RNA, and protein contents in the pancreas, and developed more pronounced azaserine (30 mg/kg once a week for 5 weeks)-induced nodular hyperplasia compared with rats fed a heat-treated soy flour diet [Morgan RG et al, 1990].

Known modulating factors

Trypsin inhibition promotes acinar cell tumor formation.

TI-enhanced growth of azaserine-induced pancreatic preneoplastic lesions were reduced especially in size by the CCK receptor antagonist lorglumide (CR-1409) [Douglas BR et al, 1989].

Pancreatic growth was induced by cholestyramine, similar to that by TIs, presumably because of the bile salt-binding properties of cholestyramine. This finding suggests that removal of proteases and bile salts from the upper small intestine results in pancreatic growths, which may become neoplastic [McGuinness EE et al, 1985].

The thrombin inhibitor ximelagatran induced focal/multifocal acinar cell hyperplasia and adenomas in the pancreas of rats after 24 months of oral administration at 240 µmol/kg/day. However, in mice, no tumors formed after 18 months of treatment with ximelagatran. Treatment with dabigatran, which is in the same class as ximelagatran, showed no carcinogenicity in mice or rats [Stong DB et al, 2012].

Unsaturated fat (corn oil) was reported to promote the growth of azaserine-induced preneoplastic lesions and acinar cell tumors, without inducing pancreatic hypertrophy, in the rat pancreas [Woutersen RA et al, 1991].

Known Feedforward/Feedback loops influencing this KER

TBD

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