Relationships between leucine and the pancreatic exocrine function for improving starch digestibility in ruminants

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ABSTRACT

Four Holstein heifers (215 ± 7 kg; means $\pm$ SD), fitted with one pancreatic pouch, duodenal re-entrant cannulas, and duodenal infusion catheters, were used in this experiment. In phase 1, the 24-h profile of pancreatic fluid was determined. Pancreatic fluid flow peaked 1 h after feeding, but peaks of similar magnitude also occurred before the morning feed, necessitating 24-h collection of pancreatic fluid to estimate daily excretion. In phase 2, the effects of duodenal infusions of 0, 10, 20, or 30 g of leucine on pancreatic fluid flow were determined in a 4×4 Latin square design. The leucine was infused for 12 h in 2,500 mL of the infusate, and samples of pancreatic fluid and jugular blood were collected in 1-h intervals from the beginning of the infusion for 36 h. The results showed that the secretion rate of pancreatic fluid (mL/h) was significantly higher in 10-g leucine group than the other groups (mL/h). Protein concentration (mg/mL) in pancreatic fluid was elevated proportional to the amount of leucine infused. Leucine infusions increased both the concentration (U/mL) and secretion rate (U/h) of α -amylase. Infusion of 10 g of leucine also increased the secretion rates (U/h) of trypsin, chymotrypsin, and lipase, but did not change their concentrations. No significant effects of leucine infusions on plasma glucose and insulin concentrations were found. The results indicate that leucine could act as a nutrient signal to stimulate α -amylase production and pancreatic exocrine function in dairy heifers.

Key words: amino acid, duodenal infusion, digestive enzyme, insulin, cow

INTRODUCTION

Starch is a major component in concentrate feed-stuffs, providing a substantial energy source to highly

productive ruminants. Starch is mainly degraded by the rumen fauna and flora, but starch also has a considerable capacity to be digested in the small intestine. Reynolds (2006) suggested that starch digestion averages 695 g/kg of duodenal starch appearance in the small intestine. However, the capacity may be limited; for example, Matthé et al. (2001), in a summary of published data from nonlactating cattle, concluded that with increasing starch flow to the small intestine (from 250 to 1,800 g/d), starch digestibility in the small intestine decreased from 800 to 500 g/kg, respectively. When the capacity for starch digestion is limited, the part of starch reaching the small intestine that is digested before the ileal-cecal junction may decrease, and undigested starch is then fermented in the hindgut, resulting in energy waste and health risks (Gressley et al., 2011). Many factors limit starch digestion in the small intestine, among them, a lack of pancreatic α -amylase. Harmon et al. (2004) found that increasing α -amylase secretion might be a way to improve postruminal starch digestion in cattle.

Leucine is one of the essential amino acids for mammals as a substrate for protein synthesis and is considered an efficient nutrient signal regulating protein synthesis (Suryawan et al., 2011). Leucine, either administered orally or injected, increases protein synthesis in skeletal muscle, liver, heart, pancreas, and other tissues (Escobar et al., 2005; Yin et al., 2010; Wilson et al., 2011). However, Hashimoto and Hara (2004) found that leucine reduces the expression and activity of pancreatic α -amylase in rats. Thus, the role of leucine in protein synthesis is still not clear (Proud, 2007), and little information is available on the effect of leucine on digestive enzymes in ruminants. Rulquin and Pisulewski (2006) showed that a duodenal infusion of leucine affected milk protein yield in a curvilinear manner, with milk protein yield reaching a peak value of 82 g/d at an infusion rate of 40 g/d; then, protein yield was depressed at higher rates of infusion. This paper addresses the hypothesis that leucine administration influences pancreatic flow and alters the secretion of pancreatic enzymes in Holstein heifers.

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MATERIALS AND METHODS

The use of the animals and experimental protocols was approved by the Animal Care and Use Committee of the College of Animal Science and Technology of the Northwest A&F University, China.

Animals and Surgery

Four Chinese Holstein heifers (215 ± 7 kg; mean $\pm$ SD) were surgically prepared with a pancreatic pouch into which the main pancreatic duct drains, and 2 duodenal cannulas. Surgical procedures used were as described by St. Jean et al. (1992) with some modifications as follows.

General anesthesia was induced with a mixture of cyclopropane, methoxyflurane, and isoflurane. A 150 to 200 mm incision was made over the last rib and 8 cm from the lumbar vertebral transverse process, and extended down the rib to the costro-chondral junction. The periosteum was stripped and a segment of the last rib was removed, and the incision deepened into the abdominal cavity. The anatomical relationships between the duodenum, liver, and pancreas were explored by blunt dissection and the cranial lobe of the pancreas with pancreatic duct was located.

Pancreatic fluid was collected by placing a cannula into a 40 to 60 mm pouch of the duodenum into which the main pancreatic duct drained. The duodenal pouch was formed by clamping the duodenum 30 to 40 mm anterior and posterior to the pancreatic duct opening. One end of the segment was closed by oversewing the clamp, and a T-shaped cannula was inserted from the other end and pulled through a small incision in the duodenal pouch, which was opposite to the duct opening within the intestine canal. The other end of the pouch was closed, and both ends of the duodenal pouch were secured with a purse-string suture. The continuity of the intestine was restored by an end-to-end anastomosis.

After the cannulated pouch had been formed, another 2 cannulas were inserted into the duodenum, one 10 cm from the pylorus for infusions and the other with 10 to 15 cm posterior to the anastomosis for the return of pancreatic fluid. All 3 cannulas were exteriorized through small incisions in the abdomen wall, and the abdominal cavity was closed.

Diets

Heifers were fed at $1.2 \times NE_M$ requirement for cattle (NRC, 1996) on an alfalfa hay-based diet plus a supplement (Tables 1 and 2). Feed offered was adjusted on a BW basis for each period. The daily feed allowance was

Table 1. Composition and nutrient levels of experimental diets (DM basis)

Item	Content
Ingredient, %	
Alfalfa hay	80.0
Concentrate	20.0
Total	100.0
Nutrient level	
CP, %	12.57
MCP, ¹ %	9.4
ME, ¹ MJ/kg	9.62
Ca, %	0.92
P, %	0.33
Ash, %	5.65
Alfalfa hay composition, % of DM	
DM	86.16
OM	90.48
CP	11.97
NDF	55.13
ADF	40.07

¹MCP = metabolizable protein. The values of MCP and ME were estimated based on NRC (1996).

divided into 4 equal portions and fed at 0800, 1200, 1600, and 2000 h, respectively, and orts were removed daily before the 0800 h feeding. Heifers were housed in individual pens (3 m $\times$ 3 m) at a constant ambient temperature of 20°C with daily length of 16 h. Fresh water was supplied ad libitum.

Phase 1

Pancreatic fluid flow was measured continuously over a 7-d period at hourly intervals. The volume of the pancreatic fluid was measured, and the fluid was returned to the duodenum. The substantial variations

Table 2. Ingredients and nutrient contents of the concentrate supplement (DM basis)

Item	Value
Ingredient, % of DM	
Corn	70.00
DDGS	5.00
Corn germ feed	19.00
Limestone	1.40
CaHPO ₄	1.00
Maltose	0.60
NaHCO ₃	1.00
Salt	1.00
Mineral and vitamin premix ¹	1.00
Nutrient content, ² %	
CP	18.01
Ca	1.17
P	0.55
Ash	10.20

¹One kilogram of the premix contains 50 mg of Mn, 50 mg of Zn, 10 mg of Cu, 0.5 mg of I, 0.2 mg of Se, 2,200 IU of vitamin A, 275 IU of vitamin D, 25 IU of vitamin E, and 2,000 mg of nicotinic acid.

²Based on analyses of the samples.

in the fluid volume with multiple pulse patterns were observed, which necessitated continuous collections of the pancreatic fluid for 24 h to accurately represent daily pancreatic secretion. The heifers were then allowed to recover to the initial state for 7 d before the next infusion experiment.

Phase 2

The heifers were randomly assigned to a 4×4 Latin square design to determine the effects of duodenal infusion of leucine on pancreatic function. Infusions started at 0700 h using a peristaltic pump at the rate of 208 mL/h for 12 h. The infusate was either 0, 10, 20, or 30 g leucine dissolved in 2,500 mL of water, where 20 g was similar to the dose used by Rulquin and Pisulewski (2006).

The pancreatic fluid was continuously collected at hourly intervals for 12 h from the beginning of the infusion. The volume and pH of the fluid were recorded, and a 10% sub-sample was collected and stored at -30°C until analysis. The remaining fluid was returned into the duodenum. At the completion of the sampling period, the heifers were allowed a 7-d rest period to allow the cattle to return to a stable basal body state.

During the infusion and pancreatic collection period, blood samples (10 mL each) were collected hourly by jugular puncture into a heparinized syringe, immediately centrifuged at $2,500 \times g$ for 15 min at 4°C , and then plasma was collected and stored at -80°C until analysis.

Sample Analysis

Pancreatic fluid samples were analyzed for total protein concentration (Smith et al., 1985) and activities of α -amylase (Walker and Harmon, 1996), trypsin (Geiger and Fritz, 1986), and lipase (Xu et al., 2009). One unit of enzyme activity was defined as 1 μmol of the product released per minute at 39°C . Plasma insulin concentrations were measured using a commercial kit (Beijing North Institute of Biological Technology, Beijing, China).

Data Calculation and Statistical Analysis

In phase 1, the means and standard deviations of pancreatic fluid flow rate from the 4 heifers over 7 d were calculated for each sampling times.

In phase 2, the means of 12 measures, unless otherwise specified, were calculated, and then used for statistical analysis. Data were analyzed as a 4×4 Latin square design using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC) to examine effects

of treatments, animals, and periods. When the effect of treatment was significant ($P < 0.05$), the comparison was made between the means using Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Phase 1

Phase 1 was undertaken to determine daily patterns of pancreatic fluid secretion in heifers fed 4 times per day. The secretion of pancreatic fluid showed a multiple pulse pattern, and was stimulated an hour after feeding (Figure 1). Peak pancreatic flow decreased linearly until the early hours of the morning.

Phase 2

The secretion rate (mL/h) of pancreatic fluid was significantly increased by an infusion of 10 g of leucine ($P = 0.008$), whereas no significant differences were found between the control and 20 or 30 g leucine infusions (Table 3). Infusions of leucine had no influence on pH of pancreatic fluid ($P > 0.05$). Protein concentration (mg/mL) in pancreatic fluid was elevated ($P = 0.044$) proportional to the amount of leucine infused, and protein secretion rate (mg/h) was increased ($P = 0.024$) by leucine infusions, with the peak value for 10 g of leucine due to its highest flow rate of pancreatic fluid (Table 3).

As shown in Table 4, both the concentration (U/mL) and secretion rate (U/h) of α -amylase were increased by infusions of 10, 20, and 30 g of leucine ($P = 0.050$ and 0.007 respectively). For trypsin, infusion of 10 g of leucine increased the secretion rate (U/h; $P = 0.018$), but not the concentration (U/L). The secretion rate (U/h) of chymotrypsin was increased by infusions of 10 and 30 g of leucine ($P = 0.037$), but the concentration (U/L) of chymotrypsin was not affected ($P > 0.05$). The secretion rate (U/L) of lipase was increased by infusions of 10 and 20 g of leucine ($P < 0.001$) with no effect on lipase concentration ($P > 0.05$) (Table 4).

Infusions of leucine had no influence on plasma glucose concentration ($P > 0.05$), whereas an increased tendency was found for insulin concentration ($P = 0.091$; Table 5).

DISCUSSION

Diurnal pancreatic secretions in heifers were pulsatory with multiple pulses over 24 h and responding to feeding. Therefore, extended periods for collection of pancreatic fluid are essential to obtain representative samples of studies on pancreatic functions (Xu et

Table 3. Effects of duodenal infusion of leucine on the secretion rate, pH, and protein concentration of pancreatic fluid

Item	Leucine infusion, g				SEM ¹	P-value
	0	10	20	30		
Secretion, mL/h	122.3 ^b	175.2 ^a	131.1 ^b	128.4 ^b	7.74	0.008
pH	7.21	7.22	7.05	7.25	0.032	0.275
Protein						
mg/mL	6.03 ^c	6.93 ^{bc}	7.81 ^{ab}	8.54 ^a	0.351	0.044
mg/h	738 ^c	1,214 ^a	1,024 ^b	1,096 ^b	61.3	0.024

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Pooled standard error of the means, $n = 4$.

al., 2009). In phase 1, pancreatic secretion (Figure 1) increased significantly after each feeding, which is supported by previous reports (McCormick and Stewart, 1967; Xu et al., 2009). The reason may be related to the intermittent feeding and the resultant outflow to the small intestine (Xu et al., 2006). In contrary to our results, pancreatic fluid secretion had little or no post-prandial fluctuation in sheep (Shimomura et al., 2004, 2006). Similar results were also observed in steers fed 12 times a day (Walker and Harmon, 1995). Pancreatic fluid plays an important role in buffering and maintaining optimum conditions for the activities of pancreatic

enzymes in the small intestine for nutrient digestion, and any increase in secretion of pancreatic fluid may result in more efficient digestion of nutrients.

The duodenal infusion of 10 g of leucine significantly increased the pancreatic fluid secretion in this experiment. The secretion rate of pancreatic fluid, when expressed per unit of BW, ranged from 0.55 to 0.61 mL·kg⁻¹·h⁻¹, except for 0.81 mL·kg⁻¹·h⁻¹ for the 10 g of leucine group, in the current experiment, similar to previous reports (0.23 to 0.60 mL·kg⁻¹·h⁻¹; Swanson et al., 2002, 2004; Richards et al., 2003). Previous studies in our laboratory showed that infusions of 3,

Figure 1. Daily profile of pancreatic fluid flow rate in Holstein heifers in phase 1. The fluid was collected at hourly intervals for 24 h for a period of 7 d. Arrows indicate feeding times (0800, 1200, 1600, and 2000 h, respectively). Data are represented as means $\pm$ standard deviations ($n = 4$).

Table 4. Effects of duodenal infusion of leucine on secretion rate and activities of α -amylase, trypsin, chymotrypsin, and lipase in pancreatic fluid¹

Item	Leucine infusion, g				SEM ²	P-value
	0	10	20	30		
α -Amylase						
U/mL	157 ^b	205 ^a	193 ^a	219 ^a	8.3	0.050
U/mg of protein	26.1	29.6	24.8	25.7	3.10	0.326
U/h	18,714 ^c	35,261 ^a	25,380 ^b	27,990 ^b	2,985.3	0.007
Trypsin						
U/L	1,458	1,539	1,554	1,578	136.0	0.122
U/mg of protein	0.242	0.222	0.199	0.185	0.0101	0.212
U/h	178 ^b	270 ^a	204 ^b	203 ^b	37.2	0.018
Chymotrypsin						
U/L	1,187	1,331	1,246	1,488	128.9	0.106
U/mg of protein	0.197	0.192	0.160	0.174	0.037	0.461
U/h	145 ^c	233 ^a	163 ^c	191 ^b	22.8	0.037
Lipase						
U/mL	336	368	427	396	40.5	0.069
U/mg of protein	55.72	53.10	54.67	46.37	10.140	0.158
U/h	41,099 ^c	64,483 ^a	55,975 ^{ab}	50,840 ^{bc}	2,033.0	<0.001

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹One unit of enzyme is defined as 1 μ mol of the product formed per minute at 39°C.

²Pooled standard error of the means, $n = 4$.

6, or 9 g/d of leucine in goats for a period of 10 h affected secretion of pancreatic fluid in goats with a quadratic dose-response curve, and the greatest value was obtained with 3 g/d leucine infusion (Yu et al., 2014a). Another study in rats revealed that luminal nutrient signals could change pancreatic secretion by up to 50% (Li et al., 2000). However, no changes in pancreatic fluid secretion were observed when starch or casein was infused into the abomasum (Richards et al., 2003; Swanson et al., 2004). A direct comparison between species needs to be treated with caution due to the difference in digestive systems between rats and ruminant species. Further research will be required to verify if this increase in pancreatic secretion is from stimulating function of leucine.

In this experiment, protein excretion rate (mg/h, the product of protein concentration in the fluid with flow rate) was significantly increased by leucine infusion, especially the 10 g level. The increase of protein concentration was likely attributed to the function of leucine as an anabolic agent that stimulates protein synthesis by modulating the activation of the mTOR pathway and signaling components of translation initiation in various tissues of species (Yin et al., 2010; Suryawan et

al., 2011; Wilson et al., 2011). Rulquin and Pisulewski (2006) reported that duodenal infusions of leucine promoted mammary protein output. However, no responses in milk yield and composition have also been reported in cows supplemented with rumen-protected leucine tablets (Krizova et al., 2008). It should be pointed out that the effect of leucine on protein synthesis seems to be tissue specific, and leucine stimulates protein synthesis in muscles and liver, but not in heart, kidney, and jejunum (Wilson et al., 2011). The protein concentration in pancreatic fluid and the flow rate in current experiment ranged from 6.03 to 8.54 mg/mL and 738 to 1,212 mg/h, respectively, lower than the values in previous reports. For example, Richards et al. (2003) reported protein concentration in pancreatic fluid ranged of 9.65 to 10.87 mg/mL and the flow rate from 1,414 to 1,645 mg/h. The results from Swanson et al. (2004) were even higher (24.1 to 32.2 mg/mL and 1,600 to 2,130 mg/h, respectively). One possible reason for the difference between our results and the reported values is the physiological stages of the animals used, and young heifers were used in this experiment. Calves have low pancreatic protein secretion, and the secretion increases with age (McCormick and Stewart, 1967).

Table 5. Effects of duodenal infusion of leucine on plasma glucose and insulin concentrations

Item	Leucine infusion, g				SEM ¹	P-value
	0	10	20	30		
Glucose, mmol/L	3.37	3.69	3.53	3.55	0.211	0.254
Insulin, pmol/L	78.4	82.9	86.1	75.3	8.46	0.091

¹Pooled standard error of the means, $n = 4$.

In addition, different nutritional status among various studies may also spare effects on protein secretion in pancreatic fluid.

The increase in dietary concentrations of amino acids could stimulate muscle (Dreyer et al., 2008) and milk (Burgos et al., 2010) protein synthesis, and among amino acids, leucine is the most effective by regulating the mTOR (mammalian target of rapamycin) networks in different organs (Kim, 2009; Vianna et al., 2010), such as the pancreas (Sans et al., 2006).

Walker and Harmon (1995) identified that the lack of α -amylase activity may be a major factor limiting the efficiency of starch digestion in the small intestine. This experiment clearly demonstrated leucine infusions significantly increased α -amylase activity, including both the concentration and the secretion rate. The results of this experiment are supported by a previous report from this laboratory that the secretion rate of α -amylase in goats was increased by infusions of 3 or 6 g/d of leucine over a period of 10 h (Yu et al., 2014a).

Leucine infusions had no effects on the concentrations (U/L or U/mL and U/mg protein) of trypsin, chymotrypsin, and lipase in the pancreatic fluid, but infusion of 10 g of leucine increased the secretion rate (U/h) of all 3 enzymes. The increases in the secretion rate with no significant changes in their concentrations may indicate that infused leucine had more profound effects on stimulation of pancreatic fluid secretion over enhancement of synthesis of these proteins. Amino acids play an essential role in provision of the substrate for the synthesis of all the enzymes. In contrast, Swanson et al. (2003) identified that provision of amino acids alone decreased trypsin release from pancreas tissue of calves *in vitro*, but supply of amino acids plus casein increased trypsin release. Yu et al. (2014a) reported reductions of the trypsin secretion rate in goats with leucine infused for a period of 10 h, no changes with leucine infused for 14 d, but when leucine was infused for 21 d the trypsin secretion rate was increased (Yu et al., 2014b). These reports imply that the response of trypsin secretion to leucine appears to be a slow process.

The information about effects of amino acids on activities of chymotrypsin and lipase in ruminants is scarce. Yu et al. (2014a) reported that leucine infusion had no effect on lipase activity in pancreatic fluid of goats (Yu et al., 2014a). One possible explanation for the contradictory effects of leucine on the activities of protease and lipase could be that the dosages of leucine differed in different experiments, which could affect the balance of amino acids and the synthesis of protein in the small intestine. Besides, the ages of animals, the length of infusion period, and the diet composition could also result in variations in outcomes. More studies are required, therefore, to reveal the effects of amino

acids on activities of digestive enzymes, particularly proteases, in ruminants.

Our results showed that duodenal infusion of leucine had no significant effect on plasma insulin concentration. Insulin is an important component of pancreatic α -amylase regulation in nonruminants (Brannon, 1990), but little information is available on the relationship between insulin and pancreatic α -amylase secretion in ruminants. Anthony et al. (2002) found that increases in serum insulin were involved in the leucine-induced stimulation of muscle protein synthesis in young adult rats. Pancreatic enzyme secretion was decreased in diabetic rats, but restored to the normal level when insulin was infused (Otsuki and Williams, 1982), suggesting that insulin plays a role in the regulation of α -amylase secretion. Leucine acts as an insulin secretagogue and can increase circulating insulin levels. Swanson et al. (2004) suggested that plasma insulin concentrations might not be related to α -amylase secretion. Although the plasma insulin concentration did not mirror the pancreatic secretion of α -amylase in this study, it does not rule out roles of insulin in protein synthesis. Insulin plays differential roles in modulating protein synthesis in various tissues in the neonatal pigs (Suryawan et al., 2009). Further studies are needed to evaluate the regulatory mechanisms of leucine, via insulin, on pancreatic enzymes in ruminants.

CONCLUSIONS

Intestinal supply of leucine enhanced the concentration and secretion of pancreatic α -amylase in heifers. Leucine could act as a nutrient signal to stimulate the secretion of α -amylase, trypsin, chymotrypsin, and lipase, and the responses appeared to be dose and time dependent. Defining specific mechanisms through which leucine regulates secretion of α -amylase could lead to the development of applicable means to increase α -amylase secretion for starch digestion in small intestines.

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