Long-term maintenance of low concentrations of fructose for the study of hepatic glucose phosphorylation

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The stimulation of glucose phosphorylation in isolated hepatocytes by low fructose concentrations is transient due to the rapid metabolism of fructose. To prolong this stimulatory effect fructose was enzymically generated in the incubation medium from either sucrose with invertase or inulin with inulinase. A maximal rate of glucose phosphorylation was achieved when fructose was formed at at least 0.01 $\mu\text{mol}/\text{min}$, which maintained a concentration of 70 μM fructose in the medium. In the presence of a fructose concentration of 70 μM , the rate of phosphorylation with 5 mM glucose was doubled and remained constant over a 2.5 h period. Under these conditions the rate of glycolysis was increased more than 3-fold. The stimulation of flux through

glucokinase by low concentrations of fructose decreased the proportion of glucose phosphorylated, which was cycled between glucose and glucose 6-phosphate, and increased the proportion that was glycolysed. The method described for maintaining the stimulation of glucose phosphorylation by isolated hepatocytes over prolonged incubation periods is especially suited to the further study of the control of glucokinase activity, in particular how the variation of flux through glucokinase affects the flux through all the pathways that utilize the product, glucose 6-phosphate.

Key words: glucokinase, hepatocyte, inulinase.

INTRODUCTION

It is well established that the low rates of hepatic glucose phosphorylation at glucose concentrations below 10 mM are stimulated in the presence of low concentrations of fructose [1,2]. The stimulatory effect of fructose is mediated through the formation of fructose 1-phosphate, which antagonizes the inhibition of glucokinase caused by a specific regulatory protein [3,4]. This regulatory agent is a constitutive nuclear protein [5] that binds and inhibits glucokinase, the binding being greatest at physiological glucose concentrations [6,7]. When the binding between glucokinase and its regulatory protein is disrupted, glucokinase translocates from the nucleus to the cytoplasm [5,8,9] where it catalyses the phosphorylation of glucose.

Studies with fructose *in vitro*, employing isolated hepatocytes, have been important in understanding the control of hepatic glucose phosphorylation, but the use of fructose in hepatocyte incubations has some limitations. The stimulation of glucose phosphorylation is maximal in the presence of fructose concentrations < 0.2 mM [2,10,11]. However, because of the rapid metabolism of fructose by isolated hepatocytes [12], this stimulatory effect on glucose metabolism is transient [11]. Thus fructose addition is not satisfactory when the objective is to study hepatic metabolism over longer incubation periods. Attempts to prolong the stimulatory effect of fructose by making a second addition to the incubation medium have proved to be ineffective [11]. Furthermore, it is not possible to use higher initial concentrations of fructose as this results in inhibition of glucose metabolism [1,13] and a dramatic reduction in cellular ATP concentrations [1].

In this paper we describe two methods for maintaining low fructose concentrations in hepatocyte incubations, whereby the stimulatory effect of fructose on the phosphorylation and utilization of glucose at physiological concentrations may be studied for periods up to at least 150 min.

MATERIALS AND METHODS

Materials

Collagenase, invertase and enzymes necessary for the assay of metabolites were purchased from Boehringer-Mannheim (Castle Hill, NSW, Australia) as was BSA (fraction V), which was defatted according to Chen [14]. Inulin was obtained from Sigma (St. Louis, MO, U.S.A.) and inulinase (Novozym 230) was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). All other chemicals were of the highest purity commercially available. HPLC-purified [2-³H] glucose, [6-³H] glucose and [U-¹⁴C] glucose were obtained from New England Nuclear (Boston, MA, U.S.A.). For the separation of radiolabelled glucose and its metabolic products Dowex AG50-X8 (H⁺, 100–200 mesh) and Dowex AG1-X8 (Cl⁻, 100–200 mesh) were purchased from Bio-Rad (Hercules, CA, U.S.A.).

Preparation and incubation of hepatocytes

Hepatocytes were prepared from male Hooded Wistar rats (280–300 g body weight), which had been starved for 24 h to deplete liver glycogen, by a modification [15] of the method of Berry and Friend [16], in which 1 mM Ca²⁺ was added to the washing media. The hepatocytes (100 mg wet weight) were incubated at 37 °C in 2 ml of a balanced bicarbonate/saline solution containing 5 mM glucose and 2.25 % (w/v) albumin, with a gas phase of 95 % O₂, 5 % CO₂ [17,18]. Where added, the initial concentrations of inulin, sucrose and fructose were 0.12 % (w/v), 5 mM and 0.2 mM respectively. For the measurement of glucose phosphorylation the incubation mixture initially contained 1 μCi of [2-³H] glucose [19]. In experiments where the rates of glycolysis and glucose cycling were determined, the initial incubation contained 1 μCi of [6-³H] glucose and 0.5 μCi of [U-¹⁴C] glucose [20].

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Long-term maintenance of low concentrations of fructose

Fructose was generated enzymically from sucrose with invertase or from inulin with inulinase, under standard conditions for the incubation of hepatocytes (37 °C, pH 7.4). Under these conditions both enzymes displayed < 10% of the activity measured at pH 4.6 under optimal assay conditions. The commercial inulin preparation was found to contain traces of free fructose. In order to remove this contamination, a saturated solution of inulin was prepared and the undissolved inulin was recovered by filtration and subsequently dried in a desiccator. The addition of invertase or inulinase to hepatocyte incubations had no detrimental effect on hepatocyte quality, judged by the degree of lactate dehydrogenase leakage and Trypan Blue uptake, or on the metabolic capacities. Stock aqueous solutions of the enzymes were prepared immediately before use and further adjustment of the specific activity of the working enzyme solutions were made by dilution. Aliquots (10 μ l) were added to incubation mixtures using a Hamilton syringe, fitted with a Chaney adaptor, at the commencement of the incubation period.

Analytical procedures

For the measurement of radioisotopic products of glucose metabolism, a 0.5 ml sample was deproteinized with 1.5 ml of ice-cold ethanol at the completion of the incubation period. The hepatocytes from a 0.3 ml sample were recovered by centrifugation (12000 *g* for 10 s) for the determination of radioactive label incorporated into glycogen [19]. The remaining portion of the incubation mixture was deproteinized with an equal volume of ice-cold 1 M perchloric acid and then neutralized before metabolites were measured by standard enzymic techniques [21]. Fructose was measured enzymically according to Bernt and Bergmeyer [22]. The analysis is based on the oxidation of glucose 6-phosphate, which is formed following the phosphorylation of fructose to fructose 6-phosphate by hexokinase, and its subsequent isomerization by phosphoglucosomerase. Thus it was essential to remove any glucose 6-phosphate present in the cells, or formed directly from the phosphorylation of glucose, by the addition of phosphoglucosomerase before the fructose analysis was commenced. With this approach it was feasible to measure fructose concentrations as low as 5 μ M in incubation mixtures containing up to 5 mM glucose. The separation of radiolabelled glucose and water was achieved by ion-exchange chromatography [13,23].

Measurement of glucose phosphorylation and glycolysis

The rates of hepatic glucose phosphorylation and glycolysis were measured isotopically as previously described [19,24] using [2-³H], [6-³H] and [U-¹⁴C]glucose respectively. However, in the experiments described in the present work, the specific radioactivity of the glucose fell in the presence of each fructose-generating system as a result of glucose cycling between glucose and glucose 6-phosphate and the metabolism of fructose to glucose. This decline in the specific radioactivity of glucose was greatest when invertase was employed, due to the direct formation of glucose from sucrose. In order to correct for this change, incubations were terminated at 20 min intervals over the course of each experiment. The average specific radioactivity of glucose and the change in isotope present in the products of glucose metabolism were determined for each interval. From these measurements the amount of glucose metabolized and the products formed were determined for each time period.

RESULTS

Enzymic formation of fructose under conditions of hepatocyte incubation

Both invertase and inulinase have a pH optimum of about 4.6. It was necessary, therefore, to determine, under standard hepatocyte incubation conditions, the amount of each enzyme required to establish and maintain concentrations of < 0.2 mM fructose in the medium, which has previously been shown to be optimal for the stimulation of glucose phosphorylation [2,10]. Figure 1 indicates that, in initial studies with these fructose-generating systems, measurable levels of fructose in the medium were rapidly established in incubations containing hepatocytes when the fructose was formed enzymically from sucrose and inulin and that by 40 min steady-state fructose concentrations of 130 μ M and 70 μ M respectively were attained. The higher concentration of fructose achieved using the sucrose/invertase system reflected the difference in the activities of the invertase and inulinase added to the incubation mixtures. Under these conditions, the rates of fructose formation in the absence of hepatocytes were 0.020 ± 0.004 and 0.010 ± 0.002 μ mol/min by the sucrose/invertase and the inulin/inulinase systems respectively.

Effect of maintaining low fructose concentrations on hepatic glucose phosphorylation

The rate of glucose phosphorylation by hepatocytes, when incubated in the presence of 5 mM glucose, was 0.27 ± 0.01 μ mol/min/g wet weight ($n = 17$), which remained constant over an 80 min period (Figure 2). In confirmation of previous studies, the addition of 0.2 mM fructose to the incubation mixture doubled the initial rate of glucose phosphorylation, however, this effect was short lived and by 40 min the phosphorylation rate had reverted to that observed in the presence of glucose alone (Figure 2). In these experiments there was no measurable fructose in the incubation mixtures by 30 min (results not shown). In contrast, in experiments in which fructose was formed enzymically from sucrose or inulin, the stimulation of glucose phos-

Figure 1 Enzymic formation of fructose under conditions of hepatocyte incubation

Hepatocytes from fasted rats were incubated with 5 mM [2-³H]glucose in the presence of either 5 mM sucrose plus 20 m-units of invertase (●) or 0.12% (w/v) inulin plus 10 m-units of inulinase (■) as described in the Materials and methods section. The concentrations of fructose in the incubation medium for each combination are presented as the means $\pm$ S.E.M. of seven individual experiments.

Figure 2 Stimulation of hepatic glucose phosphorylation by fructose

Hepatocytes from fasted rats were incubated with 5 mM $[2\text{-}^3\text{H}]\text{glucose}$ ($1\ \mu\text{Ci}$) in the absence (●) or in the presence of 0.2 mM fructose (□), 5 mM sucrose plus 20 m-units of invertase (△) or 0.12% (w/v) inulin plus 10 m-units inulinase (■). The ^3H -label from $[2\text{-}^3\text{H}]\text{glucose}$ appearing in water and glycogen is represented as glucose phosphorylation. The data for each time point are the means $\pm$ S.E.M. of seven separate experiments.

phorylation was maintained for the whole of the incubation period (Figure 2). The stimulation of phosphorylation was maximal about 10 min after the addition of fructose to, or its formation in, the medium. Each fructose-generating system stimulated the rate of glucose phosphorylation to the same degree, even though the rate of formation and concentration of fructose maintained in the medium were higher with the sucrose/invertase system.

Effect of varying the rate of fructose formation on hepatic glucose phosphorylation

In order to determine the minimal rate of fructose formation required to produce a constant and maximal stimulation of glucose phosphorylation, hepatocytes were incubated in the presence of 5 mM $[2\text{-}^3\text{H}]\text{glucose}$ and a range of inulinase activities. The inulin/inulinase system alone was used in this series of experiments to ensure that the changes in the specific radioactivity of $[2\text{-}^3\text{H}]\text{glucose}$ over the incubation period were minimal. The rate of glucose phosphorylation increased with additions of inulinase up to 10 m-units; no further stimulation of phosphorylation was observed with higher inulinase activities (Figure 3a). The concentration of fructose in the medium, measured at 40 min, was found to be a linear function of the inulinase activity (Figure 3b). There was a significant stimulation of glucose phosphorylation by 0.5 m-unit of inulinase, even though the concentration of fructose in the medium was too low to be measured with the method employed. Glucose phosphorylation was stimulated when fructose was generated in the medium at rates of up to $0.01\ \mu\text{mol}/\text{min}$. Although higher rates of fructose formation produced correspondingly higher fructose concentrations in the medium, there was no further stimulation of the rate of phosphorylation when the fructose concentration was $\geq 70\ \mu\text{M}$ (Figure 3). From these studies it was concluded that the lowest amount of inulinase required to achieve a maximal rate of glucose phosphorylation in the presence of 5 mM glucose was 10 m-units. In another series of experiments, in which hepatocytes were incubated with 10 mM glucose, a maximal stimulation of glucose phosphorylation was also achieved in the presence of 10 m-units of inulinase (results not shown).

Figure 3 Glucose phosphorylation and fructose formation in hepatocytes incubated with $[2\text{-}^3\text{H}]\text{glucose}$ and inulinase

Hepatocytes from fasted rats were incubated with 5 mM $[2\text{-}^3\text{H}]\text{glucose}$ ($1\ \mu\text{Ci}$) and 0.12% (w/v) inulin in the presence of a range of inulinase activities from 0–20 m-units. The rate of glucose phosphorylation (a) and the concentration of fructose established in the medium at 40 min (b) for each inulinase activity are expressed as the mean $\pm$ S.E.M. of four separate experiments. Glucose phosphorylation represents ^3H -label recovered from $[2\text{-}^3\text{H}]\text{glucose}$ in water and glycogen. One unit of inulinase activity is the amount of enzyme which forms $1\ \mu\text{mol}$ of fructose per min from inulin under the conditions described for the incubation of hepatocytes.

In the presence of glucose alone, the rate of glucose phosphorylation was 0.27 ± 0.01 ($n = 17$) and the rate of net glucose removal was $0.07 \pm 0.01\ \mu\text{mol}/\text{min}/\text{g}$ wet weight. Experiments that included $[2\text{-}^3\text{H}]$, $[6\text{-}^3\text{H}]$ and $[\text{U}\text{-}^{14}\text{C}]\text{glucose}$ demonstrated [19,20] that, in incubations containing glucose alone, the difference between the rates of glucose phosphorylation and net removal ($0.20 \pm 0.02\ \mu\text{mol}/\text{min}/\text{g}$ wet weight) was the result of glucose cycling, mainly between glucose and glucose 6-phosphate. In consequence, in the presence of a cell mass of 100 mg wet weight, the initial pool of $10\ \mu\text{mol}$ of added $[2\text{-}^3\text{H}]\text{glucose}$ was diluted at a rate of $0.02\ \mu\text{mol}/\text{min}$ by unlabelled glucose that had been detritiated as a result of cycling, representing a decrease in specific radioactivity of only 4% during a 20 min incubation.

An optimal addition of inulinase increased the rate of glucose phosphorylation to $0.53 \pm 0.03\ \mu\text{mol}/\text{min}/\text{g}$ wet weight ($n = 10$) and raised the net rate of glucose removal to $0.13 \pm 0.02\ \mu\text{mol}/\text{min}/\text{g}$ wet weight. The rate of glucose cycling was $0.30 \pm 0.03\ \mu\text{mol}/\text{min}/\text{g}$ wet weight, which thus failed to account for the difference of $0.40\ \mu\text{mol}/\text{min}/\text{g}$ wet weight between the rates of glucose phosphorylation and glucose removal. Control incubations of hepatocytes, to which no glucose was added, demonstrated that, in the presence of inulin and inulinase, there was a net formation of glucose of $0.13 \pm 0.02\ \mu\text{mol}/\text{min}/\text{g}$ wet weight ($n = 10$) but in the presence of cells it was not possible to determine the relative contribution of this glucose derived directly from fructose or from endogenous substrates. The overall effect of the formation of unlabelled glucose was to lower the specific radioactivity of the glucose by 9% over a 20 min period. As described in the Materials and methods section, an appropriate

Figure 4 Glucose metabolism and lactate + pyruvate accumulation during hepatocyte incubation

Hepatocytes from fasted rats were incubated for up to 150 min with 5 mM [$2\text{-}^3\text{H}$]glucose ($1\text{ }\mu\text{Ci}$) or 5 mM [$6\text{-}^3\text{H}$]glucose ($1\text{ }\mu\text{Ci}$) plus [$1\text{-}^{14}\text{C}$]glucose ($0.5\text{ }\mu\text{Ci}$) in the absence (open symbols) or presence of 0.12% (w/v) inulin plus 10 m-units of inulinase (closed symbols). The data presented is a representative of three separate experiments in which glucose phosphorylation (□, ■) and glycolysis, determined as the accumulation of labelled products from [$6\text{-}^3\text{H}$]glucose (△, ▲) and [$1\text{-}^{14}\text{C}$]glucose (○, ●), were measured (a). The concentration of lactate + pyruvate in the medium in the absence (▽) or presence (▼) of the fructose-generating system in the same experiment are shown in (b). In this representative experiment 85 mg wet weight of hepatocytes were present in each incubation. Mean rates of glucose phosphorylation for three separate experiments in the absence (□) and presence (■) of inulin/inulinase were 0.26 ± 0.03 and $0.56 \pm 0.05\text{ }\mu\text{mol}/\text{min}/\text{g}$ wet weight respectively. The rates of glycolysis measured with [$6\text{-}^3\text{H}$] and [$1\text{-}^{14}\text{C}$]glucose showed the same variation.

correction for the dilution of [$2\text{-}^3\text{H}$]glucose was made regularly throughout each incubation period and this change in glucose specific radioactivity was taken into account when calculating the rate of glucose phosphorylation.

Long-term maintenance of the stimulation of glucose phosphorylation by fructose

An objective of this study was to establish conditions for the maintenance of stimulated rates of glucose phosphorylation over prolonged incubation periods in order to determine the influence of changes in flux through glucokinase on the various pathways that utilize glucose 6-phosphate. Figure 4(a) shows that the rate of phosphorylation with 5 mM glucose was stimulated 2-fold over a 150 min period at a fructose formation rate of $0.01\text{ }\mu\text{mol}/\text{min}$, which maintained a concentration of $70\text{ }\mu\text{M}$. Under these conditions the rate of glycolysis measured using either [$6\text{-}^3\text{H}$] or [$1\text{-}^{14}\text{C}$]glucose was increased more than 3-fold; the sustained discrepancy between the two measures of glycolysis indicated a constant rate of substrate cycling between glucose and pyruvate over this extended period [25,26]. The stimulation of flux through glucokinase by fructose had a pronounced effect on the ac-

cumulation of lactate and pyruvate in the medium. In the absence of the fructose-generating system a low, constant rate of lactate + pyruvate accumulation was measured over the entire incubation period; in the presence of fructose the rate of lactate + pyruvate accumulation was stimulated 10-fold but this rate was maintained only for the initial 90 min period (Figure 4b). At longer incubation times, the rate of accumulation decreased so that by 120 min a steady-state lactate + pyruvate concentration of 1.4 mM was established (Figure 4b). The changes in the rate of lactate + pyruvate accumulation over the 150 min period occurred under conditions where rates of glucose phosphorylation and utilization were constant (Figure 4a). No lactate + pyruvate accumulated in the presence of the fructose-generating system alone (results not shown).

From the data in Figure 4(a), under conditions where the flux through glucokinase was stimulated, it was calculated that about 60% of the glucose phosphorylated was glycolysed compared with only 40% in the absence of the fructose-generating system. As a consequence of this increased flux through glucokinase, the production of glucose phosphorylated that was recycled to glucose fell by 20%. Even though glycogen synthesis was doubled in the presence of fructose, this represented only 6% of the glucose phosphorylated (results not shown).

DISCUSSION

The stimulation of glucose metabolism by fructose has been important in elucidating features of the control of glucokinase activity in the cell; however, its application in the study of hepatic glucose metabolism has some major limitations. Van Schaftingen's group has shown that the translocation (and activity) of glucokinase is proportional to the cellular concentration of fructose 1-phosphate [12]. As a consequence of the rapid metabolism of fructose by hepatocytes the concentration of fructose 1-phosphate initially rises, then falls as the fructose is utilized to levels that are unable to maintain the activation of glucokinase. This inability of a single addition of fructose to sustain glucose phosphorylation has been described previously [11] and is confirmed in the present study. Several experimental approaches have been made in order to maintain the stimulatory effect of fructose on glucokinase activity over extended incubation periods, however these approaches have proved unsuccessful. The use of higher concentrations of fructose is precluded as these inhibit glucose metabolism [1,13] and decrease the cellular concentration of ATP. Sequential additions of fructose to the incubation medium are also ineffective in maintaining increased rates of glucose phosphorylation [11]. In the present study, two enzymic methods for generating and maintaining low concentrations of fructose in incubations of hepatocytes have been described, which enable the rates of glucose phosphorylation and metabolism from physiological concentrations to be stimulated and to remain elevated for incubation periods up to 150 min.

Of the two enzymic systems tested, the enzymic degradation of inulin by inulinase was the more advantageous as fructose was the only product of the reaction. This is an important consideration in experiments where isotopes and low concentrations of glucose are used, because the fructose-generating system itself does not directly contribute to the lowering of the specific radioactivity of the glucose in the medium. The subsequent conversion of part of the fructose formed into glucose by hepatocytes does contribute to a fall in the specific radioactivity of glucose. However, the contribution of this process is small in comparison with the recycling of glucose through the glucose/glucose 6-phosphate cycle [23], which accounted for 70% of the unlabelled glucose formed in these incubations.

A rate of fructose formation of 0.01 $\mu\text{mol}/\text{min}$ was required to maximally stimulate hepatic glucose phosphorylation. Under these conditions, a fructose concentration of 70 μM was maintained in the medium as a consequence of the balance between the rate of fructose formation in the inulinase reaction and its rate of utilization catalysed by hepatocyte fructokinase. The K_m for fructose of hepatic fructokinase is reported to be approx. 0.35 mM [12]. Thus in the presence of an optimal inulinase activity for the stimulation of glucose phosphorylation the concentration of fructose attained in the incubation medium was 20 % of the K_m of the enzyme for fructose. It would be expected, therefore, that the concentration of fructose 1-phosphate would also increase in proportion to the rate of fructose production; yet there was a point at which no further stimulation of glucose phosphorylation was observed, suggesting that sufficient fructose 1-phosphate was produced to interfere maximally with the interaction between glucokinase and its regulatory protein. It is clear however from our study that a continual production of fructose 1-phosphate was needed in order to maintain the stimulation of glucose phosphorylation.

There are numerous studies which demonstrate that physiological concentrations of glucose are poorly utilized by isolated hepatocytes from fasted rats [27–29]. Therefore in order to study hepatic glucose metabolism, supraphysiological glucose concentrations have often been employed [11,20]. In this study we have shown that the utilization of glucose at physiological glucose concentrations can be investigated successfully when the flux through glucokinase is stimulated by fructose generated at a constant low rate from inulin by inulinase. Under these circumstances, hepatocytes in suspension develop a physiological steady-state concentration of lactate in the medium when exposed to a 5 mM concentration of glucose. This behaviour has been observed previously only when supraphysiological glucose concentrations were employed [20].

This simple method for maintaining the stimulation of glucose phosphorylation by isolated hepatocytes over prolonged incubation periods allows the degree of stimulation to be varied readily by enzymically controlling the rate of fructose formation in the medium. The system is especially suited to the further study of the control of glucokinase activity and of how the variation of flux through glucokinase affects the flux through all the pathways that utilize the product, glucose 6-phosphate [19].

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