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Enteral Pancreatic-like Enzymes of Microbial Origin affect Insulin Release during an Intravenous Glucose Tolerance Test

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Abstract

We have previously shown that the presence of pancreatic enzymes in the gut lumen of exocrine pancreatic insufficient pigs influences blood glucose and insulin levels during an intravenous glucose tolerance test (IVGTT). The present study aims to highlight the effects of orally applied pancreatic-like enzymes on blood glucose and plasma insulin levels during an IVGTT in young intact pigs.

Five, 7-week old pigs were fed with pancreatic-like enzymes of microbial origin, a proteinase (from *Aspergillus melleus*), α-amylase (from *Aspergillus oryzae*) or lipase (from *Burkholderia cepacia*) alone or in combination with the Ca/Na salts of α-ketoglutaric acid (AKG). One hour following administration of the various supplements an IVGTT was performed. Blood samples were withdrawn during the 2 hours of IVGTT for glucose and insulin analyses.

Blood glucose during the IVGTT was identical following administration of all combinations of the various enzymes or enzyme mixtures. Enteral loading of amylase or any amylase containing mixture resulted in reduced insulin secretion while administration of proteinase or any proteinase containing mixture resulted in enhanced insulin secretion during IVGTT, as compared to the control water vehicle. Lipase or AKG and lipase or AKG containing mixtures did not affect insulin secretion. Thus, it can be suggested that host amylase/protease ratio and their amount in pancreatic juice can participate in regulation of insulin release, thus, possibly affecting development of obesity and diabetes type 2.

Keywords: α-amylase; Proteinase; Insulin

Introduction

Insulin misregulation has considerable impact on health status [1]. Increased consumption of diets rich in carbohydrates and proteins results in the overproduction of insulin, leading to the development of insulin resistance and undesirable anabolic activity. Since the assimilation of nutrients is regulated in multiple stages, one possible way to improve insulin regulation would be to improve the clearance of glucose from the blood stream, in an insulin independent way. This could be achieved by using appropriate functional dietary supplements, which in turn could trigger alternative mechanisms of glucose assimilation. Results from previous studies in our lab have suggested that both pancreatic enzymes and AKG are involved in the regulation of blood glucose [2,3].

The role of pancreatic enzymes in the digestion of nutrients in the intestine is well studied and recognized. E.g., enzymatic activity of pancreatic α-amylase is responsible for formation of postprandial hyperglycaemia [1,4]. However, the involvement of the pancreatic enzymes in the regulatory mechanisms responsible for the regulation of the postprandial glycaemia seems to be overlooked. Blood glucose regulation is believed to be accomplished mostly by the endocrine pancreas, liver and kidneys, as well as various gastrointestinal hormones which influence insulin secretion [5,6]. However, glucose intolerance, together with a delayed insulin release has been observed during exocrine pancreatic insufficiency in both humans and pigs [3,7]. Such changes are recognized as a pancreatogenic type of diabetes (type 3c) and are suggested to occur together with exocrine pancreatic insufficiency or pancreas injury [8]. In humans, the influence of pancreatic enzyme replacement therapy (PERT) on glucose metabolism remains controversial. Improved blood glucose control and reduced glycated haemoglobin, following PERT, has previously been observed [7,9]. However, other studies have observed no effect [10-12]. By using young pigs with induced exocrine pancreatic insufficiency, we found that the presence of pancreatic enzymes in the gut lumen resulted in improved direct utilization of blood glucose and improved growth performance, without reinforcing insulin release [3]. Thus, we were interested to investigate whether exogenous digestive enzymes could alter glucose tolerance or insulin response in healthy pigs, especially during the growth period, using microbial enzymes, which mimic endogenous digestive pancreatic enzymes and are widely available. We also hypothesized that another potential supplement, α-ketoglutaric acid (AKG), could have an impact on post-loading glycaemic control, based on several previous reports [2,13,14]. An intravenous glucose tolerance test (IVGTT) was chosen as a convenient and appropriate method for testing the glucose-stimulated insulin response and capacity for blood glucose clearance [15]. The main advantages of an IVGTT is that it can reveal the loss of glucose tolerance at an early stage of metabolic syndrome and type 2 diabetes development by

detecting lower blood glucose utilization. An IVGTT excludes the oral route of glucose delivery together with the associated enteral hormone secretion. Young healthy pigs were used as the experimental model because of their physiological similarity to humans, good reproducibility, and ability to be trained, which decreases stress under experimental conditions [16,17]. For those reasons, we studied the effects of supplementation of pancreatic-like enzymes of microbial origin alone, and in combination with AKG, on blood glucose tolerance and plasma insulin response during an IVGTT in young healthy pigs.

What is the central question of this study?

Do pancreatic-like enzymes influence glucose tolerance during an IVGTT?

What is the main finding and its importance?

Oral supplementation with α-amylase of microbial origin prior to an intravenous glucose tolerance test resulted in decreased insulin release with further increased insulin sensitivity while supplementation with proteinase decreased insulin sensitivity by increasing insulin release. The mechanism behind this finding is not understood, but pancreatic – like enzyme dietary supplementation may be considered as an alternative for improving blood glucose regulation under healthy, diabetic/obese conditions.

Materials and Methods

Animals

The study was approved by the Malmo/Lund Ethical Review Committee on Animal Experiments. The experiments were performed on 5 crossbred ((Yorkshire × Swedish Landrace) × Hampshire) pigs at an age of 7 weeks (9.6 ± 0.7 kg). The pigs were housed individually in pens in the same stable at 20 ± 2°C and with lights on from 7.00-19.00. The pigs had free access to water via a drinking nipple and were fed a commercial pelleted pig feed (Vaxtill 320, Lantmännen, Sweden). In total, the feed was offered in an amount of 4 % of the pigs body weight (0.504 MJ/kg/day) which was divided between the morning and afternoon meal. The pigs were accustomed to the experimental conditions for 1 week prior to commencement of the experiment in order to prevent stress during the experimental period.

In order to collect blood samples during the experiments, surgery was performed to implant a catheter into the anterior *vena cava*, via the external jugular vein. After an overnight fasting period the pigs were pre-medicated with azaperone (Stresnil®, Janssen Pharmaceutica, Belgium, 2.2 mg/kg, intramuscularly) and washed using surgical soap. The pigs were then anaesthetized via an inhalation mask with a 0.5-1.5% air mixture of Fluothane® (Astra Läkemedel, Sodertälje, Sweden) in O_2 as a carrier gas at approximately 0.5-1 L/min, using a close-circuit respiratory flow system (Komesaroff Medical Developments, Melbourne, Australia). Surgical anaesthesia was indicated by a lack of corneal reflex. Post-operative pain was prevented by administration of buprenorphine (Temgesic®, Schering-Plough AB, 0.01 mg/kg, intramuscularly) for 1 day. Ampicillin (Doktacillin, Astra Läkemedel, Södertälje, Sweden) was administered i.v. (15 mg/kg) and at the incision site (250-500 mg).

At the end of the experimental period, the pigs were euthanized by an i.v. injection of an overdose of pentobarbital sodium (Allfatal Vet. Omnidea, Stockholm, Sweden, 100 mg/kg,) and post-mortem examinations were performed.

Experiments and design

Pancreatic-like enzymes of microbial origin (Proteinase P4032 from *Aspergillus melleus*; Amylase A9857 from *Aspergillus oryzae*; and Lipase 534641 from *Burkholderia cepacia,* all from Sigma-Aldrich) were tested separately, as a mixture and in combination with AKG, as oral supplements (A – α-amylase; P – proteinase; L – lipase, PAL – mixture of proteinase+ amylase+ lipase, PA – mixure of proteinase+ amylase, PL – mixture of proteinase+ lipase; AL – mixture of amylase+ lipase; AKG – α-ketoglutaric acid; +AKG – mixture of enzyme, or enzyme combination with α-ketoglutaric acid). The dose of the enzymes per pig (3.37 g of α-amylase, 100 mg of proteinase and 100 mg of microbial lipase), was chosen to match the corresponding enzymatic activities of 4 capsules of commercial pancrelipase (Creon® 10,000, Abbott Healthcare Products Ltd, Southampton, United Kingdom) as used in a previous study in pigs [18-20].

The proteolytic activity was measured using casein as a substrate and with Folin's reagent detecting the released tyrosine. Amylolytic activity was analysed using ethylidene-pNP-G7 (4,6-ethylidene-p-nitrophenyl-alpha, D-maltoheptaoside) as the substrate, according to the manufacturer's instructions (Infinity Amylase Liquid Stable Reagent; Thermo Electron, Victoria, Australia). Lipase activity was analysed using 1,2-o-dilauryl-rac-glycero-3-glutaric acid (6'-ethylresorufin) ester as the substrate (Randox Laboratories, Crumlin Co. Antrim, Northern Ireland, United Kingdom), according to the manufacturer's instructions. The specific activity in 1 mg of individual enzymes were measured at 37°C and compared with the activity of 1 pancrelipase capsule dissolved in PBS buffer (pH 7.2).

The AKG supplement consisted of 50% of Na_2-AKG × $2H_2O$ (0.144 g/1 kg body weight), and 50% of Ca-AKG (0.127 g/1 kg body weight) which is equivalent to 1% of a single feed portion (equal to 0.02% of body weight). The dose of AKG was based on that used in previous publications involving porcine models [21,22].

The lyophilized microbial enzymes, and the AKG salts were dissolved in 30 ml of tap water and sweetened with saccharin (3 tablets of Hermesetas mini sweeteners, Valora Trade Denmark Food, Herlev, Denmark).

These enzyme solutions were administered orally using a syringe within 2 minutes after which the pigs received another 40 ml of tap water to rinse the mouth and throat. As a control solution, 30 ml of sweetened water, without enzymes or AKG, was administered. Control feedings were performed on 3 occasions during the experimental period and the average value of these was used since no significant differences between control data were seen. The experimental design is shown in Table 1.

IVGTT

The IVGTT was performed on the pigs following an overnight fast of about 20 hours. One hour prior to the glucose infusion, oral administration of the enzyme, enzyme+AKG or control solutions was performed. Glucose loading was carried out with a sterile 50% D-glucose solution (0.5 g/kg body weight) infused via the venous catheter during 60 seconds. Immediately after this, the catheter was flushed with 5 ml of 0.9% sterile saline solution. Blood samples were drawn from the jugular catheter at 1 minute before (time point 0), and at 5, 15, 30, 45, 60 and 120 minutes after the glucose infusion. Blood was collected into 5 ml syringes containing EDTA (0.20 mg) and a protease inhibitor, aprotinin (1000 kIU, Bayer, Leverkusen, Germany) and immediately cooled on ice. The blood was then centrifuged at 3000

Pig / Day	1	3	5	7	9	11
I	Control	Amylase	AKG	Amylase+AKG	Control	Lipase
II	Control	Amylase	AKG	Amylase+AKG	Control	Lipase
III	Control	AKG	Amylase+AKG	Amylase	Control	Lipase
IV	Control	AKG	Amylase+AKG	Amylase	Control	Lipase+AKG
V	Control	Amylase+AKG	Amylase	AKG	Control	Lipase+AKG

Pig / Day	13	15	17	19	21	23
I	Lipase+AKG	Proteinase	Proteinase+AKG	Control	PAL	PAL+AKG
II	Lipase+AKG	Proteinase	Proteinase+AKG	Control	PAL	PAL+AKG
III	Lipase+AKG	Proteinase	Proteinase+AKG	Control	PAL	PAL+AKG
IV	Lipase	Proteinase+AKG	Proteinase	Control	PAL+AKG	PAL
V	Lipase	Proteinase+AKG	Proteinase	Control	PAL+AKG	PAL

Pig / Day	25	27	31	31	33	35
I	PA	PA+AKG	AL	AL+AKG	PL	PL+AKG
II	PA	PA+AKG	AL	AL+AKG	PL	PL+AKG
III	PA	PA+AKG	AL	AL+AKG	PL	PL+AKG
IV	PA+AKG	PA	AL+AKG	AL	PL+AKG	PL
V	PA+AKG	PA	AL+AKG	AL	PL+AKG	PL

Table 1: Experimental design. AKG - α-ketoglutaric acid salt (50% of Na_2-AKG *$2H_2O$+50% of Ca-AKG), PAL - pancreatic-like enzymes mixture, PA –proteinase+amylase; PL – proteinase+lipase; AL – amylase+lipase.

$\times g$ for 15 min at 4°C and plasma was separated and stored at -20°C until analyses.

Analysis of blood glucose and plasma insulin and C-peptide

Glucose was measured in the fresh blood samples using a glucose-meter with test strips (Accu-Chek® Aviva, Roche Diagnostics, Mannheim, Germany). Plasma insulin and C-peptide levels were measured using porcine insulin or C-peptide ELISA kits, respectively (Mercodia, Uppsala, Sweden), mainly according to manufacturer's protocol, but with an increased sample volume (50 μL) and an additional standard point at the lower end of the standard curve, obtaining a lower detection limit of 0.2 pmol/L for insulin and 2 pmol/L for C-peptide.

Statistical analysis and calculations

The total area under the curve (AUC) was calculated for the post-infusion glucose, insulin and C-peptide curves using the trapezoidal rule.

Insulin sensitivity was calculated as a surrogate index (S2), using the classic glucose disappearance rate (k) related to released insulin concentration during 0-30 minutes, $30*k/_{0-30}AUC_{insulin}$. The glucose disappearance rate is the negative slope of the linear regression of the logarithm of glucose, in a 25-min interval from 5 to 30 min $(k = \frac{Ln1 - Ln2}{T2 - T1})$. The incremental area under the curve of insulin concentration from 0 to 30 min was calculated using the trapezoidal rule ($_{0-30}AUC_{insulin}$). The units of the index are 1/min*pM [15].

Statistical analysis was performed using Prism, version 6 (GraphPad Software, Inc, San Diego, CA, USA) and R (v. 3.0.1) programming environment. The differences for the AUC and S2 between treatments and control values were determined using a one-way Anova with a Dunnett's multiple comparison post-hoc test. Summarized effect of enzyme action presented as aggregated data obtained from supplements containing specific enzyme in comparison to supplements not containing enzyme. We used the repeated measures, mixed-effect model to compare the enzyme presence as a fixed effect, with pig and supplement as random effects. A p-value of < 0.05 was considered to be statistically significant while a p ≤ 0.1 was considered a tendency.

Results

Effect of different supplements on glucose utilization during the IVGTT

In general, glucose utilization during the IVGTT followed a similar pattern on all experimental days and blood glucose levels returned to baseline levels 30 minutes following the glucose infusion (Table 2). $AUC_{glucose}$ represents the total glucose utilization during the IVGTT. Oral administration of the supplements tested 1h prior to the IVGTT had no significant influence on total blood glucose utilization (Table 3, $AUC_{glucose}$, Figures 1-3).

Effect of different supplements on insulin response and insulin sensitivity during the IVGTT

Plasma insulin levels peaked at 5 and 15 minutes following the glucose infusion and returned to baseline levels 30 minutes following the glucose infusion (Table 2). Analysis of plasma C-peptide levels was performed only to confirm the insulin response after α-amylase supplementation (Table 2). The plasma C-peptide levels following administration of α-amylase were significantly lower 30 minutes following glucose infusion, than those observed following administration of the control solution ($AUC_{C-peptide}$ 3.3 ± 0.3 and 4.4 ± 0.1, with p=0.01).

The total insulin response observed during the IVGTT was reflected in the $AUC_{insulin}$ (Table 3) and α-amylase supplementation resulted in a significantly lower $AUC_{insulin}$ and higher surrogate insulin sensitivity index S2 compared to that observed following administration of the control solution (p=0.03). Tendency to lower $AUC_{insulin}$ was also found after supplementation of amylase together with AKG (A+AKG, p=0.1). Oral administration of a combined supplement, containing proteinase+lipase+AKG (PL+AKG) resulted in a significantly higher insulin response (p=0.002). The mixture of proteinase+lipase (PL) had a tendency to increase the insulin response (p=0.09).

Supplement	IVGTT						
	Fasting baseline	0'	5'	15'	30'	60'	120'
	Glucose, mmol/L						
Control	4.5 ± 0.1	4.1 ± 0.2	15.9 ± 0.2	7.4 ± 0.1	2.9 ± 0.1	3.1 ± 0.1	3.9 ± 0.1
PAL	4.7 ± 0.2	4.2 ± 0.2	15.6 ± 0.2	7.5 ± 0.1	2.3 ± 0.3	3.3 ± 0.2	4.0 ± 0.3
P	4.6 ± 0.3	4.4 ± 0.2	16.0 ± 0.6	7.5 ± 0.3	2.8 ± 0.3	3.2 ± 0.3	4.1 ± 0.4
A	4.3 ± 0.1	3.1 ± 0.2	13.9 ± 0.2	8.2 ± 0.4	3.1 ± 0.2	2.9 ± 0.1	3.9 ± 0.3
L	4.6 ± 0.2	4.2 ± 0.2	16.4 ± 0.4	9.0 ± 0.4	3.5 ± 0.3	2.9 ± 0.2	3.8 ± 0.3
PA	4.7 ± 0.1	4.4 ± 0.1	16.8 ± 0.1	7.1 ± 0.4	2.1 ± 0.1	3.2 ± 0.2	3.9 ± 0.1
PL	4.8 ± 0.2	4.4 ± 0.1	17.3 ± 0.3	7.7 ± 0.4	2.7 ± 0.3	2.8 ± 0.2	3.5 ± 0.3
AL	4.5 ± 0.3	4.8 ± 0.2	16.0 ± 0.5	7.0 ± 0.3	2.1 ± 0.2	3.0 ± 0.2	3.8 ± 0.3
AKG	4.3 ± 0.2	4.2 ± 0.2	16.4 ± 0.9	9.7 ± 0.8	4.4 ± 1.0	2.8 ± 0.3	3.9 ± 0.2
PAL+AKG	4.5 ± 0.3	4.3 ± 0.5	16.1 ± 0.5	7.5 ± 0.3	2.1 ± 0.2	3.3 ± 0.2	3.9 ± 0.2
P+AKG	4.4 ± 0.2	4.3 ± 0.1	16.1 ± 0.8	7.6 ± 0.2	3.2 ± 0.1	2.8 ± 0.2	3.6 ± 0.4
A+AKG	4.3 ± 0.1	3.6 ± 0.4	15.2 ± 0.4	8.4 ± 0.7	3.4 ± 0.6	3.2 ± 0.2	4.2 ± 0.1
L+AKG	4.8 ± 0.1	4.4 ± 0.1	15.3 ± 0.4	7.8 ± 0.2	2.9 ± 0.2	2.8 ± 0.1	3.9 ± 0.1
PA+AKG	4.8 ± 0.2	4.7 ± 0.2	17.4 ± 1.1	7.5 ± 0.3	2.1 ± 0.1	3.3 ± 0.3	4.0 ± 0.3
PL+AKG	4.8 ± 0.1	4.6 ± 0.1	17.2 ± 0.5	8.3 ± 0.3	2.8 ± 0.3	2.9 ± 0.2	3.5 ± 0.1
AL+AKG	4.7 ± 0.2	4.1 ± 0.3	16.1 ± 0.9	7.0 ± 0.6	2.2 ± 0.3	3.1 ± 0.1	4.0 ± 0.3
	Insulin, pmol/L						
Control	1.3 ± 0.2	1.7 ± 0.2	34.5 ± 2.9	37.3 ± 0.8	4.0 ± 0.1	1.0 ± 0.1	0.6 ± 0.1
PAL	2.1 ± 0.8	0.8 ± 0.3	29.6 ± 2.4	37.5 ± 5.4	3.2 ± 0.3	0.8 ± 0.1	0.7 ± 0.2
P	1.3 ± 0.2	1.2 ± 0.1	33.0 ± 5.6	35.2 ± 3.7	4.0 ± 0.4	0.9 ± 0.1	2.0 ± 0.9
A	2.9 ± 0.6	1.1 ± 0.2	15.8 ± 2.6	24.1 ± 3.8	3.1 ± 0.4	1.1 ± 0.2	1.4 ± 0.3
L	1.8 ± 0.4	1.3 ± 0.3	26.0 ± 3.0	34.7 ± 2.3	4.7 ± 0.9	0.9 ± 0.1	0.9 ± 0.1
PA	1.9 ± 0.7	1.3 ± 0.4	30.9 ± 4.0	44.1 ± 2.8	3.4 ± 0.2	0.6 ± 0.0	0.5 ± 0.1
PL	2.0 ± 0.4	1.8 ± 0.1	42.3 ± 3.1	48.5 ± 5.4	5.3 ± 0.4	1.5 ± 0.2	1.4 ± 0.3
AL	1.5 ± 0.4	1.6 ± 0.5	45.4 ± 6.1	35.2 ± 5.9	4.1 ± 0.3	1.0 ± 0.1	0.9 ± 0.3
AKG	2.9 ± 0.8	1.6 ± 0.4	33.3 ± 4.8	37.5 ± 5.3	4.8 ± 0.6	0.9 ± 0.1	0.7 ± 0.1
PAL+AKG	1.6 ± 0.6	0.6 ± 0.1	28.5 ± 3.8	37.7 ± 4.5	3.0 ± 0.3	0.6 ± 0.0	1.0 ± 0.3
P+AKG	1.5 ± 0.4	1.2 ± 0.4	37.1 ± 4.7	43.3 ± 5.7	4.3 ± 0.3	0.8 ± 0.1	0.8 ± 0.2
A+AKG	1.3 ± 0.1	1.7 ± 0.5	20.4 ± 3.5	27.9 ± 2.2	2.9 ± 0.5	1.0 ± 0.2	0.9 ± 0.2
L+AKG	1.4 ± 0.3	1.3 ± 0.2	32.0 ± 4.3	35.3 ± 6.0	4.1 ± 0.6	0.7 ± 0.1	1.2 ± 0.4
PA+AKG	1.0 ± 0.4	1.9 ± 0.9	34.3 ± 5.5	42.6 ± 3.7	3.6 ± 0.4	0.6 ± 0.1	0.7 ± 0.2
PL+AKG	1.3 ± 0.2	1.8 ± 0.4	45.5 ± 4.8	54.5 ± 6.6	5.3 ± 0.6	3.1 ± 1.9	1.1 ± 0.2
AL+AKG	1.3 ± 0.2	2.4 ± 1.0	33.9 ± 4.6	31.3 ± 5.6	3.6 ± 0.0	1.1 ± 0.2	1.2 ± 0.3
	C-peptide, pmol/L						
Control	10.3 ± 2.2	175.7 ± 11.3	212.3 ± 6.9	51.0 ± 4.6	N/M	N/M	N/M
Amylase	4.6 ± 1.0	106.0 ± 12.0	174.8 ± 17.1	37.3 ± 5	N/M	N/M	N/M

Table 2: Blood glucose, plasma insulin and C-peptide levels during an IVGTT in pigs 1 hour after oral administration of different supplements. Control – water solution; A –amylase; P–proteinase; L–lipase, PAL – mixture of proteinase+amylase+lipase, PA – mixure of proteinase+amylase, PL – mixture of proteinase+lipase; AL – mixture of amylase+lipase; AKG – α-ketoglutaric acid;+AKG – mixture of enzyme, or enzymes combination with α-ketoglutaric acid. The results are means ± SEM, (n=5). N/M– not measured.
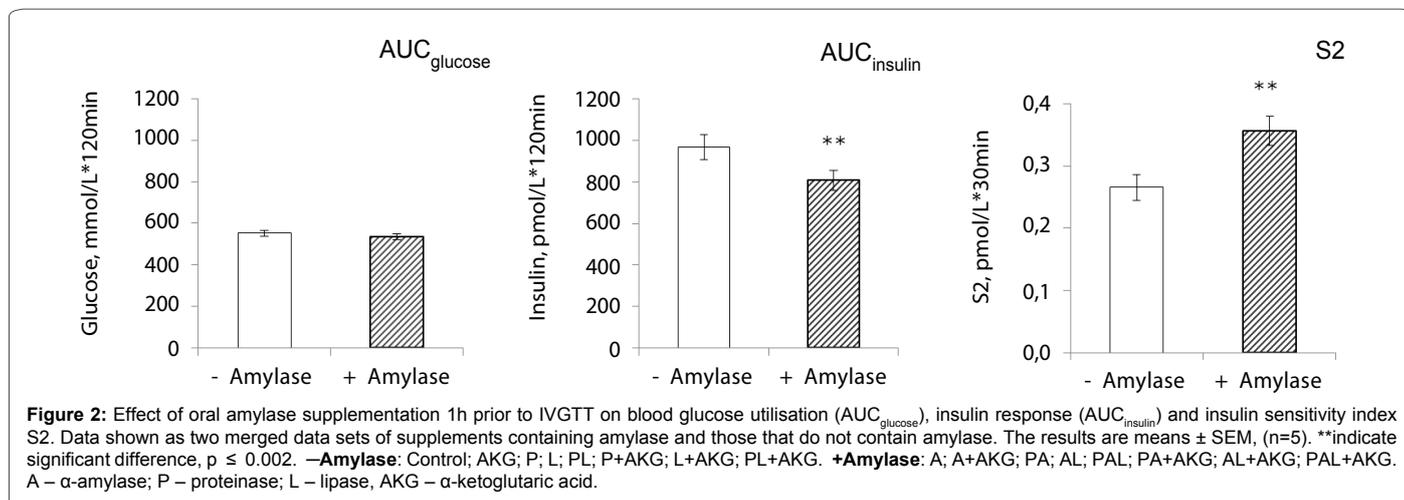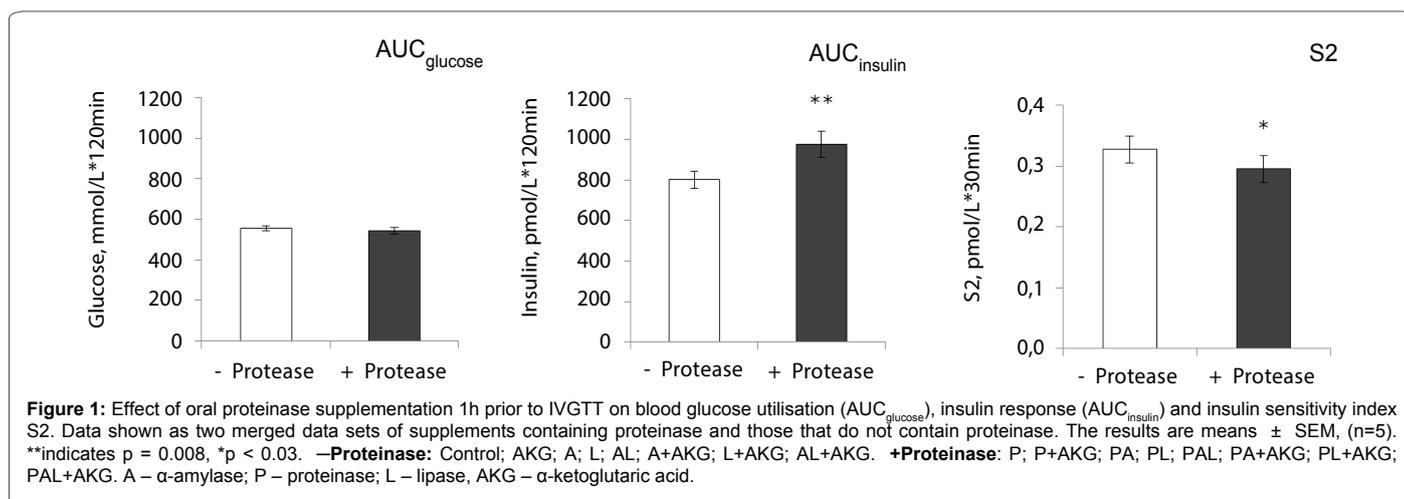
The complex enzyme effect on oral feeding insulin response, calculated as an average from the data obtained for different supplements, contained the reviewed enzymes. As a result, the presence of proteinase in the supplements led to a higher $AUC_{insulin}$ and lower insulin sensitivity index S2 during the IVGTT (Figures 1 and 4). The presence of microbial α-amylase resulted in a lower total insulin response and increased insulin sensitivity during the IVGTT, compared to that obtained following administration of the supplements which did not contain amylase ($AUC_{glucose}$, S2, Figure 2). Comparison of proteinase and amylase administrations showed contrary effects of both enzymes on insulin sensitivity (S2, Figure 4). Lipase presence had a tendency to increase the total insulin response with no effect on insulin sensitivity during the IVGTT (S2, Figure 3). AKG addition to enzyme supplementation had no effect on the parameters investigated.
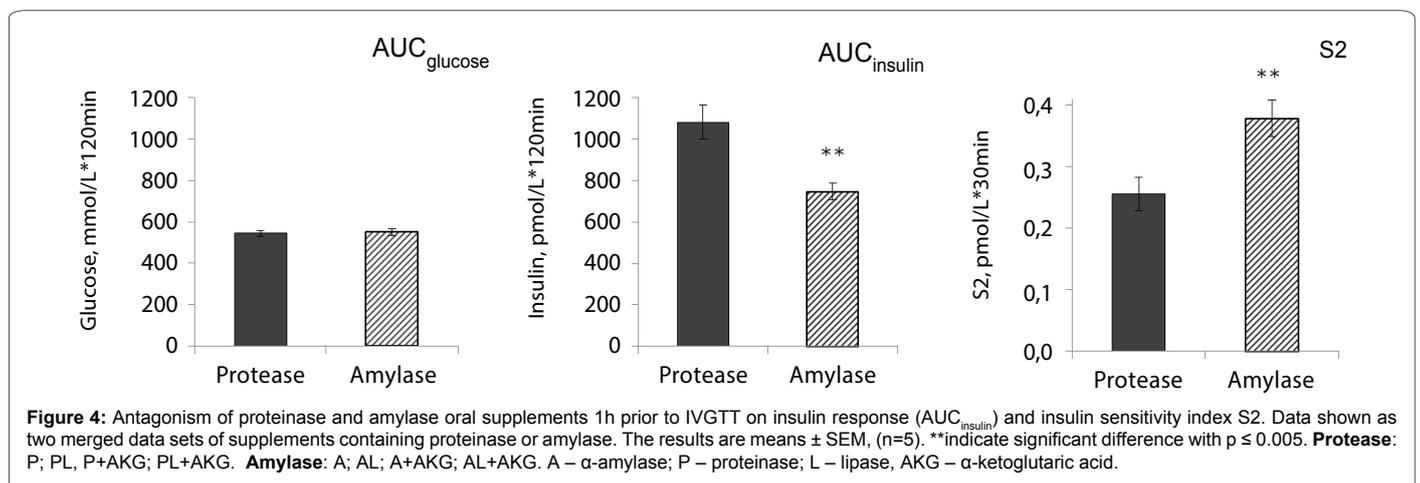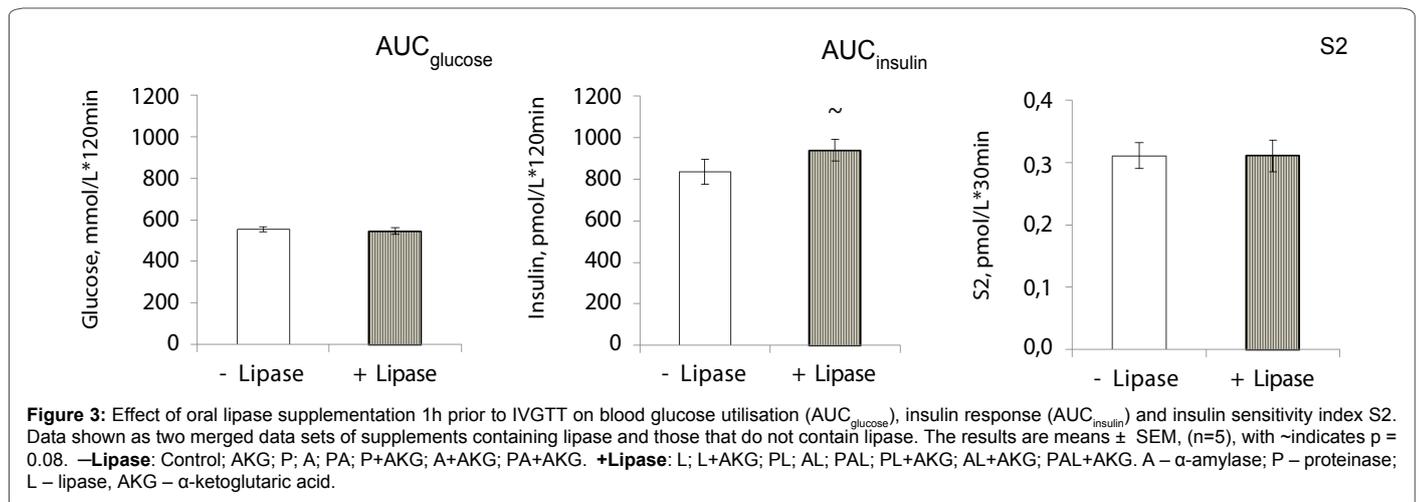
Discussion

Blood glucose and insulin control is dependent on a variety of factors, and can be influenced at various different stages [6]. The recognition of compounds, which would trigger nutrient assimilation, is important for the prevention of metabolic dysregulation and for the treatment of different forms of diabetes. Previous studies have found that pancreatic enzyme supplementation can have a measurable influence on insulin efficiency [9]. Moreover, a considerable number of publications have reported the development of metabolic syndrome in humans with a lower activity of pancreatic amylase in their plasma [23-26]. In the majority of cases, lower plasma pancreatic amylase activity also represents a reduced supply of pancreatic amylase to the duodenum. In addition, type 3c diabetes is exocrine pancreas-related,

Supplement	AUC glucose mmol/L/120min	AUC insulin pmol/L/120min	S2, pmol/L/30min
Control (Water)	544 ± 9	881 ± 17	0.27 ± 0.01
PAL	542 ± 15	814 ± 90	0.34 ± 0.04
P	558 ± 27	882 ± 84	0.31 ± 0.04
A	531 ± 24	581 ± 80*	0.43 ± 0.05*
L	569 ± 16	804 ± 68	0.29 ± 0.04
PA	546 ± 22	905 ± 68	0.32 ± 0.03
PL	543 ± 20	1157 ± 86~	0.25 ± 0.03
AL	541 ± 26	949 ± 89	0.32 ± 0.05
AKG	597 ± 40	892 ± 92	0.22 ± 0.01
PAL+AKG	535 ± 12	812 ± 60	0.36 ± 0.02~
P+AKG	530 ± 19	976 ± 119	0.25 ± 0.04
A+AKG	572 ± 30	642 ± 58~	0.37 ± 0.05~
L+AKG	534 ± 9	844 ± 109	0.32 ± 0.06
PA+AKG	560 ± 31	938 ± 100	0.32 ± 0.03
PL+AKG	546 ± 13	1316 ± 115**	0.22 ± 0.03
AL+AKG	559 ± 23	818 ± 61	0.40 ± 0.05~

Table 3: Blood glucose utilization, plasma insulin response during IVGTT and insulin sensitivity index S2 in pigs 1 hour after oral administration of different supplements. Control – water solution; A –amylase; P – proteinase; L – lipase, PAL – mixture of proteinase+amylase+lipase, PA – mixure of proteinase+amylase, PL – mixture of proteinase+lipase; AL – mixture of amylase+lipase; AKG – α-ketoglutaric acid; +AKG – mixture of enzyme, or enzymes combination with α-ketoglutaric acid. The results are means ± SEM, (n=5). *indicate significant difference, $p < 0.05$, **$p = 0.001$ as compared to the Control value.

Figure 1: Effect of oral proteinase supplementation 1h prior to IVGTT on blood glucose utilisation ($AUC_{glucose}$), insulin response ($AUC_{insulin}$) and insulin sensitivity index S2. Data shown as two merged data sets of supplements containing proteinase and those that do not contain proteinase. The results are means ± SEM, (n=5). **indicates $p = 0.008$, *$p < 0.03$. **─Proteinase:** Control; AKG; A; L; AL; A+AKG; L+AKG; AL+AKG. **+Proteinase**: P; P+AKG; PA; PL; PAL; PA+AKG; PL+AKG; PAL+AKG. A – α-amylase; P – proteinase; L – lipase, AKG – α-ketoglutaric acid.

Figure 2: Effect of oral amylase supplementation 1h prior to IVGTT on blood glucose utilisation ($AUC_{glucose}$), insulin response ($AUC_{insulin}$) and insulin sensitivity index S2. Data shown as two merged data sets of supplements containing amylase and those that do not contain amylase. The results are means ± SEM, (n=5). **indicate significant difference, $p ≤ 0.002$. **─Amylase:** Control; AKG; P; L; PL; P+AKG; L+AKG; PL+AKG. **+Amylase:** A; A+AKG; PA; AL; PAL; PA+AKG; AL+AKG; PAL+AKG. A – α-amylase; P – proteinase; L – lipase, AKG – α-ketoglutaric acid.

Figure 3: Effect of oral lipase supplementation 1h prior to IVGTT on blood glucose utilisation (AUC$_{glucose}$), insulin response (AUC$_{insulin}$) and insulin sensitivity index S2. Data shown as two merged data sets of supplements containing lipase and those that do not contain lipase. The results are means ± SEM, (n=5), with ~indicates p = 0.08. −**Lipase**: Control; AKG; P; A; PA; P+AKG; A+AKG; PA+AKG. +**Lipase**: L; L+AKG; PL; AL; PAL; PL+AKG; AL+AKG; PAL+AKG. A – α-amylase; P – proteinase; L – lipase, AKG – α-ketoglutaric acid.

Figure 4: Antagonism of proteinase and amylase oral supplements 1h prior to IVGTT on insulin response (AUC$_{insulin}$) and insulin sensitivity index S2. Data shown as two merged data sets of supplements containing proteinase or amylase. The results are means ± SEM, (n=5). **indicate significant difference with p ≤ 0.005. **Protease**: P; PL, P+AKG; PL+AKG. **Amylase**: A; AL; A+AKG; AL+AKG. A – α-amylase; P – proteinase; L – lipase, AKG – α-ketoglutaric acid.

which has also been confirmed by our lab, using a pig model [3]. In order to understand the purpose of the current study, as well as our hypothesis, one should consider that pancreatic enzymes are not only responsible for digestion, but also exhibit a positive influence on the luminal intestinal mucosa [27,28].

In the present study we investigated the effects of oral administration of the main digestive components usually found within pancreatic juice (proteinase, amylase and lipase), on blood glucose and plasma insulin levels during an IVGTT, in order to validate our assumptions. In addition, the proteinase, amylase, and lipase of microbial origin are actually tested and used as alternatives to pancreatic enzymes of porcine origin (pancrelipase Creon®) to treat pancreatic insufficiency [29].

Lipase administration in comparison to that of proteinase and amylase was found to have no influence in altering the insulin response. The ability of microbial proteinase to influence insulin augmentation requires additional investigation. This influence of microbial-derived proteinase could be due to the different substrate-specific activity with porcine trypsin (the main component of pancreatic juice) and different electrophoretic separations upon charge (data not shown). It was shown, that different proteases vary in cleavage sites on the protease-activated receptors (PAR) [30]. In addition, in experiments using EPI pigs, no increases in insulin response were detected following oral administration of a commercial mixture of pancreatic digestive

enzymes [3]. The ability of proteinase to attenuate insulin sensitivity is perhaps related to its ability to potentiate inflammation [31]. The opposed results obtained following administration of proteinase and amylase reveal difference in enzyme action. Oral administration of microbial amylase alone, resulted in a reduced requirement for insulin in response to an intravenous glucose infusion, compared to that observed after administration of the control solution. The mixture of microbial α-amylase together with lipase, proteinase and AKG, slightly deteriorated the impact of amylase on insulin release. However, a significantly decreased level of required insulin as an effect of oral amylase administration prior to IVGTT was detected in merged data sets.

Fungal amylase has sequences homologous to that of porcine and human amylases in its enzymatically functional regions [32]. The microbial α-amylase used in the present study has been shown to remain active in the duodenum for as long as 1h after oral administration Considerable degradation of the microbial α-amylase is observed within the ileum of pigs with exocrine pancreatic insufficiency following oral administration of the microbial α-amylase, together with proteinase and lipase in the form of pills [33]. However, healthy animals such as those used in the present study, retain the high proteolytic activity of their endogenous trypsin and other endo- and exopeptidases, which also implies a possible higher rate of proteinase activity, which may in turn affect the action of amylase. We are also not excluding the role

of peptides possibly derived following the digestion of α-amylase or that of other enzymes through the action of endo- and exo-peptidases which are present in the gut lumen.

The effect of oral α-amylase administration on lowering the insulin response was verified by the lower C-peptide levels. We should point out that we recognize the amelioration of the insulin sensitivity together with its lower release and possible attenuated insulin resistance (what is the clue for obesity, diabetes type II and metabolic syndrome) by dietary α-amylase as primary finding of our study. It has been postulated that results obtained can suggest that amylase food supplementation facilitates/promotes enterocyte glucose uptake from the blood. Insulin sensitivity index S2, which has been proposed to be relevant for pigs as an accurate representative insulin sensitivity index based on the IVGTT [15] was also highest following amylase administration.

Based on current results we can speculate, that pancreatic α-amylase, by interacting with the luminal epithelial cells, increases glucose consumption by enterocytes from the blood via insulin independent GLUT2 transporters on their basolateral side. Other mechanisms of α-amylase action are not excluded e.g., via peptides appearing after the digestion of α-amylase by trypsin or other proteinases. The phenomenon of blood glucose regulation by amylase is probably of importance during the early stages of development in neonates, who produce quite a high amount of pancreatic amylase as they are being nursed exclusively by mother's milk. The presence of pancreatic amylase in the gut lumen during the suckling period is unclear since there is no substrate in the milk for α-amylase to act on during that time [34]. Thus, maybe the regulation of glucose is the primary duty of pancreatic α-amylase in neonates, taking into account the well-known fact that plasma insulin levels in neonatal pigs are essentially [34].

It has been shown that the enteral application of pancreatic enzymes (Creon®) never leads to increased levels of the corresponding enzymes in the blood, suggesting that no enzymes are absorbed from the lumen [18]. Also, to our knowledge, no connection between digestive enzyme activity in the gut and β-cell function has been reported. Moreover, in previous experiments on young pigs with induced exocrine pancreatic insufficiency, blood glucose clearance without reinforced insulin release was observed during an IVGTT, following pancrelipase feeding [3]. So, we are inclined to link any changes in blood parameters with the enzyme's influences on intestinal cells and their function.

Based on our previous research, AKG could also have an indirect influence on the parameters studied, due to its positive effect on the intestinal epithelium [22,35]. It was found that AKG, as well as digestive enzymes, interacts with enterocytes and is involved in amino acid biosynthesis, offering advantages in a catabolic state and thus may also affect the rate of gluconeogenesis in mucosa cells [2,21,34,36-38]. On the other hand, AKG in enterocytes undergoes amination and appears in the plasma as glutamate [36] which may stimulate insulin exocytosis from β-cells in response to glucose stimulation [39]. However, the oral supplementation of AKG in the amount used in our studies seemed not to be involved in the regulation of blood glucose utilization and insulin sensitivity.

In conclusion, the experiment shows that oral administration of microbial α-amylase 1h prior to the IVGTT can have a significant influence on the release of insulin required for glucose clearance in growing healthy pigs. The results suggest a para-digestive, insulin-like anabolic action of amylase on glucose utilization, which occurs via some unidentified mechanism in response to the presence of α-amylase in gut lumen. Microbial proteinase as opposed to amylase attenuated insulin sensitivity increasing its release, whereas lipase had no significant effect. Thus, α-amylase, can be suggested as the most important component of pancreatic juice, which, acting from the gut, regulates blood glucose utilization in an insulin independent manner, and in that way postpones the development of glucose intolerance. Obtained data point out the necessity for further investigations highlighting role of amylase and proteinase in post-prandial glucose utilization.

Author Contributions

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