Increased Serum Immunoreactive Insulin in Rabbits Fed a Protein- and Mineral-Rich Diet.

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Summary. In rabbits fed a diet of pellets containing approximately 12% protein, 4% fat, 44% nitrogen-free extracts and 1% calcium carbonate, serum immunoreactive insulin (IRI) was found to increase to values as high as 1134 μ U/ml three hours after the intake of food. After oral glucose (1.75 g/kg), glucose increased by an average of 105 mg% and IRI by 57 μ U/ml, whereas after 100 g of pellets these levels increased on the average by 45 mg% and 210 μ U/ml, respectively. There was a significant correlation between the IRI levels and the amount of food consumed, and a significant correlation ($P < 0.001$)

between the glucose and IRI levels after intake of food. In rabbits that were fed natural diet such as sugar beet and oats, the increases in glucose and IRI were small. Addition of meat and bone meal as well as the mineral-vitamin supplement containing up to 2 g calcium carbonate caused a considerable increase in glucose and IRI. It is suggested that the high amount of calcium in the feed stimulated the glucose-induced insulin release.

Key-words: Insulin release, protein, glucose, minerals, CaCO_3 , rabbits.

Introduction

During routine control of blood sugar and serum insulin in a large number of normal rabbits fed on a laboratory pellet diet, very high immunoreactive insulin (IRI) levels were observed in the fed animals. Therefore, a systematic investigation was undertaken of the effect of handling and composition of the diet on the serum glucose and insulin in normal rabbits. The IRI and glucose responses to oral glucose, to natural feed and to a synthetic diet (consisting of a mixture of protein, fat, carbohydrate, minerals and vitamins) are reported. The IRI values after increased food intake and the correlation between serum IRI and glucose are presented.

Materials and Methods

Normal, untreated albino rabbits (Rex) were used throughout the study, they were kept in cages 41 cm high, 62 cm long and 44 cm wide. Blood was obtained from a puncture of the marginal ear vein and allowed to clot for an hour at 0° before centrifugation. Serum was stored frozen at -18° until used. Oral glucose tolerance tests (OGTT) were performed after a 16 h fast by administration of 1.75 g of glucose/kg as a 35% solution, by stomach tube. Serum glucose was determined by the glucose oxidase method after an uranyl acetate precipitation of the serum proteins (the Boehringer, Mannheim method). Serum insulin was determined according to Heding (1972) using anti-pork insulin guinea-pig serum (M 10), human insulin, lot MC 12569 with a biological activity of 25.7 i. U/mg (NOVO Insulin Laboratory) as the standard, and ^{125}I -labeled pork insulin as the tracer. It was shown that human and rabbit insulins, whose amino acid compositions differ only in regard to one amino acid, gave identical standard curves in the immunoassay system used. The rabbits were routinely fed at 7 a.m. every day and had water *ad libitum*. The composition of the pellets is shown in Table 1. The natural food consisted of sugar beet, hay, oats and lettuce. The vitamin-mineral mix (for composi-

tion see Table 1) was used as additive together with CaCO_3 .

Table 1. *Composition of pellets for rabbits (Boserup OH feed)*

Meat-and-bone meal	1.0 %
Fish meal	1.0 %
Sunflower seed, peeled	6.0 %
Soybean oil cake	1.5 %
Lucerne green meal	40.0 %
Maize	10.0 %
Barley	20.5 %
Wheat bran	18.25 %
Calcium carbonate	1.0 %
Vitamin/mineral mix 306 ^a	0.75 %
	100.00 %

^a Vitamin/mineral mix 306 contains A, D₃, B₁, B₁₂ and E-vitamins as well as niacinamide, D-pantothenic acid and choline chloride, mixed in: 76.25% wheat flour, 3.8% mangan oxide, 4.8% ferr. sulphate monohydrate, 0.36% copper sulphate, 0.072% cobalt sulphate, 1.65% zinc sulphate monohydrate, 0.05% potassium iodide, 9.268% calcium carbonate, 3.75% ethoxyquin (Santoquin).

Each gram of this mix contains: 18 int. units of A-vitamin, 6 int. units of D₃-vitamin, 5 μ g of B₁-vitamin, 12 μ g of B₂-vitamin, 68 μ g of niacinamide, 30 μ g of D-pantothenic acid, 650 μ g of choline chloride, 25 μ g of E-vitamin, 15 mg of B₁₂-vitamin.

Results

Fasting Glucose and IRI Values in Normal Rabbits, Effect of Repeated Blood Sampling

Table 2 shows glucose and IRI values in 6 normal fasting rabbits under the stress of 3 venous punctures performed by an experienced technician. It appears that glucose increases significantly whereas IRI tends to decrease.

Glucose and IRI after OGTT

Table 3 shows the results of OGTT in 6 normal rabbits. The mean glucose increase observed during a period of from 0 to 30 min was 105 mg%, and the increase in IRI averaged 57 μ U/ml.

Table 2. Effect of repeated punctures of the ear vein on serum glucose and IRI in 6 normal, untreated, fasting rabbits

Blood sampling at	Serum glucose, mg% mean $\pm$ S.E.M.	IRI, μ U/ml mean $\pm$ S.E.M.
0	98 $\pm$ 4.0	13.6 $\pm$ 3.7
30 min	110 $\pm$ 2.3	10.3 $\pm$ 1.0
60 min	122 $\pm$ 5.4	9.0 $\pm$ 1.2

of the pellets see Table 1) is shown in Table 3. Ingestion of pellets (between 50 and 100 g within the first 3 h) induced significant rises both in serum glucose and IRI averaging 30 mg% and 148 μ U/ml, respectively. The variation in IRI was substantial even between animals that had consumed the same amount of feed.

Table 5 shows how the amounts of pellets consumed influenced the glucose and IRI levels. 94 rabbits were given 100 g of pellets *ad libitum*. Their food intake was measured 3 h later, and blood samples were drawn for IRI and glucose determinations. Three rabbits had consumed approximately 25 g, 19 rabbits approximately 50 g, 55 rabbits approximately 75 g and 17 rabbits had eaten 100 g, corresponding to a protein

Table 3. Variation in serum glucose and IRI in normal rabbits after intake of pellets and oral glucose (1.75 g/kg) as a function of time

			fasting	1/2 h	1 h	2 h	3 h	4 h	5 h
Glucose mg%	mean $\pm$ S.E.M.	pellets (12 rabbits)	131 $\pm$ 2	—	142 $\pm$ 5	159 $\pm$ 9	158 $\pm$ 6	161 $\pm$ 7	156 $\pm$ 6
IRI μ U/ml	mean $\pm$ S.E.M.		8 $\pm$ 2	—	137 $\pm$ 25	134 $\pm$ 28	143 $\pm$ 24	151 $\pm$ 58	99 $\pm$ 29
Glucose mg%	mean $\pm$ S.E.M.	glucose (6 rabbits)	120 $\pm$ 2	225 $\pm$ 7	216 $\pm$ 9	147 $\pm$ 12	—	145 $\pm$ 3	
IRI μ U/ml	mean $\pm$ S.E.M.		9 $\pm$ 2	66 $\pm$ 10	68 $\pm$ 19	13 $\pm$ 4	—	6 $\pm$ 2	

Table 4. Effect of oats + sugar beet + hay feed (50 g + 250 g + 50 g) on serum glucose and IRI in 12 normal rabbits

No. of Weeks since start of oats + beet + hay feed	Prior to feeding				3 h after feeding			
	Serum glucose mean $\pm$ S.E.M.	mg% (range)	IRI, mean $\pm$ S.E.M.	μ U/ml (range)	Serum glucose mean $\pm$ S.E.M.	mg% (range)	IRI, mean $\pm$ S.E.M.	μ U/ml (range)
1 week	122 $\pm$ 4.5	(100–165)	15 $\pm$ 2.2	(6–30)	125 $\pm$ 5.2	(106–173)	29.7 $\pm$ 6.4	(7–68)
2 weeks	120 $\pm$ 3.9	(109–157)	18 $\pm$ 2.2	(11–34)	121 $\pm$ 1.9	(107–134)	37.6 $\pm$ 6.8	(10–160)
3 weeks	115 $\pm$ 4.5	(98–158)	12 $\pm$ 1.8	(3–25)	132 $\pm$ 4.9	(111–161)	52 ^a $\pm$ 2.6	(10–155)

^a change from prior-to-feed level significant, $p < 0.01$ (paired t-test).

Glucose and IRI after Natural Food

Rabbits fed beet and lettuce *ad libitum* showed no increase in glucose and IRI two hours after they started eating. When rabbits were fed oats, sugar beet and hay (50 g + 250 g + 50 g), variations were recorded in glucose and IRI as shown in Table 4. The glucose increase after intake of food was small and insignificant. A significant increase in IRI was recorded only on one out of three occasions, after 3 weeks of feeding oats and beet. During the 3 h, the rabbits had eaten between one-quarter and one-half of the feed.

Glucose and IRI after Pellets

The variation in glucose and IRI as a function of time after intake of 100 g of pellets (for composition

intake of 3–12 g. All the animals showed an increase in glucose and all except one showed an increase in IRI. It appears from Table 5 that IRI levels rise significantly with increasing food intake. The correlation between interdependent glucose and IRI concentrations in serum 3 h after the intake of food was determined in 140 cases (none was left out) and the correlation coefficient found to be 0.59, which is highly significant ($P < 0.001$).

IRI Increase after Single Pellet Constituents

Some of the components of pellets (bone meal and vitamin-mineral mix + CaCO_3) were added to the sugarbeet-and-oats feed in order to see if it was possible to reproduce the increase in the IRI release. The

powdered components were placed on top of the oats, but they dropped to the bottom of the feeding trough to a greater or lesser degree in the course of feeding so that the intake of components was not reproducible, for which reason these experiments were discontinued. Nevertheless, it could be concluded (Table 6) that there was a considerable and significant rise in the IRI levels in rabbits that had access to bone meal and vitamin + mineral mix containing CaCO_3 . A few efforts were made to force the components on the rabbits either in form of a slurry or capsulated, but the animals refused to eat. Gastric tube feeding was also tried but this involved increases in blood glucose due to insertion of the tube, and this method was abandoned.

estimates. Finally, 1640 μU of extracted IRI was electrophoresed in polyacrylamide gel at pH 8.7. After electrophoresis, the 6 cm long gel was cut into 32 pieces and each piece eluted with the immunoassay buffer and assayed for IRI. Only one peak of IRI was found, which contained 1540 μU of IRI at the site of insulin. However, the position of rabbit proinsulin is not known in this electrophoresis system.

Discussion

It is well known that many ingredients of food other than carbohydrates induce the release of insulin.

Table 5. Variation in serum glucose and IRI as a function of the amount of pellets eaten three hours after food intake

		amount of pellets eaten in g (number of rabbits)				
		0 (94)	25 (3)	50 (19)	75 (55)	100 (17)
Glucose	mean $\pm$ S.E.M.	117 $\pm$ 1	141 $\pm$ 9	136 $\pm$ 3	145 $\pm$ 3	158 $\pm$ 5
mg%	(range)	(94–139)	(132–158)	(117–158)	(116–201)	(119–188)
IRI	mean $\pm$ S.E.M.	6 $\pm$ 1	21 $\pm$ 7	48 $\pm$ 12	128 $\pm$ 18	216 $\pm$ 66
$\mu\text{U}/\text{ml}$	(range)	(0–30)	(10–34)	(14–246)	(4–636)	(44–498)

Table 6. Increase in IRI 90 min after intake of bone meal or vitamin + mineral + CaCO_3 mix as a supplement to sugar beet and oats diet

Composition of feed	No. of rabbits	Serum glucose, mg% mean $\pm$ S.E.M.	IRI, $\mu\text{U}/\text{ml}$ mean $\pm$ S.E.M.
sugar beet + oats	12	114 $\pm$ 4	24 $\pm$ 10
sugar beet + oats + 1 g bone meal ^a	6	112 $\pm$ 6	48 $\pm$ 16
sugar beet + oats + 2 g bone meal	6	123 $\pm$ 10	137 $\pm$ 53
sugar beet + oats + 0.75 g vitamin + mineral mix ^b + 1 g CaCO_3 ^c	6	120 $\pm$ 3	58 $\pm$ 17
sugar beet + oats + 1.50 g vitamin + mineral mix ^b + 2 g CaCO_3 ^c	6	145 $\pm$ 10	268 $\pm$ 86

^a 1 g of bone meal corresponds to the amount in 100 g of pellets

^b 0.75 g of vitamin + mineral mix corresponds to the amount in 100 g of pellets

^c 1 g of CaCO_3 corresponds to the extra amount of this component that is added to the pellets

Characterization of IRI in Rabbit Serum after Pellets

The very high IRI values (up to 1134 $\mu\text{U}/\text{ml}$; this value was measured in a series of rabbits not shown in this paper) measured in the rabbits 3 h after intake of food were checked in order to ensure that the immuno-reactivity really was due to insulin. Serial dilutions and recovery experiments yielded the expected values. Acid ethanol extraction (Heding 1972), which separates insulin from the bulk of serum proteins including proteolytic enzymes, gave IRI values in the extracts that were not significantly different from the direct

Certain amino acids as well as proteins induce a release of insulin in normal persons. Knopf *et al.* (1966), Floyd *et al.* (1966a) and Fajans *et al.* (1972) showed that the infusion of 30 g of arginine, lysine or leucine in normal subjects increased the (basal) IRI levels from about 10 $\mu\text{U}/\text{ml}$ to approximately 80, 60 and 30 $\mu\text{U}/\text{ml}$, respectively, whereas valine and phenyl-alanine caused only small increases and histidine and methionine no increase at all. Floyd *et al.* (1966b) reported an increase of 10–40 $\mu\text{U}/\text{ml}$ in the IRI level after a 500-g-protein meal consisting of either liver or ground beef. Similar

findings were reported by Sukkar *et al.* (1967) and Copinschi *et al.* (1967). Rabinowitz *et al.* (1966) compared the IRI release after oral glucose, beef and glucose plus beef in normal human subjects and found a substantially augmented rise in plasma insulin after glucose plus beef (max. serum IRI approx. 150 μ U/ml) as compared to glucose alone (70 μ U/ml) and beef alone (30 μ U/ml). Chick and Like (1971) fed various diets to mice with hereditary diabetes and found that a synthetic diet consisting of a combination of carbohydrates, proteins and fat gave the highest serum IRI levels. The exact composition of this diet and the IRI values in normal mice were not reported.

Although it has been recognized that many food ingredients induce the release of IRI, glucose is still considered to be the most important stimulant of insulin release.

A non-sedated rabbit will respond to stress such as a venous puncture with a quick rise in blood glucose. This response is especially pronounced in inexperienced laboratory animals. We have observed an increase of about 50 mg% glucose within a minute during a painful venous puncture. Table 2 shows that venous puncture performed by an experienced technician causes an increase in blood glucose of about 10 mg% 30 min after sampling. Thus, increases in glucose between two blood samplings should be evaluated taking this point into consideration.

Following an oral glucose load (6 rabbits), the serum glucose increased by approximately 105 mg% while the insulin increased by approximately 45 μ U/ml. After 100 g of pellets (19 rabbits), the average increases in glucose and IRI were 45 mg% and 210 μ U. There was a significant correlation ($P \leq 0.001$) between the IRI level and the glucose concentration after pellets. Since IRI secretion is stimulated by oral protein intake (Floyd *et al.* (1966), Rabinowitz *et al.* (1966)), it is probable that the 12% protein content of the pellets enhances the insulin release. It is possible that the pellets increased the level of glucagon, which has been shown to rise after protein in humans (Unger (1972)). However, Unger did not find any significant increase in glucose and reported only a small increase in IRI.

The protein content of the pellets cannot be solely responsible for the IRI-increases, since an insulin increase was observed after oats + sugar beet + vitamins and minerals (including CaCO_3). In the animals fed only oats and sugar beet, the changes in glucose and IRI were minor, whereas the vitamin + mineral + CaCO_3 supplement resulted in a rise in glucose and an enormous IRI release. The same was observed, though to a lesser degree, after a bone-meal supplement, which is rich in minerals (e.g. calcium) and protein. Although it was difficult to quantitate the amount of bone meal or vitamin and mineral mix (including CaCO_3) eaten by the rabbits, there is no doubt that these additions caused the IRI increases. Malaisse *et al.* (1970), Malaisse & Malaisse (1971) and Malaisse (1972) have

shown that enhanced insulin secretion from isolated rat islets was associated with an increased calcium uptake by the islets. The *in vitro* rate of insulin secretion increased as a linear function of calcium uptake, and Malaisse *et al.* (1970) put forward the hypothesis that Ca^{++} influx in the β -cell might trigger off the release of insulin. Recently, Böttger *et al.* (1972) have shown that an intraduodenal load of calcium chloride (4.5 mmol/kg dog) caused a rise in gut GLI, whereas no changes were observed in IRI and glucose. The mineral-vitamin mix administered to the rabbits contained 10–20 mmol CaCO_3 , yielding a dose similar to the one given by Böttger *et al.* The reason for the absence of an IRI increase in the dogs could be either due to species differences or to the fact that other food constituents such as carbohydrate or protein are needed along with the CaCO_3 to cause enhanced IRI release. The pancreatic and gut GLI and gastrin responses are now being investigated.

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