A Probing Dose of Phenylacetate Does Not Affect Glucose Production and Gluconeogenesis in Humans

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Phenylacetate ingestion has been used to probe Krebs cycle metabolism and to augment waste nitrogen excretion in urea cycle disorders. Phenylalkanoic acids, including phenylacetate, have been proposed as potential therapeutic agents in the treatment of diabetes. They inhibit gluconeogenesis in the liver in vitro and reduce the blood glucose concentration in diabetic rats. The effect of sodium phenylacetate ingestion on blood glucose and the contribution of gluconeogenesis to glucose production have now been studied in 7 type 2 diabetic patients. The study was not designed to test whether the changes in glucose metabolism observed in the rat could be reproduced in humans. After an overnight fast, over a period of 1 hour, 4.8 g phenylacetate was ingested, which is the highest dose used to probe Krebs cycle metabolism. Glucose production was measured by tracer kinetics using [6,6-$^2H_2$]glucose and gluconeogenesis by the labeling of the hydrogens of blood glucose on ^2H_2O ingestion. The concentration of phenylacetate in plasma peaked by 2 hours after its ingestion, and about 40% of the dose was excreted in 5 hours. The plasma glucose concentration and production, and the contribution of gluconeogenesis to glucose production, were unaffected by phenylacetate ingestion at the highest dose used to probe Krebs cycle metabolism.

PHENYLACETATE is conjugated to glutamine in human liver and the conjugate is excreted in urine.[1,2] Therefore, phenylacetate has been administered to augment waste nitrogen excretion in individuals with urea cycle disorders.[3-6] As a probe, it has been administered to normal subjects[7-9] and diabetics[10-12] along with ^{13}C- and ^{14}C-labeled gluconeogenic substrates. From the distribution of label in the carbons of the glutamine from the excreted conjugate phenylacetylglutamine, by assuming the same distribution as in hepatic α-ketoglutarate, the rates of the reactions of the Krebs cycle and the contribution of gluconeogenesis to glucose production have been estimated. The required dose of phenylacetate is dependent on the method used to measure the distribution. Its administration is assumed not to alter the contributions. Similar distributions of label in glutamate from the liver in animal studies and in glutamine from the conjugate excreted by humans provide limited support for this assumption.[13]

Phenylalkanoic acids, including phenylacetate, inhibit gluconeogenesis in isolated perfused rat liver.[14] Recently, phenylacetate also was shown to inhibit gluconeogenesis in isolated rat liver cells.[15] This appears to occur by the inhibition of pyruvate carboxylase through the formation of phenylacetyl-coenzyme A (CoA). In accordance with that effect, the infusion of phenylpropionate in normal and streptozotocin-induced diabetic rats decreased the glucose concentration and glucose production under conditions of liver glycogen depletion.[15] A lack of significant toxicity observed in the use of phenylacetate in vivo, encouraged the suggestion that phenylalkanoic acids might be therapeutic agents in the treatment of hyperglycemia.[15]

The present study was intended only to test whether phenylacetate at the highest dose used in tracer studies in humans[8-10] alters glucose production and/or the contribution of gluconeogenesis to glucose production in diabetic subjects.

SUBJECTS AND METHODS

Subjects

Seven type 2 diabetics, 2 women and 5 men, were studied during treatment with phenylacetate (treatment series). Six of the 7 were also studied identically, except without phenylacetate (control series). The mean age was 63.4 ± 3.0 years and the body mass index was 27.8 ± 1.3 kg/m^2 when they received phenylacetate. The known duration of diabetes was 10.0 ± 2.4 years. All subjects were treated in our outpatient clinic. They were on a diabetic diet and antidiabetic medication. One subject was on sulfonylurea (SU) treatment only. Another was on SU during the day and NPH insulin before bedtime. One was using SU combined with metformin and another with acarbose. One subject was treated with metformin only and another with metformin together with NPH insulin before bedtime. One was treated with 4 doses of insulin. Therapy with SU, metformin, and acarbose was discontinued 1 week before study. Daytime insulin was administered as usual, but bedtime insulin was omitted the day before the study. Blood hemoglobin A_{1c} was 7.2% ± 0.2% on therapy (normal, <5.8%). Controls were performed 3 to 11 months (mean, 6 months) after phenylacetate administration to ensure that ^2H_2O given in the treatment series had been excreted.[16] The treatment was not changed. Their weight was stable, except for one subject whose weight declined from 100 to 96 kg and another from 84 to 81 kg.

Procedure

Subjects were admitted to the Endocrine Research Unit of Karolinska Hospital in the afternoon. Between 5:00 and 6:00 PM, each subject ate a dinner of 14 kcal/kg body weight, composed of 48% carbohydrate, 19% protein, and 33% fat. The subjects then fasted except for water ingestion ad libitum until the study was complete. At 11:00 PM, they drank 2.5 mL ^2H_2O/kg body water (99.9% ^2H; Isotec, Miamisburg, OH), and again at 2:00 AM.[16,17] Body water was estimated to be 60% of body weight in the men and 50% in the women. At 8:00 AM, a bolus of [6,6-$^2H_2$]glucose (99% enriched; Cambridge Isotope Laboratories, Andover, MA) was injected into an antecubital vein that was cannulated. The bolus equaled 0.46 mmol [6,6-$^2H_2$]glucose times the subject's plasma glucose concen-

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Submitted December 30, 1999; accepted February 16, 2000.

Supported by Grant No. 72X-00034 from the Swedish Medical Research Council and Grants No. DK-14507 and DK-35543 from the National Institutes of Health.

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0026-0495/00/4909-0003$10.00/0

doi:10.1053/meta.2000.8601

tration at 8:00 AM in millimolars divided by 5 mmol/L.[18] An infusion of [6,6-$^2H_2$]glucose 4.6 μmol/min was started via the cannula as soon as the bolus injection was complete, and continued until 4:00 PM.

At 11:00 AM, repeated at 11:30 AM and again at noon, each subject ingested 1.6 g sodium phenylacetate prepared from phenylacetic acid (Sigma Chemical, St Louis, MO), dissolved to mask its taste in 150 mL Coca-Cola Light (Coca-Cola, Atlanta, GA), a diet soda devoid of calories.[7] One hundred fifty milliliters contains 78 mg aspartame, which upon hydrolysis releases 44 mg phenylalanine. Only a small portion of that is converted to phenylacetate, as is the 200 to 500 mg phenylalanine estimated to be released daily by endogenous proteolysis.[19] Blood (20 mL) was drawn from a cannula in an antecubital vein in the arm opposite to the one used for the infusion. Blood sampling occurred at 9:00 AM, 11:00 AM, 12:30 PM, 2:00 PM and 4:00 PM for determination of the ratio of ^2H bound to carbons 2 and 5 of blood.[16,17] Blood (5 mL) was also drawn at 10:40, 10:50, and 11:00 AM and 3:40, 3:50, and 4:00 PM to determine the percent of glucose molecules with 2 ^2H atoms bound at carbon 6. Glucose, insulin, C-peptide, and glucagon concentrations in plasma were determined at regular intervals throughout the 8-hour period of infusion. The 6 subjects, readmitted to the research unit, were studied under an identical protocol (control series) except that Coca-Cola Light without phenylacetate was ingested.

Phenylacetate concentrations were determined in plasma for 5 of the subjects before phenylacetate administration at 10:00 and 11:00 AM, and after, at noon, 2:00 PM, and 4:00 PM. Two other mild diabetics aged 57 and 62 were treated identically, except the concentration of phenylacetate was also determined at 1:00 PM. Their urine was collected before phenylacetate administration and after its administration, from 11:00 AM to 4:00 PM, for measurement of phenylacetate excretion.

Analyses

Plasma glucose was determined in duplicate using glucose oxidase (YSI 2300; Yellow Springs Instrument, Yellow Springs, OH). Enrichment of the hydrogen bound to carbons 2, 5, and 6 of blood glucose was determined as previously detailed.[16,17] Briefly, the supernatant obtained after deproteinizing a blood sample with $ZnSO_4$-$Ba(OH)_2$ was deionized by passage through cation- and anion-exchange resins. Glucose in the effluent was isolated by high-performance liquid chromatography. To determine the percentage of glucose molecules with 2 ^2H atoms at carbon 6, a sample of the glucose was oxidized with periodate and the formaldehyde formed, which contained carbon 6 with its 2 hydrogens, was converted to hexamethylenetetramine (HMT) by the addition of ammonia. The HMT was analyzed by gas chromatography–mass spectrometry for mass 142, ie, m + 2.

To determine the enrichment of the hydrogen bound to carbon 2, carbon 1 of a portion of the glucose was removed to form ribulose-5-phosphate. The ribulose-5-P was reduced to a mixture of the polyol phosphates, ribitol-5-P and arabitol-5-P. These were oxidized with periodate, yielding formaldehyde, which contained carbon 2 with its hydrogen. HMT was made from the formaldehyde and analyzed for mass 141, ie, m + 1. To determine the enrichment of the hydrogen bound to carbon 5, another portion of the glucose was oxidized to remove its carbon 6. The resulting xylose was also oxidized with periodate, yielding carbon 5 with its hydrogen in formaldehyde. Again, HMT was formed and analyzed for mass 141.

Background enrichment was measured in blood glucose before ^2H_2O ingestion. HMTs formed from formaldehyde from [6,6-$^2H_2$]glucose and [1-^2H]sorbitol of known enrichment served as standards in the assays. [1-^2H]sorbitol was prepared by reduction with $NaBH_4$ of [1-^2H]glucose, 98% enriched, also purchased from Cambridge Isotope Laboratories.[17]

Heparinized blood was kept on ice until centrifugation at 4°C. An aliquot of the plasma was used for determination of glucose. The remainder was frozen at −20°C for later analyses of C-peptide and insulin. The C-peptide level was measured by radioimmunoassay (RIA) using a commercially available kit (Novo Nordisk Research, Bagsvaerd, Denmark). Immunoreactive insulin was determined by RIA using our own antibodies, human insulin as a standard, and the addition of charcoal to separate antibody-bound and free insulin.[20] Blood samples for glucagon assay were collected in prechilled tubes containing Trasylol (Bayer, West Haven, CT) and EDTA. Glucagon was analyzed by the method of Faloona and Unger[21] using the 30K antibody. The phenylacetate concentration in plasma and urine was measured by gas chromatography–mass spectrometry as described previously,[22] except that a *t*-butyl dimethylsilyl derivative was used. Quantitation was achieved by isotopic dilution with an internal standard of [$^2H_7$]phenylacetate (Isotec). Ions were monitored at *m/z* 193 and 200. The urinary concentration of total phenylacetate, ie, free phenylacetate + conjugated phenylacetate (phenylacetylglutamine), was measured after treating 0.1 mL urine with 1 mL 6-mol/L HCl for 4 hours at 100°C. This treatment hydrolyzed phenylacetylglutamine to free phenylacetate.

Calculations

The rate of glucose production was calculated equal to the percent enrichment of [6,6-$^2H_2$]glucose infused, ie, 99%, times its rate of infusion, 4.6 μmol/min, divided by the percent [6,6-$^2H_2$]glucose enrichment in the blood glucose, determined by the assay of m + 2 at carbon 6, minus the rate of infusion. The results are expressed in micromoles per kilogram body weight per minute. Enrichment at carbon 6, used in the calculation, was the mean of 3 determinations, ie, from samples collected at 10:40, 10:50, and 11:00 AM or at 3:40, 3:50, and 4:00 PM. The percent contribution of gluconeogenesis to glucose production was set equal to 100 times the excess enrichment of the hydrogen bound to carbon 5 versus carbon 2 of the glucose, ie, the C5/C2 ratio.[16,17] The rate of glucose production by gluconeogenesis was calculated by multiplying the rate of glucose production by the percent contribution.

Statistics

The mean ± SEM are presented. Student's *t* test was used, with a *P* value less than .05 considered significant. The coefficient of variation was calculated by dividing the standard deviation of the mean by the mean.

RESULTS

There was no significant difference in plasma glucose concentrations during the 8-hour period with or without ingestion of phenylacetate (Table 1). Fasting plasma glucose was about 10 mmol/L after the 14 hours of fasting (8:00 AM), and during the 8 additional hours of fasting it declined to about 7 mmol/L. At 11:00 AM, when the first measurement of glucose production was complete and the first portion of the dose of phenylacetate was ingested, plasma concentrations were about 9.5 mmol/L.

There was no difference in the percent contribution of gluconeogenesis to glucose production at any time with or without ingestion of phenylacetate (Table 2). At 11:00 AM, when phenylacetate ingestion began, the contribution was 65.6% ±

Table 1. Plasma Glucose (mmol/L) in Diabetics With Phenylacetate Ingestion From 11:00 AM to Noon and Without

Condition	8:00 AM	9:00 AM	10:00 AM	11:00 AM	12:30 PM	2:00 PM	3:00 PM	4:00 PM
With PA	10.4 ± 0.6	10.2 ± 0.5	10.0 ± 0.5	9.8 ± 0.5	8.3 ± 0.5	7.5 ± 0.4	7.1 ± 0.5	6.9 ± 0.4
Without PA	9.6 ± 0.9	9.6 ± 0.9	9.7 ± 1.0	9.2 ± 1.0	8.6 ± 1.0	8.2 ± 1.0	7.9 ± 0.9	7.5 ± 0.9

Abbreviation: PA, phenylacetate.

2.0%, and 68.8% ± 2.2% when it was not ingested. At 4:00 PM, when the second measurement of glucose production was complete, it was 72.2% ± 3.5% when phenylacetate was ingested and 74.3% ± 4.2% when not ingested.

There was also no difference in the rate of glucose production with or without phenylacetate ingestion. Production at 11:00 AM was 10.3 ± 0.3 with it and 10.6 ± 0.5 μmol/kg/min without phenylacetate and at 4:00 PM, 7.6 ± 0.3 with and 7.7 ± 0.3 μmol/kg/min without phenylacetate. These estimates are made assuming that the equation used for the steady state holds. The m + 2 excess enrichment at carbon 6 of blood glucose at 10:40 AM was 99.1% ± 1.7% of that at 11:00 AM with and 96.4% ± 0.8% without phenylacetate ingestion; at 3:40 PM, it was 103.5% ± 1.3% of that at 4:00 PM with phenylacetate and 99.0% ± 1.2% without. The coefficient of variation for the measurements in the subjects at 10:40, 10:50, and 11:00 AM was 3.2% ± 0.5%, and 2.1% ± 0.3% at 3:40, 3:50, and 4:00 PM. Glucose production with phenylacetate declined to 73.6% ± 3.3% from 11:00 AM to 4:00 PM, and without phenylacetate, to 73.5% ± 0.8%. By multiplying the glucose production at 11:00 AM and 4:00 PM by the percent contribution of gluconeogenesis at those times, the absolute contribution of gluconeogenesis declined at 4:00 PM to 80.6% ± 3.1% of that at 11:00 AM with phenylacetate ingestion and 79.4% ± 4.8% without, again no significant difference.

There were no significant differences in insulin, C-peptide, or glucagon concentrations with or without phenylacetate. Insulin values at 8:00 AM were 156 pmol/L before phenylacetate administration, and at the end of the experiment at 4:00 PM, 122 pmol/L. In the control series, the corresponding values were 156 and 136 pmol/L. C-peptide in the treatment group was 1.1 nmol/L at 8:00 AM and 0.9 nmol/L at 4:00 PM. The corresponding values in the control series were 1.0 and 0.9 nmol/L. Glucagon concentrations were 37.0 ng/L at 8:00 AM and 28.4 ng/L at 4:00 PM in the phenylacetate-treated group. In the controls, they were 38.7 and 27.2 ng/L. The concentration of phenylacetate in plasma increased to a peak between noon and 2:00 PM (Fig 1). Of the phenylacetate ingested, 45.4% and 31.6% was excreted by 4:00 PM in the 2 subjects in whom measurements were made.

DISCUSSION

The acute ingestion of 4.8 g sodium phenylacetate by mild diabetics, after an overnight fast, did not alter glucose production or the contribution of gluconeogenesis to glucose production. Phenylacetate administered in solution has been reported to be effectively absorbed.[1,2] This was evidenced by the quantity excreted by the 2 subjects between 11:00 AM and 4:00 PM and by the plasma concentration after phenylacetate ingestion. Since glucose production was significantly lower at 4:00 PM than at 11:00 AM, 5 hours was a sufficient time for glucose pool turnover, to allow an effect on glucose production. Therefore, an effect on gluconeogenesis, if it occurred, should have been demonstrated.

Table 2. Percent Gluconeogenesis Contribution (%) to Glucose Production in Diabetics With Phenylacetate Ingestion From 11:00 AM to Noon and Without

Condition	9:00 AM	11:00 AM	12:30 PM	2:00 PM	4:00 PM
With PA	66.0 ± 3.4	65.6 ± 2.0	67.3 ± 2.1	65.8 ± 3.2	72.2 ± 3.5
Without PA	62.8 ± 3.4	68.8 ± 2.2	70.6 ± 2.6	73.4 ± 1.8	74.3 ± 4.2

Abbreviation: PA, phenylacetate.

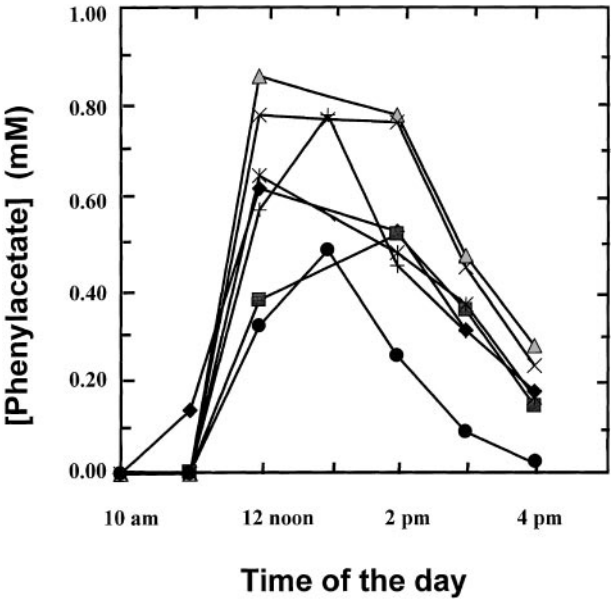

Fig 1. Phenylacetate concentrations in plasma from the 7 subjects. Plasma was collected from 10:00 AM to 4:00 PM, and sodium phenylacetate 4.8 g (30.4 mmol) was ingested between 11:00 AM and noon.

There are several possible reasons that an effect of phenylacetate was not found in humans but has been found in rats. The dose per kilogram in the rat, and intravenously, has been several-fold greater. Phenylacetate is conjugated with glutamine only in primates.[23] In the rat, its conjugation is with glycine.[23] The rate of conjugation may be faster in the human versus the rat, so an inhibiting concentration of phenylacetyl CoA is not achieved. The maximum plasma concentration of phenylacetate achieved was less than 1 mmol/L (Fig 1). In perfusions of rat liver, about 25% inhibition of gluconeogenesis by phenylacetate has been observed, but at a concentration in the perfusate of 4 mmol/L.[14]

The importance of this study, justifying the effort required, rests on reports of the use of phenylacetate and its analogs in humans, and their possible use in the future. Many years ago, a dose of 5 g was ingested by students who were taking courses in physiological chemistry.[24] Long-term therapies with phenylacetate in the treatment of urea cycle disorders have been at such a dose, but repeated at regular intervals.[3-6] A larger dose, 250 mg/kg body weight, has been administered intravenously to treat children in episodic hyperammonemia.[25] In estimating Krebs cycle flux relative to the rate of gluconeogenesis in humans, about 5 g sodium phenylacetate, at most, has been given, with measurements made 3 to 5 hours later. The present results indicate that such estimates are not compromised because of an effect of phenylacetate.

ACKNOWLEDGMENT

We thank nurses Annica Clark and Kajsa Sundquist for their care of the patients and for technical assistance, and Michelle A. Puchowicz for helping with the measurement of phenylacetate.

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