Effects of cassava cyanoglucoside, linamarin, on blood sugar levels in the dog

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This study was undertaken to examine the diabetogenicity of linamarin in dogs of different nutritional status. The total dog population was 35; 15 normals, 10 undernourished, and 10 recovered. The recovered dogs were the refed undernourished dogs. On each dog a fasting plasma insulin and insulin receptor studies were done, followed by a controlled glucose tolerance test. After feeding linamarin, 0.5 hour was allowed to elapse before these investigations were repeated. In the linamarin-treated undernourished animal, plasma insulin and insulin binding to erythrocytes and mononuclear leucocytes were significantly ($P < 0.01$) lower than in the control animals, and this resulted in abnormally high glucose levels. Linamarin administration had no effect on plasma insulin, insulin binding, or blood sugar levels in the normal and recovered dogs. These results point to the possible aetiology of diabetes in the undernourished state by "toxic" food substances such as linamarin. (J. Nutr. Biochem. 4:625–629, 1993).

Keywords: linamarin; diabetes; undernutrition

Introduction

Linamarin (hydroxyisobutyronitrile-β-D-glucose) is the main cyanogenic glucoside in cassava, which liberates hydrocyanic acid (HCN) on hydrolysis.[1] The action is mediated by linamarase, a β-glucosidase, also present in cassava. Hawksworth et al.,[2] after examining the gut flora of humans and general laboratory animals, showed that linamarin can also be hydrolysed by intestinal microorganisms.

Epidemiological observations strongly suggest an association between the global prevalence of the malnutrition-related diabetes mellitus (MRDM) sub-type, fibro-calculous pancreatic diabetes (FCPD) and the consumption of cassava.[3] However, FCPD has been reported from several developing countries where cassava is either not grown or not consumed as staple food by the population. Further, even where cassava intake is high, clear differences between its consumption in subjects with FCPD and in a matched control group may not always be demonstrable. In one study there was no significant difference in the consumption of cassava between FCPD and matched controls.[3]

Apparently, the hypothesis that cassava intake and protein malnutrition are causally related to FCPD requires further detailed study. This paper is a report of studies conducted on animal models, investigating the possible effects of linamarin on blood sugar levels.

Methods and materials

There were 15 normal dogs and 20 undernourished dogs. Ten undernourished dogs were refed. The dogs were 6–8-month-old mongrels. Normal dogs were maintained on a diet (125 g/kg body weight) of cornmeal, chicken, Purina Laboratory Chow (Purina, St. Louis, MO USA), and water ad libitum. Average weight of the normal population was 10.3 ± 0.4 kg.

Undernutrition was induced by restricting the diet to 11.3 g of only cornmeal per kg body weight for 10 days. Average weight of undernourished dogs was 6.8 ± 0.3 kg.

The recovered group was refed the normal diet with added milk and multivitamin supplements over a period of 5 days. Recovered dogs were 10.2 ± 0.6 kg in weight.

Plasma insulin, insulin receptors, and blood glucose were tested on fasting samples followed by a complete glucose tolerance test (GTT) on the control and linamarin-fed dogs. The control investigations were done on the first day of experimentation in each of the nutritional states. On the following day, linamarin in dosages ranging from 20 to 60 mg/kg body weight were dissolved in 2 mL of distilled water and administered orally to the fasting dogs. One-half hour was allowed to elapse before the investigations were repeated. After a 10–12 hour overnight fast, 10 mL of freshly drawn blood, in a tube con-

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taining EDTA (2 mg/mL), was centrifuged at 1500*g* for 5 minutes at ambient temperature. The plasma was separated and stored at −70° C for insulin radioimmunoassay using Coat-a-Count insulin diagnostic kits (Diagnostics Product Corp., Los Angeles, CA USA) and for plasma glucose determination, using the standard glucose oxidase method.[4]

The red and white blood cells were separated using a Percoll density gradient. The red cells were washed three times by centrifugation (room temperature, 600*g*, 10 min) in 10 mL of Hepes phosphate buffer, pH 8.0. After each wash, the upper 100 μL of cells were discarded with the supernatant to remove the residual mononuclear cells and reticulocytes. Finally, 3 mL of Hepes-phosphate buffer with 1% human serum albumin was added to the sedimented red cells. This was the final suspension used for incubation. The reticulocyte counts in the cell suspension were within 0.2%. The ratio of white to red cells in this final suspension was 1:7,000,000.

The method used for the study of the insulin binding properties was a modification of that of Gambhir et al.[5] One hundred μL aliquots of the washed red cell suspension were added to a series of polyethylene tubes in duplicate. Washed red cells (concentration 0.75–1.5 × 10^9/mL) were incubated at 20° C for 3 hours in the presence of 16.7 pmol/L A_{14}-[^{125}I] monoiodinated insulin (Amersham, Arlington Heights, IL USA; specific radioactivity 2000 Ci/mM) and increasing amounts of nonradioactive insulin (Sigma porcine, 23.5 U/mg, Sigma Chemical Co., St. Louis, MO USA) in the physiological range, 16.7–1670 pM. The incubation was terminated by the addition of 2.7 mL of 150 mM sodium chloride and centrifuged (20° C, 1 min, 1500 *g*). The supernatant was removed by suction and 100 μL of 40% formaldehyde was added to harden the red cell pellet. Saline was then added and the supernatant removed.

The mononuclear leucocytes insulin binding assay was performed in the same way with slight changes. These included using a Hepes-phosphate buffer (pH 8.0) containing 1% human serum albumin, centrifugation was done at 400*g*, and buffer instead of saline was used to stop the incubation.

Specific insulin binding (SB) was calculated as the percentage of radioactive insulin specifically bound by 4 × 10^9 and 1 × 10^6 cells/mL for erythrocytes and mononuclear leucocytes, respectively. Non-specific binding was assessed by the amount of radioactive insulin bound in the presence of 16.7 × 10^6 pM unlabeled insulin. Competitive binding curves were obtained for each red and white blood cell suspension. From these curves, the insulin receptor affinity and the number of receptor sites were determined by Scatchard analysis.[6]

Packed cell volume (PCV), hemoglobin (Hb), white blood cell count, white blood cell differentiation, and film appearance[7] were done on each dog.

Differences in the levels of the various measurements for the three nutritional states were examined using two-way analyses of variances (ANOVA). When differences were found, individual means were compared using linear contrasts.[8] In the cases in which the variances were not equal, logarithmic transformation was done. In addition, confidence intervals (CI) were determined to reflect the magnitude of the effect(s) of linamarin.[9]

Results

There was a significant group difference for erythrocyte (ANOVA, F = 29.5, P = 0.001) and mononuclear leucocyte (ANOVA, F = 45.6, P = 0.001) specific insulin binding. Post-analysis of variance comparison showed that these differences were between the undernourished group and the other two groups. Insulin binding in the undernourished dog erythrocytes (16.9 ± 1.1%) and mononuclear leucocytes (6.3 ± 2.0%) was significantly lower ($P < 0.05$) than in the normal dog (erythrocytes = 22.8 ± 2.3%; mononuclear leucocytes = 16.5 ± 2.7%) and in the refed dog (erythrocytes = 24.6 ± 1.1%; mononuclear leucocytes = 16.5 ± 4.2%). There was no difference (P = 0.100) in binding of insulin between the normal and recovered dogs to erythrocytes and mononuclear leucocytes (*Table 1*). The CI of the difference seen in undernourished erythrocyte binding was 4.3–6.7% and that for the difference in mononuclear leucocytes was 2.2–4.4%.

The affinity of the insulin receptor was significantly lower ($P < 0.01$) in the undernourished state (*Table 2*), for both erythrocytes and mononuclear leucocytes than in the normal and refed dogs. The difference in affinity between the control and linamarin-fed animals observed in each nutritional phase was not significant (P = 0.10). The affinities of the undernourished control and linamarin-fed dogs were lower ($P < 0.01$) than those of the other groups (*Table 2*).

On administration of linamarin, a decrease in receptor sites per cell in erythrocytes was observed in each group (*Table 3*). The decrease recorded for the normal and the recovered dogs were not significant (P = 0.10); however, in the undernourished it was marked ($P < 0.001$).

Linamarin had very little or no effect on receptor numbers in normal mononuclear leucocytes (*Table 3*). Also, the change from 4.6 ± 0.3 to 3.7 ± 0.6 × 10^4 sites recorded for the recovered animals was not statistically significant (P = 0.08). However, in the undernourished state there was a significant decrease in receptor numbers, from 4.9 ± 0.4 to 1.5 ± 0.3 × 10^4 ($P < 0.01$).

Table 4 shows the fasting plasma concentrations of insulin and corresponding glucose levels. Analysis of variance for the fasting insulin levels suggested that there may be differences (ANOVA F = 8.5, P = 0.006). Post ANOVA analysis revealed that the difference in the insulin concentrations of control and linamarin-fed normal dogs approached significance (P = 0.071), while for the undernourished group the mean difference was significant (P = 0.046). The CI of this difference was 14.5–15.03 μIU/mL. There was no real difference in the fasting insulin levels of recovered dogs with linamarin treatment.

Group interaction showed that the mean change in insulin levels on feeding linamarin to undernourished dogs was significant ($P < 0.05$) when compared with the other two groups, while the difference between the mean change in the normal and recovered states was not significant (P = 0.199).

The red blood cells were normochromic and normocytic, while the white blood cells were normal in appearance and proportions for all the dogs in the study.

Figure 1 shows the normal control glucose tolerance curve after administration of a glucose load of 1.75 g/kg body weight. The blood sugar reached its highest concentration, 6.8 ± 0.4 mM, 0.5 hr after ingestion. It slowly returned to near normal (4.1 ± 0.1 mM) 2.5 hr post-prandially.

Table 1 Insulin binding to dog erythrocytes and mononuclear leucocytes

Groups	Erythrocytes (%) Mean ± SEM		Mononuclear leucocytes (%) Mean ± SEM	
Normals (n = 10)				
Control	22.8	2.3	16.5	2.7
Linamarin-fed	20.8	1.5	14.2	3.0
Under-nourished (n = 10)				
Control	16.9	1.1	6.3	2.0
Linamarin-fed	11.4	1.0	2.8	0.2
Recovered (n = 10)				
Control	24.6	1.1	16.5	4.2
Linamarin-fed	22.2	1.6	15.1	2.4

Table 2 The affinity of dog erythrocytes and mononuclear leucocytes for insulin

	Erythrocytes ($\times 10^8$ M^{-1}) Mean ± SEM		Mononuclear leucocytes ($\times 10^8$ M^{-1}) Mean ± SEM	
Normal (n = 10)				
Control	7.4	2.0	4.7	0.6
Linamarin-fed	4.9	2.0	4.1	1.6
Undernourished (n = 10)				
Control	1.1	0.2	1.1	0.1
Linamarin-fed	0.9	0.1	0.6	0.1
Recovered (n = 10)				
Control	6.8	2.1	3.8	0.9
Linamarin-fed	4.1	1.1	4.0	1.6

Table 3 Number of receptor sites per cell in dog erythrocytes and mononuclear leucocytes

Groups	Erythrocytes Mean ± SEM		Mononuclear leucocytes ($\times 10^4$) Mean ± SEM	
Normal (n = 10)				
Control	147	11.6	3.4	1.6
Linamarin-fed	124	25.5	3.4	0.8
Undernourished (n = 10)				
Control	142	29.5	4.9	0.4
Linamarin-fed	36	0.6	1.5	0.3
Recovered (n = 10)				
Control	163	10.1	4.6	0.3
Linamarin-fed	142	29.1	3.7	0.6

Figure 1 also shows the glucose tolerance curve on administration of linamarin. The fasting blood sugar (0 hr) is essentially the same as that in the control. At the 0 hr interval, which is 0.5 hour after feeding the linamarin, the blood sugar was slightly higher. This point represents the start of the glucose tolerance curve, wherein the highest blood sugar concentration (7.4 ± 0.4 mM) occurred 1.5 hr after glucose administration. It dropped to 4.8 ± 0.5 mM at the 2.5-hr interval.

In addition, *Figure 1* shows the response of the undernourished control and linamarin-fed dogs to the glucose load. The undernourished control followed a similar course to the normal control over the first hour, after which the blood sugar climbed to a peak of 12.2 ± 1.0 mM, 1.5 hr after glucose ingestion. The blood sugar was not significantly higher, (P = 0.20), 5.6 ± 0.06 mM, than that of the normal control, 4.1 ± 0.1 mM, in the 2.5-hr sample.

On administration of linamarin, the glucose tolerance curve of the undernourished dog was elevated over its control throughout the entire course of the glucose tolerance curve. The blood sugar attained its highest levels after 1.5 hr (15.4 ± 0.5 mM) and at 2.5 hr fell to 10.1 ± 0.5 mM, which was statistically higher ($P < 0.001$) than that seen in the undernourished control. The CI of the difference was 3.4–5.6 mM. The hyperglycemic effect was seen with 20 mg of linamarin per kg body weight.

The recovered control glucose tolerance curve traced

Table 4 Plasma glucose and insulin levels of control and linamarin-fed dogs

Groups	Fasting plasma glucose (mmol/L) Mean ± SEM		Fasting plasma insulin (μIU/ml) Mean ± SEM	
Normal (n = 15)				
Control	4.3	0.1	42.5	11.2
Linamarin-fed	4.5	0.2	33.7	1.2
Undernourished				
Control	4.0	0.2	35.6	2.7
Linamarin-fed	5.8	0.2	20.8	5.5
Recovered				
Control	5.1	0.7	33.9	3.6
Linamarin-fed	5.3	0.4	26.3	3.3

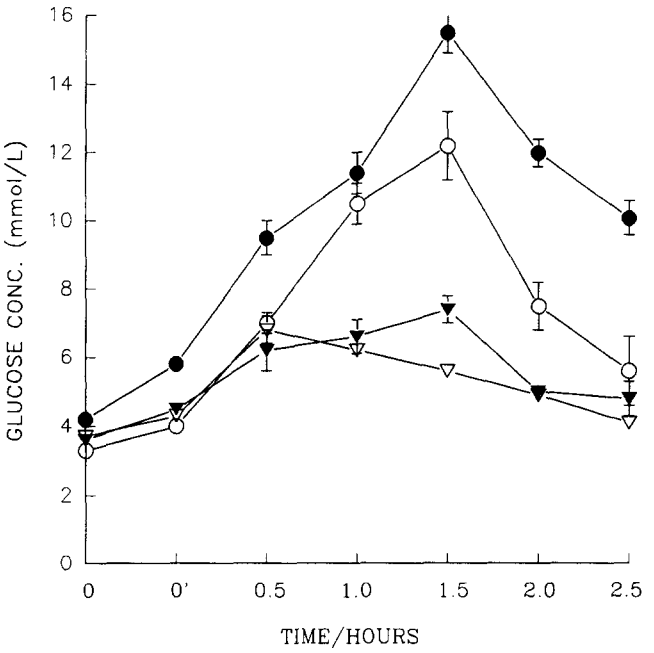

Figure 1 Glucose tolerance curve of controlled and linamarin-fed, normal, and undernourished dogs. Linamarin in 20–40 mg/kg body weight dosage dissolved in water was administered orally. After the fasting test sample was taken, the glucose load (1.75 g/kg body weight) was administered orally. There were no apparent differences in results when normal dogs were treated with lower and higher dosages of linamarin; and, as such, the data were pooled. However, in the undernourished state, 20 mg/kg showed significant changes in blood sugar. These were repeated for confirmation. ○, Undernourished, control; ●, undernourished, linamarin-fed; ∇, normal, control; ▼, normal, linamarin-fed. 0, Fasting control sample: this is the first blood sugar reading after the overnight fast. 0', Fasting test sample: this is the sample taken after administration of linamarin.

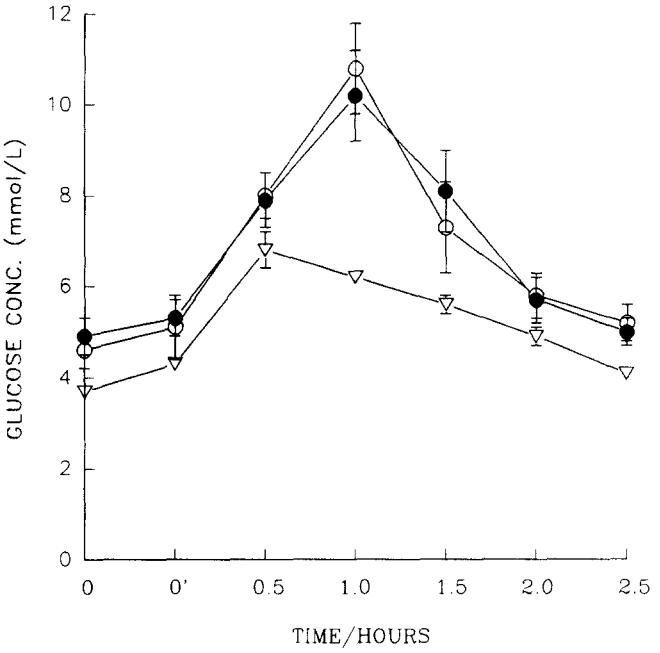

Figure 2 Glucose tolerance curve of controlled and linamarin-fed recovered dogs. Linamarin in 20–60 mg/kg body weight dosage, dissolved in water, was administered orally. After the fasting test sample was taken, the glucose load (1.75 g/kg body weight) was given. There were no apparent differences in results with low and high dosages of linamarin in recovered dogs and, as such, the data were pooled. ○, Recovered, control; ●, recovered, linamarin-fed; ∇, normal control. 0, Fasting control sample: this is the first blood sugar reading after the overnight fast. 0', Fasting test sample: this is the sample taken after administration of linamarin.

a path that was generally higher than the normal control (*Figure 2*). Interestingly, linamarin had little or no effect on the recovered dogs. Dosage as high as 60 mg of linamarin per kg body weight caused no aberration from the recovered control glucose tolerance curve.

Discussion

Linamarin has a hyperglycemic effect on the undernourished state. This is most likely due in large part to decreased insulin binding ($P < 0.05$), such as shown here for both erythrocytes and mononuclear leucocytes. The latter resulted from a significant decrease in the number of receptor sites per cell. This extremely low number of receptor sites in the linamarin-treated undernourished dogs could be the result of primary alteration in the receptor or may be secondary to some other alteration in the membrane.[10]

Linamarin is hydrolyzed by intestinal bacteria,[11] yielding hydrogen cyanide. Sublethal doses of cyanide activates the detoxification mechanisms, which ensure that the cyanide is transformed into less toxic sub-

stances, principally thiocyanates. In the protein energy-deprived state, the sulphur-containing amino acid required for the transformation is not available. The undetoxified cyanide can inhibit cytochrome oxidase, which precipitates a proliferation of superoxide free radicals. Cyanide also inhibits the copper-zinc super-oxide dismutase enzyme, as well as peroxidase, both of which are involved in quenching or safe disposal of free radicals generated in vivo.[12]

These reactive free radicals may damage cells in the short term by covalently binding to membrane components, thereby initiating lipid peroxidation, with direct effects on membrane structure and associated influences of the products of peroxidation on membrane fluidity, cross-linking, and function.[13] These changes in membrane integrity could be responsible for the decreased receptor sites. The resulting membrane dysfunction, in addition to the decrease in insulin production seen in this group, can impair transport of glucose and other metabolites across the cell membrane, resulting in the observed hyperglycemia.

Insulin receptors in the normal dogs were not seriously affected by linamarin. The receptor numbers of both erythrocytes and mononuclear leucocytes in the recovered animals were slightly higher ($P = 0.08$) than those seen in the normal state. This could be the sequelae of up-regulation that would have accompanied the period of undernutrition. Vitamins have been implicated in the scavenging of free radicals,[13,14] and may account for the negligible effect of linamarin, in the recovered state, on insulin binding. It was noteworthy that the glucose tolerance curve in the recovered dogs traced the classical "lag storage" pattern, which could possibly be related to decreased uptake of glucose by the peripheral tissues and induced by the previous state of undernutrition.

The affinity of the receptor for insulin was affected solely by the undernourished state. Interestingly, the number of receptor sites in the undernourished control were similar to that for normal dogs for both erythrocytes and mononuclear leucocytes. A study in India found similar results in malnourished rats.[15] This was also observed by Neufeld et al. in hepatic plasma membrane.[16]

Apparently, in the undernourished state there are significant changes in membrane lipid composition and physical state,[17] which accompanies the fall in plasma insulin. This may cause an up regulation of the receptors, resulting in either normal or increased numbers. However, they still bind significantly less ($P < 0.05$) because of the poor affinity for the hormone. The decrease in affinity may be the result of variations existing in fluid behavior of micro-domains other than those adjacent to the insulin receptor, and though these changes in the fluidity may not easily be detected, their effect is still felt and seen in the behavior of the receptors.[18]

It is apparent that insulin receptors are more vulnerable to linamarin toxicity in the undernourished state. The significance of any binding study with circulating blood cells is open to question. One must appreciate, however, that these are more easily accessible than cells of primary insulin target organs. Also, there may be differences in binding capacity between the target organs themselves. It seems reasonable to suggest that any changes in membrane properties, resulting in changes of receptor behavior, will be evident in some measure in blood cells as well as target tissues.

In conclusion, similar changes in receptor activity may occur in undernourished humans, who ingest cassava and other staples high in linamarin content. This could lead to impaired carbohydrate tolerance, thus lending support to this animal study as a possible model for the aetiology of malnutrition related diabetes mellitus.

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