Ex Vivo Method to Simultaneously Evaluate Glucose Utilization, Uptake, and Production in Rat Liver

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Abstract

A novel ex vivo method to simultaneously evaluate hepatic glucose utilization, uptake, and production was developed in rats. The right lateral lobe of the liver was perfused with Krebs-Henseleit bicarbonate buffer containing 5 mmol/L uniformly labeled $^{13}$C-glucose ([U-$^{13}$C]-glucose). The whole glucose concentration in the perfusate was measured by colorimetric assay, and the concentrations of [U-$^{12}$C]-glucose (natural isotope) or [U-$^{13}$C]-glucose were estimated on the basis of the abundance ratio of [U-$^{12}$C]-glucose or [U-$^{13}$C]-glucose, which were measured by GC-MS. The difference in whole glucose and [U-$^{13}$C]-glucose concentrations between the baseline and effluent perfusate represents hepatic glucose utilization and glucose uptake, respectively. The [U-$^{12}$C]-glucose concentration in the effluent perfusate corresponds to hepatic glucose production. With this method, we clarified the precise mechanism that underlies the hepatic impairment of diabetic animals and
pharmacological effects of anti-diabetic agents. Thus, this method is useful for the pathophysiological and pharmacological research of type 2 diabetes.

**Keywords:** Hepatic glucose metabolism, gas chromatography, mass spectrometry, diabetes mellitus, partial liver perfusion
Introduction

The liver has an important role in regulating whole body glucose homeostasis by controlling various pathways of glucose metabolism, including glycogen synthesis, glycolysis, glycogenolysis, and gluconeogenesis. Glycogen synthesis and glycolysis affect hepatic glucose uptake, and in contrast, glycogenolysis and gluconeogenesis affect hepatic glucose production. The difference between glucose uptake and production is determined as hepatic glucose utilization. Since these parameters directly influence blood glucose levels, the evaluation method for hepatic glucose utilization, uptake, and production is important for the pathophysiological analysis of type 2 diabetes and the development of anti-diabetic drugs.

In previous studies, methods of isolated liver perfusion have been used for the analysis of hepatic glucose metabolism in experimental animals. These methods are used to evaluate glucose utilization by perfusing a buffer containing glucose via a portal vein and calculating the difference in the glucose concentration between the baseline and effluent perfusate. However,
these methods cannot elucidate whether the change in glucose utilization is caused by the change in glucose uptake or by the change in glucose production. Another liver perfusion study evaluated glucose production by perfusing a buffer containing lactate and measuring the glucose concentration in the effluent perfusate, but this method cannot be used to evaluate glucose utilization at the same time. Thus, independent experiments or changing the experimental conditions are needed to reveal the relationship between glucose utilization, uptake, and production. However, since these results were obtained under different experimental conditions, such as different baseline perfusate compositions or liver contents of metabolites and enzymes, there is a concern regarding whether the relationship between glucose utilization, uptake, and production was appropriately examined.

For the simultaneous evaluation of glucose utilization, uptake, and production, glucose derived from the liver needs to be distinguished from glucose derived from the baseline
perfusate in the glucose measurement of the effluent perfusate. Uniformly labeled $^{13}$C-glucose ([U-$^{13}$C]-glucose) is an efficient analytical tool to investigate glucose metabolism because it can be distinguished from natural isotopes of glucose by mass spectrometry analysis, and it has been utilized in glucose clamp studies and metabolomic analyses.$^{5,6}$

In this study, we developed the first ex vivo method which enables simultaneous evaluation of hepatic glucose utilization, uptake, and production using [U-$^{13}$C]-glucose. Using this method, we evaluated hepatic glucose metabolism and the pharmacological effects of a glucokinase activator (GKA) in diabetic Goto-Kakizaki rats. The results showed that this method is useful for the analysis of the hepatic pathophysiology in diabetic rats and the effect of anti-diabetic drugs on hepatic glucose metabolism.

**Experimental**
Chemicals and materials

[U-¹³C]-D-glucose was purchased from SHOKO SCIENCE CO., LTD. (Kanagawa, Japan). TMG-123, a GKA, was synthesized in Teijin Pharma Limited (Tokyo, Japan). All other reagents were analytical grade. Ultrapure water purified by Milli-Q Integral 10 system (Merck Millipore, MA) was used throughout the study.

Animals

Male Goto-Kakizaki rats and Wistar rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed under a 12 h light-dark cycle and were fed ad libitum a CE-2 (CLEA Japan, Inc., Tokyo, Japan). All experimental procedures were approved by the Animal Care and Use Committee of Teijin Institute for Bio-Medical Research (Protocol Number: A14-047, A14-120-R). All efforts were made to minimize suffering. This laboratory animal
facility was accredited by the Center for Accreditation of Laboratory Animal Care and Use, Japan Health Sciences Foundation (Certification Number: 13-066, 15-051, 16-066).

Liver perfusion study

Rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) under ad libitum feeding condition. After the peritoneal cavity was opened, 30 units of heparin Na (Nipro Corporation, Osaka, Japan) were injected into the inferior vena cava. Blood were collected via inferior vena cava and measured the blood glucose level by GlutestAce (Sanwa Kagaku Kenkyusho, Nagoya, Japan). The left branch of the portal vein was ligated to limit the perfusion area. Subsequently, the portal vein and the infrahepatic inferior vena cava were cannulated. After cannulation, the suprahepatic inferior vena cava was ligated, and the whole rat body was soaked in a saline bath at 33 °C to maintain the liver temperature. The right lateral lobe of the
liver was perfused via the portal vein at a flow rate of 6 mL/min (3 mL/min/g liver) for 30 minutes as the stabilization period with 5 mmol/L D-glucose in Krebs-Henseleit bicarbonate buffer (KHBB) (118.5 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 24.9 mmol/L NaHCO₃, 33 ºC) bubbled with 95% O₂/5% CO₂. Following, the perfusate was switched to 5 mmol/L [U-¹³C]-glucose in KHBB bubbled with 95% O₂/5% CO₂. The effluent perfusates were collected as measurement samples from the inferior vena cava three times at 10, 15, and 20 minutes after perfusate switching. The glucose concentrations of collected samples were measured by a Glucose CII-Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The sample partial pressures of oxygen (pO₂) were measured with an i-STAT (Abbott Japan, Tokyo, Japan) and i-STAT G3+ cartridge. To obtain baseline values, glucose concentration and pO₂ of KHBB with [U-¹³C]-glucose were also measured. The lactate dehydrogenase (LDH) activities of collected samples were measured by a
LDH-Cytotoxic Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for monitoring the tissue damage, and animals were excluded when LDH activities of collected samples were high. In the pharmacological study, Goto-Kakizaki rats were orally administered TMG-123 (30 mg/kg) or vehicle (Gelucire/Polyethylene Glycol 400 (PEG400) = 3/2) 1 hour before the start of the liver perfusion.

**Instrumentation and analytical conditions for GC-MS**

GC-MS was operated with a GCMS-QP2010 plus with an AOC-20s autosampler and column inserted into the ion source of a GCMS-QP2010 mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The GC was equipped with a 30 m × 0.25 mm SH-Rtx-5MS column with 0.25 μm thickness (Shimadzu Corporation). Helium at a linear velocity of 36.8 cm/second was used as the carrier gas. The injector was used in the split mode, and its
temperature was kept at 250 ºC. The GC column temperature was programmed to increase 80 ºC to 320 ºC at a rate of 15 ºC/min. Retention time for the derivatives of [U-12C]-glucose and [U-13C]-glucose were around 13 minutes. The mass conditions were set as follows: ion source temperature, 200 ºC; scan range of 45-600 m/z, and event time 0.5 second.

Measurement of abundance ratio of [U-12C]-glucose and [U-13C]-glucose

The abundance ratio of [U-13C]-glucose and [U-13C]-glucose in the samples were measured by GC-MS. Sample derivatization was conducted according to the previous report with minor modification. Briefly, the samples (100 μL) were diluted 10 times with KHBB. Ethanol was added (diluted sample: ethanol = 100 μL: 300 μL) for deproteinization and centrifuged for 5 minutes at 1000 g. The supernatant (100 μL) was collected and evaporated to dryness under reduced pressure. The glucose in the sample was nitrated by adding 0.2 mol/L
Hydroxylamine Hydrochloride (pyridine solution, 30 μL) and heating at 90 °C for 60 minutes.

After nitration, the sample was acetylated by adding cold acetic anhydride (150 μL) and heating at 90 °C for 120 minutes. The sample was dried again and dissolved in ethyl acetate (100 μL) for GC-MS measurement. The vials were loaded into the autosampler, which was programmed to inject 1 μL of each sample into the gas chromatograph. The abundance ratio of each sample was calculated by the ratio of peak areas for [U-12C]-glucose (m/z 314.0) and for [U-13C]-glucose (m/z 319.0) in the GCMSsolution version 4.11 (Shimadzu Corporation). The m/z 242.1 and 246.1 ions were also used as qualifier ions. These ions in the mass spectrometry were shown in Fig.S1.

Calculation of hepatic glucose utilization, uptake, and production

Hepatic glucose utilisations were calculated by subtracting the average whole glucose
concentration of each sample collected at 10, 15, and 20 minutes from that of the baseline perfusate (KHBB with [U-13C]-glucose). The concentrations of [U-12C]-glucose or [U-13C]-glucose were estimated by dividing the whole glucose concentration depending on the abundance ratio of each type of glucose. Hepatic glucose uptake was calculated by subtracting the average [U-13C]-glucose concentration of the collected sample from the [U-13C]-glucose concentration of the baseline perfusate. Hepatic glucose production was calculated by subtracting [U-12C]-glucose concentration of the baseline perfusate from the average [U-12C]-glucose concentration of each collected sample.

Statistical Analysis

Statistical analysis was performed using SAS software version 9.2 (SAS Institute Inc., NC, USA). Statistical significance was assessed using the two-tailed Student’s t-test. Differences
were considered significant when $p$ values were $< 0.05$. The regression analysis was performed with Prism version 6.07 software (GraphPad, CA, USA).

Results and Discussion

Development of the partial liver perfusion method

We first developed the partial perfusion method for a rat liver. The liver perfusion study in rats required 3 mL/min/g perfusate to supply sufficient $O_2$ and maintain the viability of the tissue. Considering the weight of the rat liver, a large quantity of perfusate was needed for the whole liver perfusion. To reduce the total cost of the expensive $[U-^{13}C]$-glucose, we tried to limit the perfusion area. The left branch of the portal vein was ligated in Wistar rats at 14-15 weeks of age, and the livers were perfused with Evans blue solution (Fig.1). The area of the liver stained by Evans blue was limited to the right lateral lobe, and the flow rate of the effluent perfusate was almost the same as the infusion rate, indicating that partial liver perfusion was
achieved by this procedure. The weight of the identified perfusion area was measured (about 2 g), and the perfusate flow rate was set to 6 mL/min (3 mL/min/g liver) for the maintenance of the viability of the tissue according to a previous report. At this flow rate, the whole glucose concentration measured by colorimetric assay and pO₂ in the effluent perfusate was clearly decreased compared to the baseline perfusate (glucose concentration decreased from 90 mg/dL to about 80 mg/dL and pO₂ from about 450 mmHg to about 150 mmHg) and LDH activities of collected perfusates were stable, indicating that partial liver perfusion was achieved with maintaining hepatic viability. By this partial liver perfusion method, the perfusate was reduced by one fifth compared to the whole liver perfusion (about 10 g, 30 mL/min is required as a flow rate).

Construction of the quantification method of [U-¹²C]glucose and [U-¹³C]glucose in the
Next, we constructed the quantification method of \([\text{U}^{12}\text{C}]\)-glucose and \([\text{U}^{13}\text{C}]\)-glucose in the perfusate. First, we confirmed the procedure of the sample derivatization and the GC-MS measurement. Four standard samples containing D-glucose and \([\text{U}^{13}\text{C}]\)-glucose (2 mg/dL, respectively) in water were derivatized according to the previous report and then analyzed by GC-MS. A single peak was detected in each extracted ion chromatogram at m/z 314 and m/z 319, which represented \([\text{U}^{12}\text{C}]\)-glucose and \([\text{U}^{13}\text{C}]\)-glucose, respectively (Fig.2a). The coefficient of variation of the peak area in the four samples was 4.3% for m/z 314.0 and 3.3% for m/z 319.0, indicating that the repeatability of this procedure was favorable.

Second, we examined the performance for quantification of \([\text{U}^{12}\text{C}]\)-glucose and \([\text{U}^{13}\text{C}]\)-glucose in the perfusate by GC-MS measurement. The baseline and effluent perfusate obtained from a liver perfusion study in Wistar rats was derivatized and analyzed by GC-MS. As
shown in Fig. 2 b and c, while the peak was detected only in the extracted ion chromatogram at 
m/z 319 for the baseline perfusate analysis, the peak was detected both at m/z 314 and m/z 319 
for the effluent perfusate analysis, indicating that the effluent perfusate contained glucose 
produced by the liver. Thus, we tried to determine the [U-12C]-glucose and [U-13C]-glucose 
concentration in the effluent perfusate, respectively, using the calibration curve in the GC-MS 
measurement. However, the sum of the [U-12C]-glucose and [U-13C]-glucose concentration 
determined by GC-MS did not conform with the glucose concentration obtained in colorimetric 
assay (by a Glucose CII-Test Wako), which is generally used in clinical assays (data not shown).

We assumed that the matrix effect caused by various hepatic metabolites in the effluent 
perfusate influences GC-MS analysis performance for quantification, and that it is difficult to 
determine the [U-12C]-glucose and [U-13C]-glucose concentration using the calibration curve.

Since the mass chromatogram of the effluent perfusate shows the same retention time for
the [U-12C]-glucose and [U-13C]-glucose after derivatization (Fig. 2c), the matrix effect may not influence the ratio of the peak area of [U-12C]-glucose / [U-13C]-glucose. Next, we tried to estimate the concentration of [U-12C]-glucose and [U-13C]-glucose by dividing the whole glucose concentration measured by colorimetric assay depending on the ratio of the peak area. To validate this strategy, we first evaluated the ratio of the peak area of the sample, which contained the same amount of D-glucose and [U-13C]-glucose in KHBB by GC-MS several times. In the range of 1-100 mg/dL glucose, the ratio of peak area of [U-12C]-glucose and [U-13C]-glucose was nearly 1 and their variations were low (Table 1), indicating that the ratio of these peak areas precisely reflects the actual abundance ratio. Next, for the quantitative validation, the mixtures of [U-12C]-glucose and [U-13C]-glucose in several 90 mg/dL glucose solutions, which contained various ratios of D-glucose and [U-13C]-glucose (each concentration was 0-90 mg/dL), were measured to estimate the concentrations of [U-12C]-glucose and
[U-13C]-glucose. These analytes showed good linearity over this concentration range (Fig.3), indicating the good quantitative estimation of this method in the range of 0-90 mg/dL glucose. These results showed that estimation of the concentration of [U-13C]-glucose and [U-13C]-glucose by dividing the whole glucose concentration measured by colorimetric assay depending on the ratio of the peak area in GC-MS is appropriate for the quantification.

Pathophysiological and pharmacological analyses using the present method

To verify the novel ex vivo method, we performed analyses of the hepatic pathophysiology in Goto-Kakizaki rats, a spontaneous model of type 2 diabetes, and the effect of an anti-diabetic drug (GKA) on hepatic glucose metabolism.

At 14 weeks of age, the blood glucose levels of Goto-Kakizaki rats were significantly higher than those of Wistar rats (Fig.4a). At this time, the hepatic glucose utilization of
Goto-Kakizaki rats tended to be lower, and the hepatic glucose production of Goto-Kakizaki rats was significantly higher than that of Wistar rats (Fig.4b, d). On the other hand, the hepatic glucose uptake of Goto-Kakizaki rats was similar compared to those of Wistar rats (Fig.4c).

These results showed that hepatic glucose utilization of Goto-Kakizaki rats was impaired, which is mainly caused by the increase in hepatic glucose production.

We also evaluated the pharmacological effects of GKA on hepatic glucose metabolism. Goto-Kakizaki rats were orally administered GKA (TMG-123) or vehicle under ad libitum feeding conditions, and we demonstrated that blood glucose levels were significantly lower in the GKA-administered group at 1 hour after administration (Fig.5a). At this point, we performed the liver perfusion study. As a result, hepatic glucose utilization and uptake in the GKA-administered group was significantly higher, and hepatic glucose production in the GKA-administered group was similar compared to the vehicle-administered group (Fig.5b-d).
These results showed that the administration of GKA increases hepatic glucose utilization by
increasing hepatic glucose uptake, and these changes in hepatic glucose metabolism contribute
to the glucose-lowering effect.

In Goto-Kakizaki rats, it was reported that the increase in hepatic glucose production
relates to pathogenesis of hyperglycemia. It has also been known that pharmacological
activation of hepatic glucokinase increases hepatic glucose uptake by enhancing the first
metabolic reaction of glycolysis. In our liver perfusion study, we showed that hepatic glucose
production in Goto-Kakizaki rats was higher compared to Wistar rats and the administration of
GKA increased hepatic glucose uptake. These results were consistent with previous reports, and
therefore, we considered that our method can evaluate physiological responses of hepatic
glucose metabolism correctly. Moreover, we revealed for the first time that the increased hepatic
glucose production of GK rats mainly contributes to the impairment of hepatic glucose
utilization and that the GKA treatment increased hepatic glucose uptake only, and did not affect hepatic glucose production. Thus, since the precise mechanism that underlies the hepatic impairment of Goto-Kakizaki rats and pharmacological effects of GKA was revealed by the simultaneous evaluation of glucose utilization, uptake, and production, the present method is useful for the analysis of hepatic pathophysiology in diabetic rats and the effect of anti-diabetic drugs on hepatic glucose metabolism.

Conclusions

In the present study, we developed the first ex vivo method, which can simultaneously evaluate hepatic glucose utilization, uptake, and production using [U-13C]-glucose as a tracer and measuring the glucose concentration by colorimetric assay and the abundance ratios of [U-12C]-glucose and [U-13C]-glucose by GC-MS in the baseline and effluent perfusates. This method is useful for the pathophysiological and pharmacological research of type 2 diabetes.
Acknowledgement

We are grateful to Yoshihiro Tochino for helpful discussion and suggestions, and we pray that his soul may rest in peace.

Supporting information

Chemical structure of glucose aldonitril pentaacetate and its fragmentation pattern in the mass spectrometry are available free of charge on the Web (http://www.jsac.or.jp/analsci/)
References


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Table 1 Measurements for peak area ratio of the preparations containing equivalent amount of [U-12C]-glucose and [U-13C]-glucose

<table>
<thead>
<tr>
<th>Glucose concentration before derivatization / mg dL^{-1}</th>
<th>Ratio of peak areas [U-12C]-glucose/[U-13C]-glucose</th>
<th>Estimated [U-12C]-glucose concentration / mg dL^{-1}</th>
<th>Estimated [U-13C]-glucose concentration / mg dL^{-1}</th>
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</thead>
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<td>[U-12C]-glucose [U-13C]-glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1             1</td>
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<td>1.0 ± 0.0</td>
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<tr>
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<td>30            30</td>
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<td>30.9 ± 0.3</td>
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<tr>
<td>100           100</td>
<td>1.05 ± 0.04</td>
<td>102.6 ± 1.8</td>
<td>97.4 ± 1.8</td>
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</tbody>
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Data are expressed as mean ± standard deviation (n=6)
Figure Captions

Fig. 1 Representative image of the partial liver perfusion

The left branch of the portal vein was ligated in Wistar rats at 14-15 weeks of age, and the livers were perfused with Evans blue solution. The perfusion area was limited to the right lateral lobe of the liver (indicated by the arrow).

Fig. 2 Mass chromatogram of the derivatives of [U-12C]-glucose and [U-13C]-glucose

(a) Extracted ion chromatogram (XIC) at m/z 314 and m/z 319 of the standard samples containing D-glucose and [U-13C]-glucose (2 mg/dL, respectively). The derivatization and measurement of same sample were independently carried out four times. Total ion chromatogram (TIC) and XIC at m/z 314 and m/z 319 of the baseline (b) and effluent perfusate (c) obtained from a liver perfusion study in Wistar rats.
The abundance ratio of $\text{[U}{}^{12}\text{C}]-\text{glucose and } \text{[U}{}^{13}\text{C}]-\text{glucose}$ in several 90 mg/dL glucose solutions, which contain various ratios of D-glucose and $\text{[U}{}^{13}\text{C}]-\text{glucose}$ (shown as nominal glucose concentration), were measured by GC-MS and estimated the concentration of the $\text{[U}{}^{12}\text{C}]-\text{glucose and } \text{[U}{}^{13}\text{C}]-\text{glucose}$ in each solution (shown as calculated glucose concentration).

Fig. 4 Comparison of hepatic glucose metabolism in Goto-Kakizaki rats with Wistar rats by the liver perfusion study

(a) Blood glucose of diabetic Goto-Kakizaki and non-diabetic Wistar rats were measured before the start of the perfusion. (b) Hepatic glucose utilization, (c) hepatic glucose uptake, and (d)
hepatic glucose production of those animals were evaluated in the liver perfusion study. Mean + SEM, n=6-7. *p < 0.05.

Fig. 5 The analysis of effect of GKA on hepatic glucose metabolism with the liver perfusion study

(a) Blood glucose were measured before the start of the perfusion, and (b) hepatic glucose utilization, (c) hepatic glucose uptake, and (d) hepatic glucose production were evaluated in the liver perfusion study in Goto-Kakizaki rats 1 hour after vehicle or GKA administration. Mean + SEM, n=9-10. *p < 0.05.
Fig. 1 Representative image of the partial liver perfusion
Fig. 2 Mass chromatogram of the derivatives of [U-\(^{12}\)C]-glucose and [U-\(^{13}\)C]-glucose
Fig. 3 Relationship between nominal glucose concentration and estimated glucose concentration
Fig. 4 Comparison of hepatic glucose metabolism in Goto-Kakizaki rats with Wistar rats by the liver perfusion study.
Fig. 5 The analysis of effect of GKA on hepatic glucose metabolism with the liver perfusion study
Graphical Index

Experimental outline

Krebs-Henseleit bicarbonate buffer with $[U^{-13}C]$-glucose

Portal vein
Liver
Inferior vena cava

Effluent perfusate including $[U^{-13}C]$-glucose and $[U^{-12}C]$-glucose

Quantify with GC-MS and colorimetric assay

Definitions of hepatic glucose utilization, uptake, and production

Pre-perfusion

$[U^{-13}C]$-glucose

Post-perfusion

$[U^{-10}C]$-glucose
$[U^{-12}C]$-glucose

Hepatic glucose utilization

Hepatic glucose uptake

Hepatic glucose production