IMMUNOCHEMICAL TITRATION OF LIVER GLUCOKINASE FROM NORMAL, FASTED, AND DIABETIC RATS

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1. Introduction

Phosphorylation of glucose is an adaptive function in the liver of rodents and other mammalian species [1]. In fact, glucokinase which is one of four isoenzymes catalyzing this reaction [2], decreases in alloxan diabetic rats, and in normal rats which have been deprived of food or have been fed with a glucose-free diet [3–6]. Normal levels of the enzyme are recovered following administration of glucose and/or insulin [3–6]. In spite of the time elapsed since these observations were first made there is still no good evidence that changes in glucokinase activity parallel changes in the amount of the enzyme protein. The fact that actinomycin D, puromycin or cycloheximide, as well as amino acid and nucleotide analogs prevent glucokinase increase after depletion [4, 5, 7–9] gives support to the idea that de novo synthesis of the enzyme is implicated in that increase. Thus, the phenomenon is referred to as enzyme induction. The preferential labeling with ^{14}C-labelled amino acids of a liver protein fraction enriched with glucokinase during induction of the enzyme, gives further support to that contention [10].

Immunological evidence that changes in glucokinase activities under various experimental conditions correspond to changes in the enzyme protein is presented in this report.

2. Methods

2.1. *Immune serum*

Rat liver glucokinase used to prepare the antiserum was purified according to a previously published procedure [2], with minor modifications. An immune serum (antiglucokinase serum, *AGS*[2]) was obtained from a goat which was injected by the intramuscular route at approximately one month intervals with the purified glucokinase emulsified in complete Freund's co-adjuvant. The serum used in this work was obtained after three months of treatment. The immune proteins were purified by precipitation with ammonium sulfate [11] and dissolved in 0.15 M NaCl. Immune proteins from a non-immunized goat were similarly obtained to act as a control.

2.2. *Liver extracts*

Rats weighing about 200 g were used. Animals submitted to various experimental conditions (table 1) were decapitated and the livers excised and kept on ice. Liver extracts exhibiting the following specifications were prepared: i) Absence of low-K_m hexokinases, 6-phosphogluconate dehydrogenase and sugar dehydrogenases, all of which interfere with the glucokinase assay; and ii) quantitative and reproducible recovery of glucokinase. This was accomplished as follows. A fifty per cent (w/v) homogenate was prepared in a Tris buffer solution containing (final concentrations): 0.01 M Tris–HCl, pH 7.0; 0.001 M

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Table 1
Liver glucokinase in rats submitted to various dietary and hormonal manipulations.

Experimental conditions	Glucokinase (Units per 100 g body weight)	Key for fig. 1
Normal (3)	2.70; 3.04; 3.74	●
Fasting, 1 day (2)	1.03; 1.04	◒
Fasting, 2 days (2)*	0.30	○
Fasting, 2 days; refeeding, 1 day (1)	6.31	□
Diabetes, 1 day (2)**	1.60	△
Diabetes, 2 days (4)***	0.70; 1.38	▲

Number of rats in parentheses.

* The liver of two animals were pooled to prepare the extract.

** Diabetes was induced by the intravenous injection of streptozotocin (55 or 65 mg per kg body weight) dissolved in citrate buffer pH 4.5. Blood glucose concentrations at the time of death were between 350 and 400 mg per 100 ml.

The results of the immunochemical titration of liver extracts from these animals are presented in fig. 1.

EDTA**; 0.15 M KCl; 0.1 M glucose; 0.004 M DTT. After centrifugation at 105 000 *g* for 20 min, the supernatant fluid was chromatographed through a DEAE-cellulose (Schleicher & Schuell, lot 2108) column (1 × 17 cm) equilibrated with the buffer solution. A gradient was established from 0.15 to 0.5 M KCl in a similar buffer solution in order to elute the enzyme. The glucokinase peak was collected and concentrated in a small DEAE-cellulose column (1 × 4 cm).

2.3. *Immunochemical titration*

The liver extracts were incubated with the antiserum in a medium containing (final concentrations): 0.1 M Tris–HCl, pH 7.5; 0.001 M EDTA; 0.15 M KCl; 0.1 M glucose; 0.005 M DTT. The mixtures containing a fixed amount of *AGS* and variable amounts of glucokinase units were incubated for 30 min at 37°C and then left overnight in melting ice. After centrifugation, convenient aliquots from the supernatant fluid were taken for duplicate glucokinase assays. Glucokinase activity was measured in a Gilford spectrophotometer by following the change in absorbancy at 340 nm, in a system containing glucose 6-phosphate dehydrogenase (Boehringer) and NADP (Sigma). One unit of glucokinase is the amount of enzyme catalyzing the phosphorylation of 1 μmol of glucose in 1 min at 30°C under the conditions used [2].

3. Results and discussion

Fig. 1 shows that glucokinase obtained from animals submitted to various treatments exhibited identical immunochemical behaviour when used to titrate the specific antiserum. In fact the same equivalence point was obtained with liver extracts prepared from normal, fasted, and diabetic rats, as well as from animals refed after two days of starvation. The results indicate that glucokinase activities correspond in all cases with equivalent antigen activities.

Glucokinase incubated without *AGS* (fig. 1) or with immunoproteins obtained from a non immunized goat (not shown) did not decrease during the incubation. However, an inactivation was observed when either glucose or DTT were withdrawn from the mixture.

The goat antiserum inhibited glucokinase from other mammalian species, but neither amphibian glucokinase nor the low K_m hexokinases from rat liver, thus confirming results obtained with a rabbit antiserum [12]. A more detailed account of the properties of the antiserum will be published elsewhere.

** Abbreviations used: AGS, antiglucokinase serum; EDTA, ethylenediaminotetraacetate; DTT, dithiothreitol.

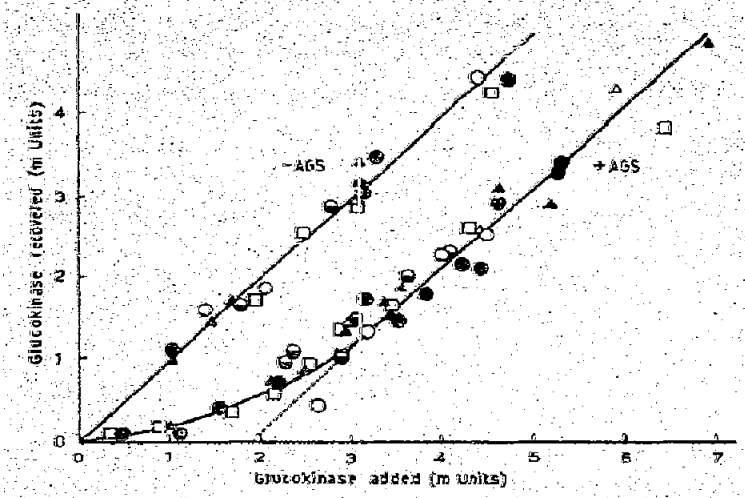

Fig. 1. Immunochemical titration of rat liver extracts. Increasing volumes of liver extracts ('glucokinase added') were incubated with a constant amount (1.92 mg) of immune protein (*AGS*) as described in the text. 'Glucokinase recovered' corresponds to the enzyme activities assayed after incubation and centrifugation. The extracts were prepared from rats submitted to various experimental conditions (see key of symbols on table 1). The control line (*–AGS*) corresponds to the theoretical complete recovery of enzyme activities after incubation in the absence of antiserum. The experimental line (*+AGS*) was drawn parallel to the control and fitting the points corresponding to normal animals. The equivalence point was obtained by extrapolation.

The results presented in this report give immunological support to the contention that changes in liver glucokinase activities observed after dietary and hormonal manipulations are the result of changes in the amount of enzyme protein.

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