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Original article

Hepatic glucokinase activity is the primary defect in alloxan-induced diabetes of mice

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Abstract

Alloxan is a classical diabetogen which is used to achieve β-cell destruction and type 1 diabetes due to its selective cytotoxic effect on pancreatic β-cells. Although alloxan-induced diabetes is widely used in the laboratory to mimic diabetic pathology and for screening antidiabetic drugs, there has not been any comprehensive research in vivo on its diabetogenicity. In our study, alloxan-induced diabetic mice were generated by a single intravenous injection of alloxan (100 mg/kg). Our data show that these mice possess hyperglycemia, hypoinsulinism and morphological characteristics of impaired pancreas that are consistent with the accepted diabetogenic effects of alloxan. Alloxan is believed to confer its diabetogenic effect by inhibiting pancreatic glucokinase activity, leading to pancreatic β-cell death. We examined the effects of alloxon on the other major site of glucokinase expression, the liver. Our results show that alloxan treatment led to an 81% reduction in glucokinase immunoreactivity and a greater than 90% reduction in glucokinase enzymatic activity in the liver, suggesting that alloxan's toxicity is not specific to the pancreas. Given the important role of glucokinase as a glucose sensor, and our findings on the effects of alloxon on liver glucokinase activity we propose that the effects on the liver are the primary contributor to pathogenesis in alloxan-induced diabetes. Alloxan-induced diabetes is thus a multifactor-promoted diabetes model which still could be used to examine the antidiabetic effects of compounds prompting insulin secretion and increasing liver-specific glucokinase activity. Despite alloxan-induced diabetes being inconsistent with the natural pathogenesis of human diabetes, further research on the causes of decreased glucokinase activity will help us to unravel the pathogenesis of diabetes and its complications.

Keywords: Alloxan; Alloxan-induced diabetes; Diabetes mellitus; Glucokinase

1. Introduction

Diabetes mellitus (DM) is a common irreversible metabolic disorder whose overall prevalence continues to increase due to population aging and improvements in the quality of life. Currently there is not a perfect approach to treat diabetes and its complications, due to variability in the causes of diabetes. Animal models provide valuable clues into understanding the underlying pathological mechanisms of diabetes and are useful for the screening of drugs for the prevention and treatment of diabetes. Currently, chemically induced models have gained widespread acceptance for pathogenesis and drug screening research due to their rapid induction of diabetes and reproducibility. Alloxan is a commonly used chemical to generate diabetic animals in the laboratory for its ability to destroy insulin-producing β-cells. It is generally accepted that free radicals, especially superoxide radicals, induced by alloxan cause cellular damage that is key to its role as a diabetogen [1,2]. Glucose transporter 2 (GLUT2) and glucokinase (GK) have been reported as are target molecules for alloxan [3–6]. Besides pancreatic β-cells, we wondered whether there are any other tissues susceptible to the toxicity of alloxan. If damage caused by alloxan exists in other tissues, it would

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affect the reliability of using alloxan-induced diabetes as a model of human diabetes. Could other alloxan-damaged tissues be related to the observed hyperglycemia? To examine the origin of hyperglycemia in alloxan treated animals we made diabetic mice by classical method and examined the serum and liver by biochemical, molecular, and morphological approaches.

2. Materials and methods

2.1. Reagents

Alloxan monohydrate, glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide (NAD) were purchased from Fluka (Fluka Scientific Research, Sigma-Aldrich, USA); polyclonal antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA). Human insulin radioimmunoassay kits were purchased from Beijing Atom High-tech Company (Beijing, China). All other chemicals were of analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China).

2.2. Animal treatments

Kunming mice of both sexes (22–24 g body weight) were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center. Mice were fed standard diet during the experimental period. Mice were randomly divided into two groups and were acclimatized for 7 days before experiments. The mice were treated to standard environmental conditions for temperature, relative humidity and dark/light cycle. Groups A and B were treated with saline, alloxan monohydrate, respectively. Mice were made diabetic by a single intravenous injection of alloxan monohydrate (100 mg/kg) after 16 h of fasting. Seventy-two hours after injection, mice with fasting plasma glucose levels of >11.1 mmol/L were included in the study. Blood samples at 8 h of fasting were collected at the end of the study. After blood sample collection, the liver and pancreas of the mice were removed and immediately frozen by liquid nitrogen and stored at −80 °C for further study.

2.3. Assessment of serum parameters

Fasting and postprandial blood glucose levels at 30 min and 120 min in control and alloxan-induced diabetic mice were determined by the glucose oxidase method [7]. In addition, serum triglyceride (TG), total cholesterol (TC) and alanine aminotransferase (ALT) were also experimentally measured using commercially available detection kits. Liver glycogen content was measured according to the anthrone-reagent method. Serum insulin levels were detected by radioimmunoassay (RIA) [8].

2.4. Measurement of mouse glucokinase and insulin receptor protein level in liver

Liver tissues were prepared and stored at −80 °C until analysis. About 100 mg liver tissue was homogenized in 1 ml lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 1 mM EDTA, 0.5% sodium deoxycholate, 100 μg/ml PMSF, 1 μg/ml leupeptin and 1% NP-40, for 30 min at 4 °C. After lysis, cellular debris was removed by centrifugation at 10,000*g* for 10 min at 4 °C. DNA in the supernatant was disrupted by sonicating briefly. Protein concentration was quantified by the Bradford protein assay using bovine serum albumin as standard [9]. Protein samples with equal volume of 2× SDS loading buffer containing 100 mM Tris–HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% (v/v) glycerol and 200 mM DTT were denaturalized by boiling for 10 min, then separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes in a cold chamber. The membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with anti-GCK polyclonal antibody (rabbit polyclonal antibody against GCK, sc-7908, Santa Cruz Biotechnology, Inc., CA) and anti-insulin Rβ polyclonal antibody (rabbit polyclonal antibody against insulin Rβ (c-19), sc-711, Santa Cruz Biotechnology, Inc., CA). After three washes with TBST for 10 min each, the membranes were incubated for 2 h with alkaline phosphatase-conjugated secondary antibodies, and finally washed thoroughly with TBST. Protein bands of interest were visualized by an NBT/BCIP reagent detection system and quantified using a Gel Doc 2000 densitometer (BIO-RAD, USA).

2.5. Determination of glucokinase activity

Glucokinase activity was measured using published methods [10,11]. About 100 mg liver tissues were homogenized in 1 ml ice-cold homogenization buffer containing 100 mM KCl, 25 mM HEPES, 7.5 mM $MgCl_2$, 4 mM dithiothreitol (pH 7.4) and then lysed overnight at 4 °C. Supernatant from the extracts was obtained after centrifugation at 3000 rpm for 10 min at 4 °C, and then supplemented with 1 mM NAD, 4 mM ATP, and 100 or 0.5 mM glucose at pH 7.4. The enzymatic reaction was started by the addition of 0.2 unit of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and incubated for 10 min at 30 °C. NADH generated by glucokinase was measured using a spectrophotometer at 340 nm. Protein concentration was quantified by the Bradford protein assay and hexokinase activities were expressed as mU/mg protein. The enzymatic activity of glucokinase was calculated as the difference between activities calculated at 100 and 0.5 mM glucose.

2.6. Morphological analysis of the pancreas and liver

Organs were quickly removed after cervical dislocation. Pancreas tissues were stained by periodic acid-Schiff (PAS) or routine hematoxylin and eosin (H&E) staining. Briefly, tissue was fixed with 4% formaldehyde, embedded in paraffin,