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Reduced insulin secretion and glucose intolerance are involved in the fasting susceptibility of common vampire bats

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ABSTRACT

Susceptibility during fasting has been reported for the common vampire bat (*Desmodus rotundus*), to the point of untimely deaths after only 2–3 nights of fasting. To investigate the underlying physiology of this critical metabolic condition, we analyzed serum insulin levels, pancreatic islets morphometry and immunocytochemistry (ICC), static insulin secretion in pancreas fragments, and insulin signaling mechanism in male vampire bats. A glucose tolerance test (*ip*GTT) was also performed. Serum insulin was found to be lower in fed vampires compared to other mammals, and was significantly reduced after 24 h fasting. Morphometrical analyses revealed small irregular pancreatic islets with reduced percentage of β-cell mass compared to other bats. Static insulin secretion analysis showed that glucose-stimulated insulin secretion was impaired, as insulin levels did not reach significance under high glucose concentrations, whereas the response to the amino acid leucin was preserved. Results from *ip*GTT showed a failure on glucose clearance, indicating glucose intolerance due to diminished pancreatic insulin secretion and/or decreased β-cell response to glucose. In conclusion, data presented here indicate lower insulinemia and impaired insulin secretion in *D. rotundus*, which is consistent with the limited ability to store body energy reserves, previously reported in these animals. Whether these metabolic and hormonal features are associated with their blood diet remains to be determined. The peculiar food sharing through blood regurgitation, reported to this species, might be an adaptive mechanism overcoming this metabolic susceptibility.

1. Introduction

It is well established that insulin is the main anabolic hormone in mammals, directly affecting carbohydrate, lipid and protein metabolism through its control of inter-organ substrate flow, stimulating substrate storage when it is abundant in the diet [7].

Glucose homeostasis in fed mammals requires proper regulation of insulin secretion from pancreatic β-cells, which is under the influence of several factors, including metabolic fuels, neurotransmitter release, paracrine mechanisms and circulating hormones [10]. The primary signal for insulin secretion is an elevation in blood glucose concentration [2], even though amino acids such as leucine can also induce insulin secretion in most mammals [28,3].

Earlier studies have shown that the common vampire bat (*Desmodus rotundus*) is highly susceptible to short-term fasting [12], showing a severe hypoglycemia followed by early deaths after 2–3 days of starvation [1]. This metabolic response to fasting is not expected for most mammals with high protein diets, which are known to be especially resistant to long periods of fasting [15,33,30]. The reasons for this unusual response are unclear, but their exclusively sanguineous diet is likely to play an important role, since blood contains 93.1% protein and only 1% carbohydrate [4]. High-protein diets induce striking early metabolic changes in human and animal models, especially when the diet contains at least 50% of energy derived from proteins [9,13].

In this context, recent studies have demonstrated that an intrauterine environment poor in glucose might decrease β-cell mass and insulin production, leading to insulin resistance and glucose intolerance [27]. Other studies have also demonstrated that high protein diets can lead to decreased serum insulin levels and reduced pancreatic islets mass, as well as insulin resistance in adult rats [20,17,18].

To elucidate these points and identify the underlying mechanisms associated with the vampire bat's susceptibility to fasting,

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we investigated the morphophysiological aspects of pancreatic islets, serum insulin levels, insulin secretion response and glucose sensitivity in the common vampire bat.

2. Materials and methods

2.1. Materials

Human recombinant insulin (Humulin®) was acquired from Lilly (São Paulo, SP, Brazil). The reagents used for the insulin secretion protocol and radioimmunoassay (RIA) were acquired from Sigma (St. Louis, MO, USA). The ^{125}I-labeled insulin (human recombinant) for RIA was purchased from PerkinElmer (Waltham, MA, USA). SDS–PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, CA, USA) and all chemicals used were from Bio-Rad (Hercules, CA, USA) and from Sigma (St. Louis, MO, USA). Anti-insulin (rabbit polyclonal), anti-serine–threonine kinase (AKT) (rabbit polyclonal), and anti-phosphorylated AKT (Ser473) (pAKT) (rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and study area

Adult male and non-pregnant female *D. rotundus* (E. Geoffroy, 1810) ($n = 37$), weighing 22–37 g, were captured using mist nets (7×3 m) from caves around Brasília-DF, Brazil (15°30′S, 48°10′W; elevation 814 m). Bats were housed in individual cages maintained in the dark at room temperature. Prior to the experiments, all bats were fed defibrinated bovine blood (40 mL of blood per bat per night) for four nights. Petri dishes containing blood were offered at 2000 pm and removed at 0730 am the next morning. Water was available *ad libitum*. For experiments with 12- or 24-h fasted bats, animals were only given water. When necessary, euthanasia was performed by cervical dislocation followed by guillotining. Free flowing blood was collected from the trunk into saline-washed tubes, centrifuged (10 min at 1500 rpm – 5810R Eppendorf) and serum was frozen at −20 °C for posterior determination of insulinemia.

2.3. Methods

2.3.1. Serum insulin levels

Serum insulin of fed ($N = 7$) and 24-h fasted ($N = 6$) vampire bats was measured by RIA using guinea pig anti-rat insulin antibody and rat insulin as standard [29].

2.3.2. Immunocytochemistry and morphometry of pancreatic islet

Seven pancreata from fed male bats were rapidly removed and immersed in 3.7% formol (phosphate buffered saline (PBS), pH 7.4) for 24 h. After fixation in Bouin's fixative for 3 h, the tissue was dehydrated and embedded in paraffin wax. The tissue was serially sectioned at 6 μm along the longitudinal axis of each pancreas. Morphometry was performed using random sections stained with hematoxylin–eosin and analyzed under a microscope-camera system (ZEISS Stemi SV MC80). For immunocytochemistry analysis, samples were treated with poly-L-lysin and incubated with anti-insulin polyclonal primary antibody and their specific secondary antibodies for pancreatic β-cell identification. To determine the proportion of endocrine tissue in relation to exocrine tissue, hematoxylin–eosin and immunocytochemistry (ICC) stained sections were examined with two softwares (Pixel View Station 4.12 and Image Pro-Plus 6.0), which also allowed the determination of the mean islets total area, diameter and size in 230 randomly and non-consecutively selected sections.

2.3.3. Cumulative static insulin secretion and pancreas insulin content

Cumulative static insulin secretion was investigated according to a previous publication [26] with modifications. Briefly, small pancreatic fragments (~2–3 mm) from fed bats were first incubated for 1 h at 37 °C in 1 mL Krebs-bicarbonate buffer solution of the following composition (in mmol/L): 115 NaCl, 5 KCl, 2.56 CaCl, 1 $MgCl_2$, 24 $NaHCO_3$, 15 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid and 5.6 glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% O2:5% carbon dioxide, pH 7.4. The medium was then replaced with 1 mL of fresh buffer with the addition of: (1) 2.8 mM glucose; (2) 5.6 mM glucose; (3) 22.4 mM glucose; (4) 2.8 mM glucose + 30 mM K^+; (5) 2.8 mM glucose + 20.0 mM leucine; or (6) 2.8 mM glucose + 100 mM tolbutamide and further incubated for 1 h. At the end of the incubation, the samples were stored at −20 °C for subsequent measurement of insulin content by RIA. During pre- and incubation periods, the medium also contained a mixture of anti-proteases (Trasylol, 2%) to avoid insulin degradation by exocrine enzymes. The insulin content in the pancreatic fragments of each well was also measured. For this purpose, the fragments were transferred into a 1.5-mL microcentrifuge tube, washed in Krebs-bicarbonate buffer solution with 2.8 mmol/L glucose, and the medium replaced with extraction solution (12 mmol/L HCl in 70% ethanol). Then, the fragments were sonicated for 15 s, maintained overnight at 4 °C, centrifuged for 10 min at 3000*g*, and the supernatant was frozen at −20 °C for subsequent analysis of insulin content by RIA.

2.3.4. Intraperitoneal glucose test (ipGTT)

For the *ip*GTT, 12-h fasted bats (F12) had blood samples collected from the wing vein at the following times: zero (T0) before i.p. glucose administration (2 g/Kg b.w., 0.5 mL) and after 60, 120, 180 and 240 min (T60, T120, T180 and T240) of glucose load. The control group received i.p. saline administration (0.9%, 0.5 mL) and was submitted to the same procedures as *ip*GTT. In all groups, blood glucose was determined with Accu Chek Active, Roche (error < 1%).

2.3.5. Protein extraction and immunoblotting for pAkt and Akt

Protein extraction and immunoblotting were carried out as previously reported [26] with modifications. Immunoblotting experiments were performed at least three times using different samples (each sample consisting of tissue obtained from one bat). Fragments of liver were obtained from the same anatomical area of each bat. Briefly, fed bats were killed after 5 or 15 min of insulin (1 unit/Kg b.w., 0.5 mL) or saline (0.9%, 0.5 mL) administration. Fragments of 200 mg of liver were first homogenized in Hanks solution using a Polytron homogenizer (PTA 20S, model PT10/35 – Brinkmann Instruments, NY, USA) (2 pulses of 15 s at the maximal speed). The extracts were then centrifuged (5804R Eppendorf) at 15,000 rpm at 4 °C for 45 min to remove insoluble material. Supernatant aliquots were used for antibody precipitation. The protein concentration in supernatants was measured by the Bradford method, according to the manufacturer (Bio-Rad, Hercules, CA, USA). The samples were treated with Laemmli sample buffer containing 10 mM dithiothreitol and boiled for 5 min. All volumes were adjusted (Multiskan EX, Labsystems) so that aliquots of similar protein concentrations were applied to a 10% polyacrylamide gel and separated by SDS–PAGE in a Bio-Rad miniature slab gel apparatus, paralleled with a molecular weight marker. The electrotransfer of proteins from the gel to nitrocellulose was done at 120 V for 50 min or 1 h in a BioRad miniature transfer apparatus. Before incubation with the primary antibody, the nitrocellulose filters were treated with a blocking buffer (5% non-fat dried milk, 10 mM Trizma, 150 mM NaCl and 0.02% Tween 20) for 2 h at 22 °C. The membranes were incubated for 4 h at 22 °C with