Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis

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[1]*Division of Pharmacology, Drug Safety and Metabolism, Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima; and* [2]*Department of Animal Science, Utsunomiya University, Utsunomiya, Tochigi, Japan*

Doi M, Yamaoka I, Nakayama M, Sugahara K, Yoshizawa F. Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 292: E1683–E1693, 2007. First published February 13, 2007; doi:10.1152/ajpendo.00609.2006.—Isoleucine, a branched chain amino acid, plays an important role in the improvement of glucose metabolism as evidenced by the increase of insulin-independent glucose uptake in vitro. This study evaluated the effect of isoleucine on glucose uptake and oxidation in fasted rats and on gluconeogenesis in vivo and in vitro. Oral administration of isoleucine decreased the plasma glucose level by 20% and significantly increased muscle glucose uptake by 71% without significant elevation of the plasma insulin level compared with controls at 60 min after administration. Furthermore, expiratory excretion of $^{14}CO_2$ from [U-^{14}C]glucose in isoleucine-administered rats was increased by 19% compared with controls. Meanwhile, isoleucine decreased AMP levels in the liver but did not affect hepatic glycogen synthesis. Under insulin-free conditions, isoleucine significantly inhibited glucose production when alanine was used as a glucogenic substrate in isolated hepatocytes. This inhibition by isoleucine was also associated with a decline in mRNA levels for phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G6Pase) and a decreased activity of G6Pase in isolated hepatocytes. These findings suggest that a reduction of gluconeogenesis in liver, along with an increase of glucose uptake in the muscle, is also involved in the hypoglycemic effect of isoleucine. In conclusion, isoleucine administration stimulates both glucose uptake in the muscle and whole body glucose oxidation, in addition to depressing gluconeogenesis in the liver, thereby leading to the hypoglycemic effect in rats.

insulin; amino acid; leucine; hepatocyte; glucose production

PREVIOUS STUDIES HAVE SHOWN that amino acids decrease insulin-stimulated glucose uptake and glucose utilization (7, 11, 33, 34). As an alternative to glucose oxidation, amino acids, including the glucogenic amino acids (alanine, valine, or glutamine), may serve as fuel, and therefore, amino acids are considered to be able to increase glucose production and blood glucose levels. Also, amino acids (especially leucine) stimulate protein synthesis via the mammalian target of rapamycin (mTOR) (1, 37), whereas amino acids can also induce degradation of the insulin receptor substrate-1 (IRS-1) by stimulating mTOR and S6 kinase-1 (S6K1), leading to desensitization of insulin signaling (20, 30, 34). In addition, leucine reduces the duration of insulin-induced IRS-1-associated phosphatidylinositol 3-kinase (PI3 kinase) in skeletal muscle (3). Given these results, it is to be expected that amino acids will decrease glucose oxidation and cause an amino acid-induced insulin resistance.

However, it has been reported that amino acid infusion causes a decrease in blood glucose levels and increases glucose oxidation in humans (31, 32), although there have as yet been only a few investigations of this hypoglycemic effect of amino acids. These changes appeared to occur via the action of insulin, since leucine, but not isoleucine or valine, stimulates insulin release from the pancreas, thereby decreasing blood glucose (10, 26). This contradicts the amino acid-induced insulin resistance described above, and thus this issue remains controversial.

In an in vitro study in C_2C_{12} skeletal muscle cells, we recently found that among the branched-chain amino acids, leucine and isoleucine increase glucose uptake in an insulin-independent manner, with the effect of isoleucine being greater than that seen for leucine (8). We also have shown that oral administration of isoleucine, which does not stimulate insulin secretion, has a potent hypoglycemic effect and increases glucose uptake in muscle in contrast to the lack of this effect after an oral administration of leucine in rats. Thus stimulation of glucose uptake and the hypoglycemic effect of isoleucine cannot be explained simply through a stimulation of insulin release. Furthermore, we found that the increase of isoleucine-induced glucose uptake occurs in a mTOR-independent manner in skeletal muscle cells through the PI3 kinase and protein kinase C (PKC) signal pathways (8). On the basis of these findings, we hypothesized that the hypoglycemic effect of amino acids was not caused only by an insulinotropic effect but also by an insulin-independent effect.

The alternations in muscle glucose metabolism by isoleucine were caused in the absence of increases in AMP-activated protein kinase activity (9), which has been thought to be associated with an insulin-independent glucose uptake. This suggests that there are other as-yet unknown signaling pathways involved in this phenomenon. In addition, it is also unknown how the glucose incorporated into the tissues by isoleucine is metabolized and what effect isoleucine has on gluconeogenesis in the liver. These actions may also be of importance.

The main purpose of this study was to clarify the mechanism of the isoleucine hypoglycemic effect. Generally, the maintenance of the blood glucose level is due to an optimal balance between the glucose uptake by peripheral tissues and the glucose production that occurs mainly in the liver. Therefore, we examined the effect of isoleucine administration on glucose uptake in peripheral tissues, glucose oxidation in the whole body, and hepatic glucose production. Glucose production is greatly affected by gluconeogenesis that occurs during the fasting state (22), and therefore, we examined the effect of

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isoleucine on the gluconeogenic rate-limiting enzymes in vivo and on the glucose production in isolated hepatocytes.

MATERIALS AND METHODS

In Vivo Experiments

Animals. Male Wistar rats (6 wk old; Clea Japan, Tokyo, Japan) were maintained under conditions of constant humidity ($55 \pm 15\%$) and temperature ($22 \pm 2°C$) and a 12:12-h light-dark cycle (0700–1900; lights on at 0700). They were fed a standard diet of AIN-93G (Nosan, Yokohama, Japan) for 10 days, and water was freely available. All experiments were carried out in accordance with the "Guidelines for the Care and Use of Laboratory Animals," as adopted by the Committee on the Care and Use of Laboratory Animals of Otsuka Pharmaceutical Factory, Inc.

Animal experimental design. In the dose-response experiment, rats were food-deprived for 16 h and then administered saline or L-isoleucine (0.3, 0.45, 0.9, or 1.35 g/kg body wt) by oral gavage, as described previously (1, 9). In the other animal experiments, on the day before the administration, a silicon catheter was inserted into the jugular vein and threaded 2.5 cm proximally from the tip in rats under anesthesia. Saline was infused continuously at a rate of 1 ml/h per rat via a vinyl tube to prevent blood coagulation. Rats were housed individually in enclosed air-conditioned cages and were undisturbed both before and during the study. Rats were food-deprived for 24 h and then administered saline, L-leucine (0.45 g /kg body wt, prepared as 18.0 g/l of the L-amino acid in distilled water), or L-isoleucine (0.45 g/kg body wt by oral gavage). The volume of saline, leucine, or isoleucine administered was 15 ml/kg body wt.

The blood samples for the measurements of the plasma concentrations of insulin and glucagon were taken from the venous catheter and collected at 10-min intervals until 60 min after administration. For the measurement of plasma concentrations of amino acids, at exactly 60 min after oral administration, rats were anesthetized with pentobarbital sodium (40 mg/kg body wt), and blood was collected via the abdominal aorta. For measurement of the AMP, ADP, and ATP contents, mRNAs, and glucose-6-phosphatase (G6Pase) activity, one portion of the liver was frozen in liquid nitrogen and then stored at $-80°C$ until assays were performed.

Glucose uptake in gastrocnemius muscle, epididymal adipose tissue, and liver. Exactly 20 min after oral administration of saline, leucine, or isoleucine, a bolus intravenous administration of 30 μCi/kg body wt each of 2-[1,2-^{3}H]deoxyglucose (2-[^{3}H]DG; Amersham Bioscience, Piscataway, NJ) was given to rats via a venous catheter. The rats were anesthetized with pentobarbital sodium at 40 min after the tracer injection (60 min after saline, leucine, or isoleucine administration), and the gastrocnemius muscle, epididymal adipose tissue, and liver were excised. All sampling for the measurement of the integral of the plasma glucose and 2-[^{3}H]DG levels were taken from the venous catheter at 2, 10, 20, 30, and 40 min after the tracer boluses, which corresponded to the disappearance period from the plasma of 2-[^{3}H]DG. Because of the marked initial decay of the 2-[^{3}H]DG in the blood, the bulk of 2-[1,2-^{3}H]deoxyglucose-6-phosphate (2-[^{3}H]DGP) was synthesized in the tissues during the 40-min labeling period (17, 18, 25). 2-[^{3}H]DGP accumulation in the tissues was measured as described previously (14). Tissues were analyzed for accumulation of 2-[^{3}H]DG and 2-[^{3}H]DGP content as described previously (9, 25). Accumulations of 2-[^{3}H]DGP in the tissues were then calculated as the difference between the total (2-[^{3}H]DG + 2-[^{3}H]DGP) and the 2-[^{3}H]DG radioactivity.

Expiratory excretion of $^{14}CO_2$ from [U-^{14}C]glucose. After administration of saline or L-isoleucine (0.45 g/kg body wt) by oral gavage, each animal was immediately placed in an acryl metabolic cage to enable the collection of expired air, and a bolus intravenous injection of 30 μCi/kg body wt of [U-^{14}C]glucose (Amersham Bioscience) was given to the rats. Normal air was drawn through the cages at 8 l/min,

although atmospheric water vapors were removed before collection of the CO_2. The expired CO_2 over a 30-min period was collected at intervals of 30, 60, and 90 min after infusion, with the collected gas subsequently aspirated slowly through 20 ml of the ethanolamine solution (Kishida Chimical, Osaka, Japan) to trap the CO_2. A 1.0-ml aliquot of this solution was then pipetted into scintillation vials, scintillation cocktail was added, and the ^{14}C radioactivity was determined. The data are expressed as the percentage of the total radioactive dose injected during each interval.

HPLC analysis of adenine nucleotide contents in the liver. Preparation of samples and measurements of ATP, ADP, and AMP using HPLC (Waters Alliance 2695 system, 2487 Dual λ absorbance detector; Empower chromatographic manager; Waters, Midford, MA), was performed as described previously (9, 24). An aliquot of each sample was passed through a TSK-GEL column (150×4.6 mm, 5-μm particle size; Tosoh, Tokyo, Japan). Detection of absorbance occurred at 260 nm, and the flow rate was set at 0.7 ml/min. A gradient was initiated by using two buffers, where *buffer A* consisted of 25 mM NaH_2PO_4 and PIC-A reagent (Waters) at pH 5 and *buffer B* consisted of a mixture of 10% (vol/vol) acetonitrile, 200 mM NaH_2PO_4, and PIC-A reagent (Waters) at pH 4. The buffers were filtered through a 0.45-μm filter (Pall, Ann Arbor, MI) and degassed in a flask linked to a vacuum pipe. The gradient used in the HPLC assay varied from 100% *buffer A* to 100% *buffer B* from 0 to 35 min and then back to 100% *buffer A* over 36 to 45 min for column reequilibration. Peaks were identified from their retention times through chromatography standards.

In Vitro Experiments

Rat hepatocytes isolation and incubation. Hepatocytes from 24-h food-deprived male Wistar rats were isolated by in situ perfusion of the liver with 0.025% collagenase, as has been described previously (29). Most of the damaged and nonparenchymal cells were removed by washing with Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4) supplemented with 0.1% BSA, 5 mM HEPES, and a centrifugation procedure. Hepatocyte viability was determined using the Trypan blue exclusion method. Hepatocytes with viability of >90% were used for all further studies. Hepatocyte concentration was 5×10^6 cells/ml, and they were maintained in oxygenated (95% O_2-5% CO_2) KHB buffer (pH 7.4) supplemented with 0.1% BSA, 5 mM HEPES, and other substances, as indicated in legends. The hepatocytes were incubated in 2-ml suspensions in 10-ml flasks at 37°C with gas (95% O_2-5% CO_2) blowing in a gyratory shaking water bath.

Measurement of glucose production, proteolysis, and mRNAs in isolated hepatocytes. The glucose production was determined after a 60-min incubation period in the presence of 10 mM alanine, which was used as the gluconeogenic substrate. Leucine, isoleucine, and phenylalanine, which were used as the neutral amino acids, as well as alanine, were added to the medium at 3 mM. The concentrations used were the same as for the plasma isoleucine levels in the L-isoleucine (0.45 g/kg)-administered rats, since this dose had exhibited the greatest hypoglycemic effect in vivo. At the end of the 60-min incubations, aliquots of the medium were taken and prepared for measurements of glucose production and the levels of amino acids. Proteolysis was measured as the production of valine (23). The hepatocyte pellets were collected by centrifugation and then stored at $-80°C$ until measurements of mRNAs and G6Pase activity were performed.

Assays

Sample measurements. Plasma glucose levels in the saline-, leucine-, or isoleucine-administered rats and the medium for incubation of rat hepatocytes were measured using the Fuji Dry-Chem 5500 (GLU-PIII; Fujifilm Medical, Tokyo, Japan). Plasma glucagon levels were measured by a double-antibody enzyme immunoassay that used rat glucagon as a standard (Glucagon EIA kit; Yanaihara Institute, Shizuoka, Japan), and plasma insulin levels were measured by a double-antibody enzyme immunoassay that used rat insulin as a

standard (Rat Insulin ELISA kit; Shibayagi, Gunma, Japan). For measurements of amino acids levels, the plasma and the incubation medium were quenched with sulfosalicylic acid. After removal of the protein by rapid centrifugation at 4°C, levels of the amino acids in the supernatants were determined using an automatic amino acid analyzer L-8800A (Hitachi High-Technologies, Tokyo, Japan). Residue alanine levels in the hepatocyte culture medium were used to express the percentage of added alanine contents to the medium.

RNA extraction and Real-Time Quantitative RT-PCR. Total RNAs were extracted from the 30-mg tissue samples using the Qiagen RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The extracted total RNAs were dissolved in 100 µl of RNase-free water and stored at −80°C until use.

Real-time quantitative RT-PCR was performed to determine the relative expression levels of the G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) enzymes in rat liver and in isolated hepatocytes as described previously (19). Oligonucleotide primers and TaqMan probes were designed using Primer Express software version 1.0 (Applied Biosystems, Tokyo, Japan), except for the TaqMan 18S ribosomal RNA (rRNA) Control Reagents kit (Applied Biosystems). The sequence for the primers and probe sets for rat G6Pase and rat PEPCK were as follows: 5′-GACCTCAGGAACGCCTTCTATG-3′ (forward), 5′-AGGAGATTGATGCCCACAGTCT-3′ (reverse), and 5′-FAM-CCTCTTTCCCATCTGGTTCCACATTCA-TAMRA-3′ (probe) for rat G6Pase; and 5′-CCCAGGAGTCACCATCACTTC-3′ (forward), 5′-GGTGCAGAATCGCGAGTTG-3′ (reverse), and 5′-FAM-CACG-GTTCCTCATCCTGTGGTCTCCA-TAMRA-3′ (probe) for rat PEPCK. RT-PCR reactions were carried out in 50-µl volume reactions using the TaqMan One-step RT-PCR Master Mix reagents kit (Applied Biosystems) in 96-well plates for the rat G6Pase and PEPCK mRNAs and for the endogenous control, 18 S rRNA. TaqMan assay reaction buffer contained 1× Master Mix reagents, 1× MultiScribe and RNase inhibitor mix (TaqMan One-step RT-PCR Master Mix reagents), 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe, and 50 ng of total RNA. However, the 18 S rRNA reaction buffer contained all the above, except for the following differences: 50 nM forward primer, 50 nM reverse primer, and 50 nM TaqMan probe. RT reaction conditions were 48°C for 30 min and 95°C for 10 min for 1 cycle. PCR conditions were 95°C for 15 s and 60°C for 1 min for 40 cycles on an ABI PRISM 7700 sequence detector (Applied Biosystems). Linearity of the standard curve for the enzyme mRNA was obtained from the 0.16- to 100-ng total RNA range by using a standard sample prepared from the tissues of control rats. Results for the expression of mRNA were normalized with a value of 18 S rRNA and are presented relative to expression levels for each of the control groups.

G6Pase activity. G6Pase activity was measured by the production of phosphate from glucose-6-phosphate. Liver and hepatocytes were homogenized using a hand-held homogenizer in 0.25 M sucrose-HEPES buffer (pH 7.4). Homogenates were centrifuged at 3,000 *g*. Supernatant (100 µl) was mixed with 10 µl of taurocholic acid (Sigma-Aldrich, St. Louis, MO), followed by incubation for 30 min on ice, after which 290 µl of sucrose-HEPES buffer was added. One aliquot (60 µl) was mixed with reaction buffer (25 µl of 0.5 M Tris buffer, pH 6.5, 20 µl of 100 mg/ml BSA, 40 µl of 0.1 M glucose-6-phosphate, and 55 µl of water) and then incubated at 37°C for 0 and 20 min, followed by the addition of 600 µl of ice-cold 3.5% trichloroacetic acid. After centrifugation (3,000 *g* for 10 min at 4°C), free phosphate in the supernatant was determined by incubation with 300 µl of phosphate reagent: 5% ammonium molybdate tetrahydrate (Sigma-Aldrich catalog no. A-7302) dissolved in 4 N HCl and 1 g of Ion (II) sulfate heptahydrate (Merck, Darmstadt, Germany) for 10 min at room temperature. The absorbance of each sample was read at 650 nm. A second aliquot was used for protein determination by the Bradford analysis.

Calculations. Glucose uptake (R_g) was calculated from the tissue accumulations of 2-[³H]DGP, the integral of the plasma 2-[³H]DG

level after a 2-[³H]DG bolus, and the plasma glucose level. The relationship was defined as follows:

$$R_g = (G)_p \times (2\text{-}[^3H]DGP)_t \bigg/ \int_0^t (2\text{-}[^3H]DG)_p dt$$

where $t = 20$ to 60 min after the administration of the amino acids, $(G)_P$ is the average arterial plasma glucose level (mM) from $t = 20$ to 60 min, and $(2\text{-}[^3H]DGP)_t$ is the accumulation of 2-[³H]DGP in the tissues at $t = 40$ min (dpm/mg wet wt). $(2\text{-}[^3H]DG)_p$ represents the plasma 2-[³H]DG level (dpm/ml), and t indicates the point where the tracer bolus was administered and was set to $t = 0$. The measurement of R_g has been described previously (17, 18, 25).

Energy charge was calculated by the following equation: [ATP + 1/2 ADP]/[ATP + ADP + AMP], where ATP, ADP, and AMP are the respective tissue concentrations (2).

Statistical analysis. Tests for statistical significance were performed using Statistical Analysis Software (version 7.0; SAS Institute, Cary, NC). Significant differences among the control, leucine, and isoleucine treatment groups were assessed using a one-way ANOVA followed by the Dunnett or Tukey-Kramer multiple comparisons test. Mean expired ¹⁴CO₂ values in the control and isoleucine treatment groups were analyzed using Student's *t*-test. Values are given as means ± SE.

RESULTS

Dose-Response Effects of Isoleucine on Plasma Concentrations of Glucose and Amino Acids in Fasted Rats

The effects of isoleucine on glucose metabolism were investigated to determine whether administered isoleucine decreases the plasma glucose level and the optimal concentrations for this effect in fasted rats. One hour after oral administration, isoleucine at a dose of 0.45 g/kg body wt significantly decreased the plasma glucose level (6.4 ± 0.2 mM; $P < 0.01$; Fig. 1*A*) compared with controls (0 g/kg body wt; 8.1 ± 0.1 mM). The administrations of isoleucine at 0.45 g/kg body wt significantly elevated the plasma isoleucine level to 3.0 mM (Fig. 1*B*). There were dose-dependent decreases in the plasma concentrations of leucine, lysine, proline, methionine, phenylalanine, and glycine, and the plasma concentrations of alanine and threonine at a dose of 0.45 g/kg body wt were significantly elevated compared with controls (Table 1). There appeared to have been reached a first peak at 0.45 g/kg body wt.

Effect of Leucine or Isoleucine on Glucose Uptake in Skeletal Muscle, Adipose Tissue, and Liver and on Plasma Glucose and 2-[³H]DG Levels in Fasted Rats

Glucose uptakes (R_g) in the gastrocnemius muscle and liver during the last 40 min are shown in Fig. 2, *B* and *C*. The R_g in the skeletal muscle of the leucine group (5.8 ± 1.4 µmol·100 g tissue⁻¹·min⁻¹) did not significantly differ from that of controls (3.6 ± 0.9 µmol·100 g tissue⁻¹·min⁻¹). However, the R_g in the skeletal muscle of isoleucine group (8.6 ± 1.1 µmol·100 g tissue⁻¹·min⁻¹; $P < 0.05$) was significantly increased compared with that of the controls (Fig. 2*B*). In contrast, no significant differences were identified among the groups in the liver (2.8 ± 0.9, 3.9 ± 0.9, and 3.2 ± 0.9 µmol·100 g tissue⁻¹·min⁻¹ in control, leucine, and isoleucine groups, respectively; Fig. 2*C*) or adipose tissue (4.3 ± 1.3, 5.2 ± 1.3, and 7.4 ± 1.5 µmol·100 g tissue⁻¹·min⁻¹ in control, leucine, and isoleucine groups, respectively; $P = 0.23$,
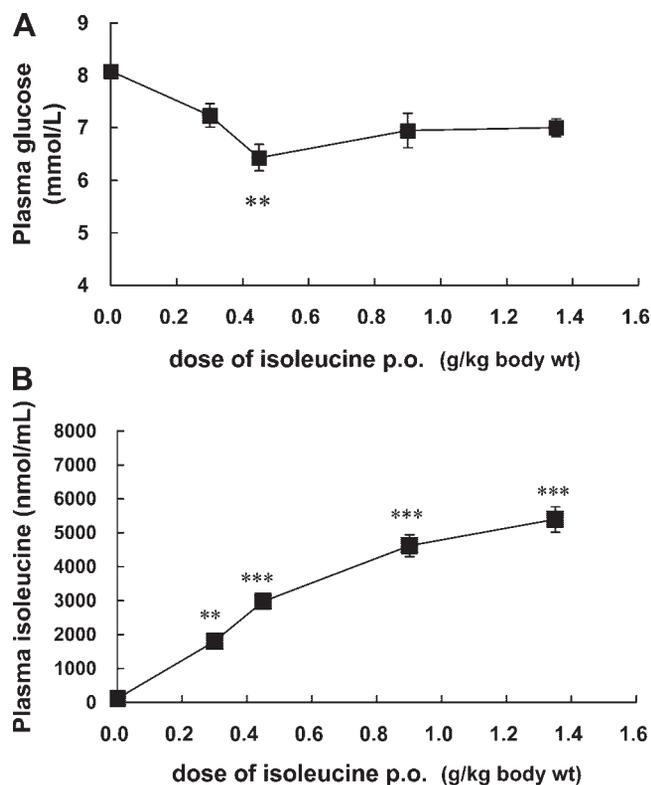
Fig. 1. Dose-response curve of plasma glucose and isoleucine concentrations in the isoleucine-administered rat. Rats were food-deprived for 16 h and administered saline or L-isoleucine (0.3, 0.45, 0.9, or 1.35 g/kg body wt) by oral gavage. One hour after oral administration (p.o.) of saline or isoleucine, plasma concentrations of glucose (*A*) and isoleucine (*B*) in administered rats were measured, as detailed in MATERIALS AND METHODS. Plasma isoleucine levels are the same as the data presented in Table 1. Data are expressed as means ± SE ($n = 4$). **$P < 0.01$; ***$P < 0.001$, significant difference from the value in the respective saline-administered rats (control); Dunnett's test.

isoleucine vs. controls). Figure 2, *D* and *E*, shows the plasma glucose and 2-[^{3}H]DG levels of saline, leucine, or isoleucine groups during the 40-min time course after the 2-[^{3}H]DG intravenous injection. Despite the fact that the plasma insulin level of the leucine group was higher than that of the isoleucine group (Fig. 3*A*), the plasma glucose level of the isoleucine group (4.7 ± 0.1 mM; $P < 0.05$) was significantly decreased at 40 min after the 2-[^{3}H]DG administration compared with that of the controls (5.6 ± 0.2 mM; Fig. 2*D*). However, the plasma glucose level of the leucine group (5.2 ± 0.1 mM) did not differ significantly compared with the control group. In terms of the disappearance of plasma 2-[^{3}H]DG, plasma 2-[^{3}H]DG levels of the isoleucine group were significantly decreased at 10 and 20 min after the 2-[^{3}H]DG injections compared with the control group (Fig. 2*E*).

Plasma Concentrations of Insulin and Glucagon in Fasted Rats

Figure 3, *A* and *B*, shows the plasma insulin and glucagon concentrations of saline, leucine, or isoleucine groups over time. A marked elevation of the plasma insulin level was observed in the leucine group (0.813 ± 0.096 ng/ml; $P < 0.001$ vs. control, $P < 0.01$ vs. isoleucine) compared with the control (0.263 ± 0.050 ng/ml) or the isoleucine group (0.386 ± 0.047 ng/ml) at 10 min after administration. In addition, at 20 or 30 min, the leucine group was also significantly elevated compared with controls. However, the plasma insulin level of the isoleucine group was not altered compared with the controls at all points. On the other hand, plasma glucagon levels did not differ among the control, leucine, or isoleucine groups at all points.

Effect of Isoleucine on Expiratory Excretion of $^{14}CO_2$ From [U-^{14}C]Glucose

To determine whether the hypoglycemic effect of isoleucine affects whole body glucose oxidation, we examined isoleu-

Table 1. *Plasma concentrations of amino acids in saline- or isoleucine-administered rats*

Amino Acids	Ile-0	Ile-0.3	Ile-0.45	Ile-0.9	Ile-1.35
Ile	108.0±6.2	1799.1±58.6†	2978.8±67.6‡	4617.7±317.6‡	5389.8±373.4‡
Leu	163.3±9.0	168.2±7.1	136.9±3.4	124.7±4.1*	122.3±11.8*
Val	196.6±9.0	218.4±9.4	197.0±2.9	181.9±4.4	194.7±16.5
Ala	300.5±12.5	364.7±8.3*	383.7±6.0*	344.8±13.8	333.1±26.8
Arg	130.2±1.5	137.1±5.0	127.7±4.0	111.6±3.5	111.8±8.2
Glu	104.1±2.0	125.1±11.1	114.8±5.6	110.8±6.1	115.4±5.2
Gln	642.7±21.3	694.2±37.1	734.7±27.9	723.1±20.4	664.9±14.0
Lys	406.0±20.1	368.1±32.1	328.9±9.6	298.3±17.9*	305.5±30.8*
Asp	6.8±0.4	9.9±1.2	9.3±0.5	9.4±1.1	9.1±1.2
Asn	60.2±0.8	72.1±7.6	69.3±5.9	61.9±1.9	56.3±2.4
Pro	129.5±3.8	127.5±3.2	122.8±5.3	98.6±4.4†	103.4±3.2†
Cys	46.8±1.0	56.2±0.7‡	47.1±0.7	48.1±0.6	47.6±0.8
Met	51.0±2.1	47.7±1.8	45.3±1.4	39.1±0.7†	39.0±3.1†
His	57.9±1.3	56.4±4.2	63.4±2.6	55.3±4.1	53.4±3.3
Phe	68.7±0.8	62.5±1.8	60.5±0.6	52.2±5.1*	48.5±6.7*
Tyr	74.6±2.3	69.3±0.9	62.6±3.6	63.2±1.6	60.9±5.2*
Trp	74.1±9.2	72.3±5.6	68.6±1.0	58.9±5.0	52.9±2.7
Gly	398.1±19.7	366.6±1.4	349.6±11.1*	256.2±2.4‡	304.5±12.3‡
Thr	223.1±25.2	272.8±15.2	321.3±7.6†	243.4±2.0	283.9±18.1
Ser	242.1±7.0	296.9±19.9*	284.8±6.5	240.8±10.4	251.3±7.8

Experimental details are the same as those presented in the legend for Fig. 1. All measurements were made 60 min after saline or isoleucine administration (in g/kg body wt per os). Rat plasma was quenched with sulfosalicylic acid. After removal of the protein by rapid centrifugation at 4°C, an automatic amino acid analyzer was used to determine the amino acid concentrations of the supernatants. Data are means ± SE in nmol/ml ($n = 4$). *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$, significant difference from the value in the respective saline-administered rats (control); Dunnett's test.

A

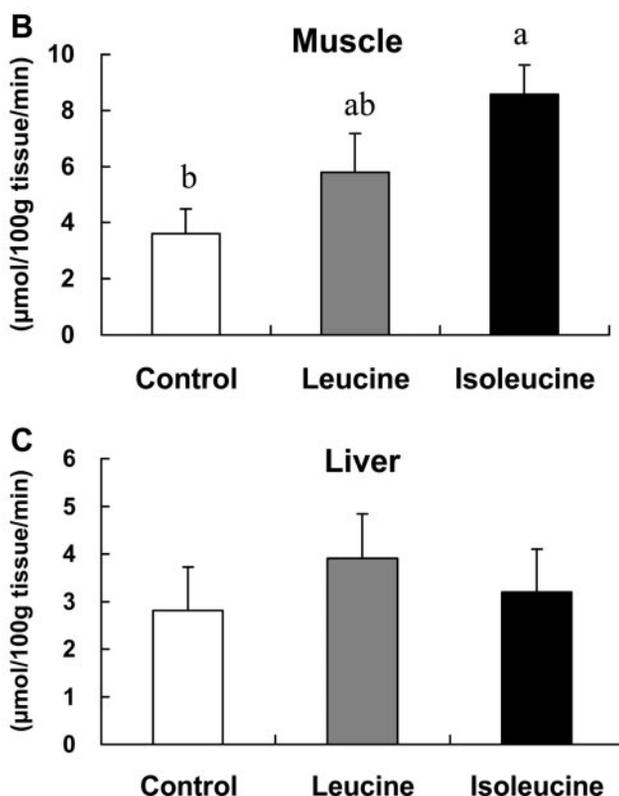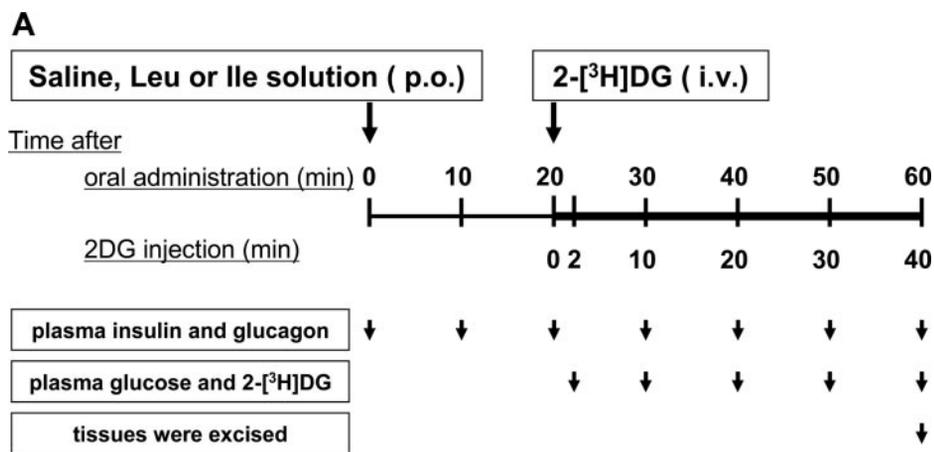Saline, Leu or Ile solution (p.o.)   2-[³H]DG (i.v.)

Time after

oral administration (min) 0   10   20   30   40   50   60

2DG injection (min)   0 2   10   20   30   40

plasma insulin and glucagon

plasma glucose and 2-[³H]DG

tissues were excised

B

Muscle

(µmol/100g tissue/min)

C

Liver

(µmol/100g tissue/min)

Fig. 2. Glucose uptake study. Protocol (*A*) and effects of leucine (Leu) or isoleucine (Ile) on glucose uptake (R_g) in skeletal muscle (*B*), liver (*C*), and plasma concentrations of glucose (*D*) and 2-[1,2-³H]deoxyglucose (2-[³H]DG; *E*). Rats were food-deprived for 24 h and administered saline, L-leucine (0.45 g/kg) or L-isoleucine (0.45 g/kg body wt) by oral gavage. Exactly 20 min after oral administration, a bolus intravenous (i.v.) injection of 30 µCi/kg body wt each of 2-[³H]DG was given to rats. The rats were anesthetized with pentobarbital sodium at 60 min after saline, Leu, or Ile administration, and the tissues were excised. Tissues were analyzed for accumulations of 2-[1,2-³H]deoxyglucose-6-phosphate (2-[³H]DGP) in the tissues, and then R_g calculations in the skeletal muscle (*B*) and the liver (*C*) were performed as described in MATERIALS AND METHODS. Data are expressed as means ± SE (*n* = 6). The samplings for the measurements of the plasma glucose concentration (*D*) and the counts of 2-[³H]DG in plasma (*E*) were obtained using a catheter at 2, 10, 20, 30, and 40 min after the tracer boluses. Data are expressed as means ± SE (*n* = 7). [a,b] $P < 0.05$, means in a column not sharing a superscript are different; determined using Tukey-Kramer test.

cine's effect on expiratory excretion of $^{14}CO_2$ from [U-^{14}C]glucose in vivo at a dose where the hypoglycemic effect was the most effective. The expiratory excretion of $^{14}CO_2$ of rats treated with isoleucine was significantly increased (6.0 ± 0.3% of the total radioactive dose injected; $P < 0.05$) between 60 and 90 min compared with the controls (5.1 ± 0.1%; Fig. 4).

Measurement of Liver Adenine Nucleotides and Energy Charge

Hepatic AMP, which indicates the cellular energy state, is reflected by changes in the energy charge in tissues such as the liver and muscle (6). Liver AMP content of the isoleucine group was lower than that seen for the control group (Table 2;

$P < 0.05$), whereas no alterations for the leucine group were noted. The liver ATP and ADP contents did not differ among each of the groups in the liver. The hepatic energy charge of the liver in the isoleucine group tended to be increased compared with that seen for controls ($P = 0.07$).

Effects of Leucine or Isoleucine on Expression of PEPCK and G6Pase mRNA and G6Pase Activity in Rat Liver

The expression of PEPCK mRNA, which is associated with gluconeogenesis, in isoleucine-administered rat liver (0.60 ± 0.14 arbitrary unit; $P < 0.05$; Fig. 5*A*) was also decreased compared with that in controls (1.01 ± 0.07) or in the leucine-administered rat liver (0.94 ± 0.09). Furthermore, the expres-
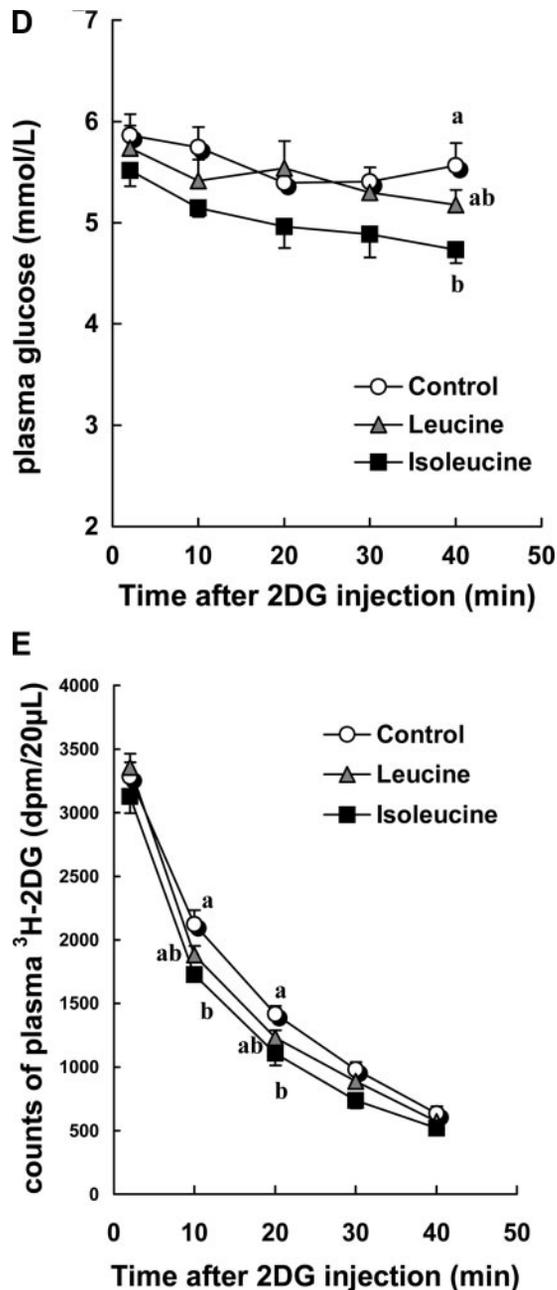
Fig. 2— *Continued*

Effects of Leucine or Isoleucine on Glucose Production, Proteolysis, and Alanine Consumption in Rat Isolated Hepatocytes

Glucose production from 10 mM alanine (1.14 ± 0.02 mM) was significantly decreased by adding 3 mM isoleucine (0.89 ± 0.02 mM) or phenylalanine (0.75 ± 0.03 mM) but not by adding leucine (1.04 ± 0.03 mM; Fig. 6).

Net valine release from the hepatocytes, which was used as an indicator of proteolysis (15), was decreased for alanine plus isoleucine compared with alanine alone during the 60-min incubation. Furthermore, the valine release from the hepatocytes with alanine plus leucine was decreased compared with alanine or alanine plus isoleucine (Table 3; $P < 0.05$). On the other hand, residual alanine in the medium after the 60-min incubation with alanine plus isoleucine was higher than that seen for alanine, although there were no alterations seen with alanine plus leucine.

Effects of Leucine or Isoleucine on Expression of PEPCK and G6Pase mRNA and G6Pase Activity in Rat Isolated Hepatocytes

The expression of PEPCK mRNA in isolated hepatocytes with alanine plus isoleucine (0.74 ± 0.04 arbitrary unit; $P < 0.05$; Fig. 7A) was significantly decreased compared with isolated hepatocytes with alanine (1.01 ± 0.06), although there
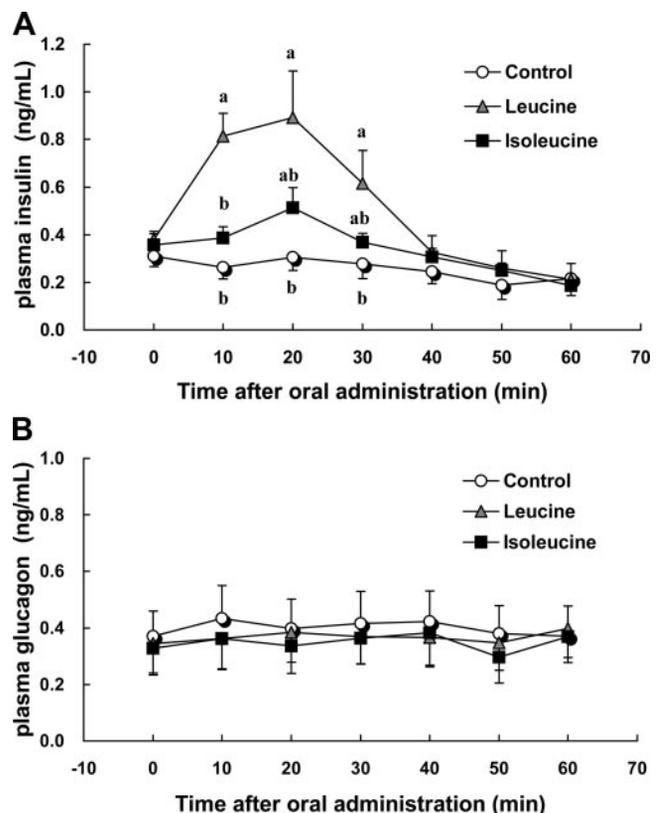

Fig. 3. Effect of leucine or isoleucine on plasma insulin (*A*) and glucagon concentrations (*B*). Experimental details are the same as in the legend for Fig. 2. The samplings for the measurements of the plasma insulin (*A*) and glucagon concentrations (*B*) were taken from the venous catheter every 10 min after the oral administrations. Data are expressed as means ± SE (*n* = 6). [a,b]$P < 0.05$, means in a column not sharing a superscript are different; determined using Tukey-Kramer test.
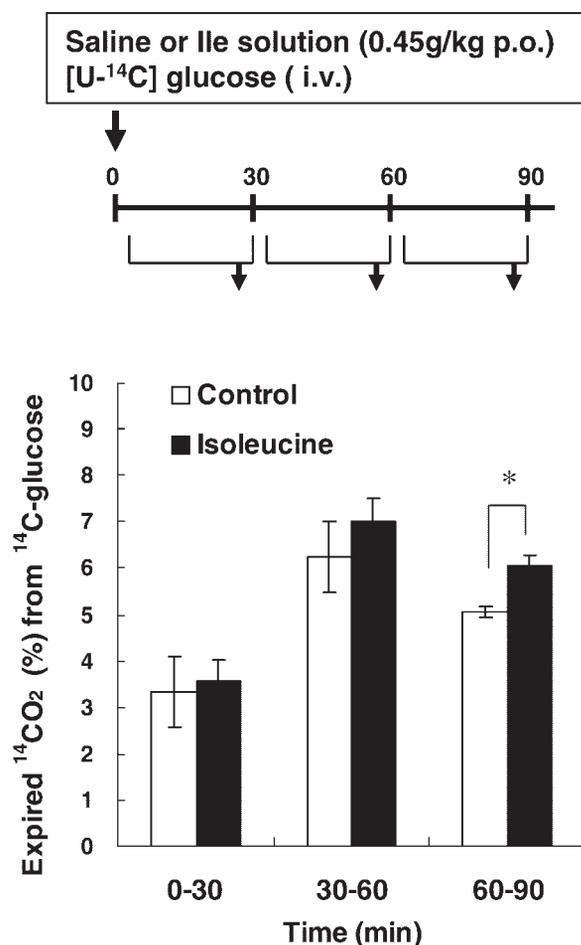
sion of G6Pase mRNA in isoleucine-administered rat liver (0.39 ± 0.07 arbitrary unit; $P < 0.05$; Fig. 5A) was decreased compared with that seen for the control (1.06 ± 0.14) or the leucine-administered rat liver (0.91 ± 0.11). On the basis of these results, we theorized that isoleucine might inhibit the glucose production through the downregulation of G6Pase activity. Thus we investigated the G6Pase activity of the liver. As expected, G6Pase activity of the isoleucine group in the liver (1.08 ± 0.08 μmol P_i/mg protein; $P < 0.05$; Fig. 5B) was decreased significantly compared with that of the controls (1.47 ± 0.09 μmol P_i/mg protein). However, G6Pase activity of the leucine group in the liver (1.28 ± 0.11 μmol P_i/mg protein) was not altered compared with that seen for the controls.

Saline or Ile solution (0.45g/kg p.o.)
[U-^{14}C] glucose (i.v.)

Fig. 4. Effect of isoleucine on expiratory excretion of $^{14}CO_2$ converted from [U-^{14}C]glucose in rat. Rats were food-deprived for 16 h and administered saline or L-isoleucine (0.45 g/kg body wt) by oral gavage. After the administration, a bolus i.v. injection of 30 μCi/kg body wt of [U-^{14}C]glucose was given to rats. The expired CO_2 for 30 min was collected at intervals from 30 to 90 min after infusion, and the ^{14}C radioactivity was determined. Data are expressed as means $\pm$ SE ($n = 4$). *$P < 0.05$, significant difference from the value in the respective control group; Student t-test.

were no alterations with alanine plus leucine (0.95 $\pm$ 0.09). Furthermore, the expression of G6Pase mRNA in isolated hepatocytes with alanine plus isoleucine (0.75 $\pm$ 0.04 arbitrary unit; $P < 0.05$; Fig. 7A) was significantly decreased compared

Table 2. *Effect of leucine or isoleucine on adenine nucleotide contents and energy charge in the liver*

Treatment Group	AMP, μmol/g liver	ADP, μmol/g liver	ATP, μmol/g liver	(ATP + 1/2ADP)/(ATP + ADP + AMP)
Control	1.911$\pm$0.077[a]	1.462$\pm$0.046	1.146$\pm$0.056	0.415$\pm$0.012
Leucine	1.794$\pm$0.076[ab]	1.530$\pm$0.068	1.203$\pm$0.103	0.434$\pm$0.017
Isoleucine	1.617$\pm$0.062[b]	1.538$\pm$0.081	1.240$\pm$0.034	0.457$\pm$0.006

Experimental details are the same as those presented in the legend for Fig. 2. All measurements were made 60 min after saline, leucine, or isoleucine administration. Preparation of samples and measurements of ATP, ADP, and AMP using HPLC were performed as described in MATERIALS AND METHODS. Energy charge was calculated using the equation (ATP + 1/2 ADP)/(ATP + ADP + AMP). Hepatic AMP, which indicates the cellular energy state, is reflected by changes in the energy charge in tissues. Data are means $\pm$ SE ($n = 6$). [a,b]$P < 0.05$, means in columns not sharing a superscript are different; determined using Tukey-Kramer test.
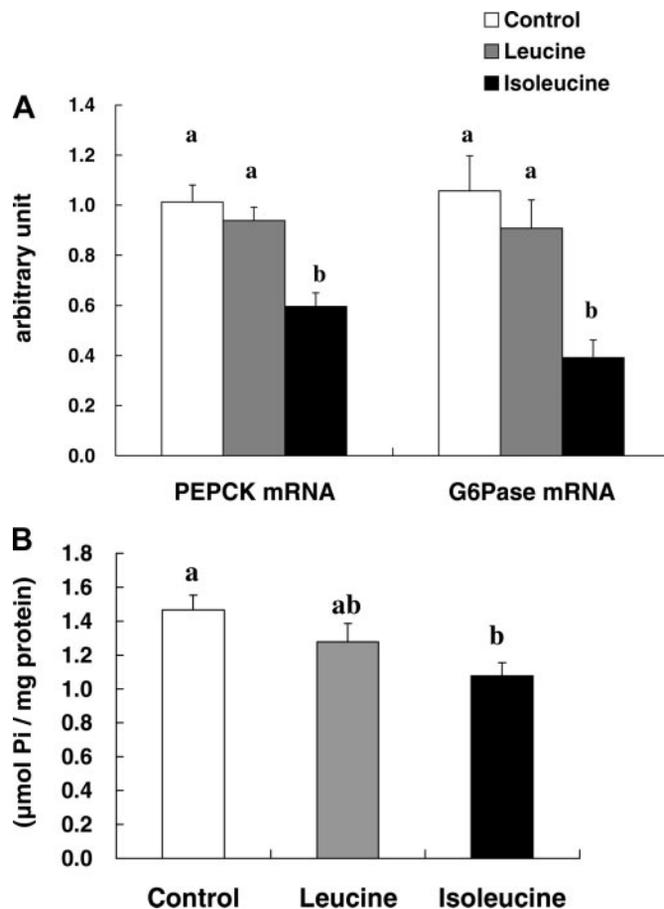
Fig. 5. Effect of Leu or Ile on the expressions of phosphoenolpyruvate carboxykinase (PEPCK) mRNA and glucose-6-phosphatase(G6Pase) mRNA (*A*) and G6Pase activity (*B*) in vivo. Livers from the saline-, Leu-, or Ile-administered rats were prepared as stated in the legend to Fig. 2. Real-time quantitative RT-PCR was performed to determine the relative expression levels of the G6Pase and PEPCK enzymes in the rat liver. RT-PCR was carried out in a 50-μl volume reaction using TaqMan One-step RT-PCR Master Mix reagents kit (Applied Biosystems) in 96-well plates for the rat G6Pase and PEPCK mRNAs and the endogenous control, 18S rRNA. G6Pase activity was measured as the production of phosphate from glucose-6-phosphate as detailed in MATERIALS AND METHODS. Data are expressed as means $\pm$ SE ($n = 6$). [a,b]$P < 0.05$, means in a column not sharing a superscript are different; determined using Tukey-Kramer test.

with isolated hepatocytes with alanine (1.01 $\pm$ 0.06) or alanine plus leucine (1.03 $\pm$ 0.09). G6Pase activity of isolated hepatocytes with alanine plus isoleucine (1.75 $\pm$ 0.12 μmol P$_i$/mg protein; $P < 0.05$; Fig. 7B) was decreased significantly compared with that seen with alanine (2.17 $\pm$ 0.07 μmol P$_i$/mg protein). However, G6Pase activity for isolated hepatocytes with alanine plus leucine (2.14 $\pm$ 0.13 μmol P$_i$/mg protein) was not altered compared with that seen for the isolated hepatocyte controls.

DISCUSSION

In the present study, we found that the hypoglycemic effect of isoleucine was not of a dose-dependent manner in fasted rats (Fig. 1A). The most effective dose of isoleucine with regard to glucose lowering was the 0.45 g/kg body wt dose, and the plasma isoleucine level at the dose was 3 mM (Fig. 1B). In another study, the ability of glucose uptake in the skeletal
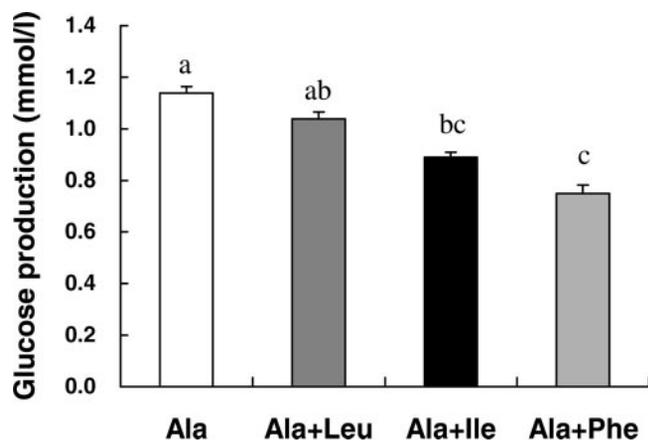
Fig. 6. Effect of Leu or Ile on glucose production from alanine in rat isolated hepatocytes. Hepatocytes from 24-h food-deprived rats were isolated by in situ perfusion of the liver with collagenase, as described previously. The glucose production was determined after a 60-min incubation period in the presence of 10 mM alanine (Ala) as the gluconeogenic substrate. Leu, Ile, or phenylalanine (Phe) was added to the medium at 3 mM. At the end of the 60-min incubations, aliquots of the medium were taken and prepared for measurements of glucose production. Data are expressed as means $\pm$ SE ($n = 6$). [a,b,c] $P < 0.05$, means in a column not sharing a superscript are different; determined using Tukey-Kramer test.
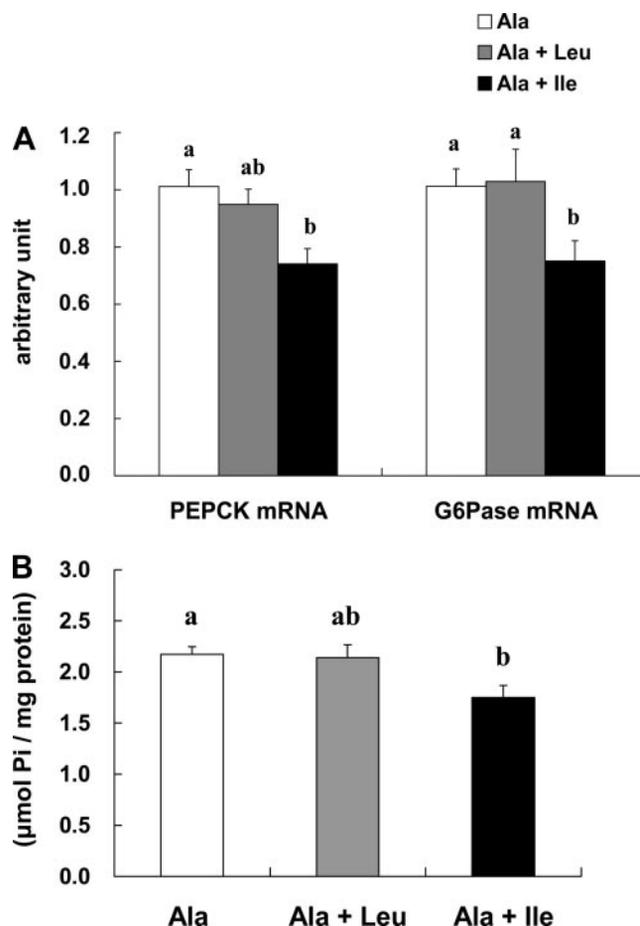

Fig. 7. Effect of Leu or Ile on the expressions of PEPCK mRNA and G6Pase mRNA (*A*) and G6Pase activity (*B*) in vitro. Isolated hepatocytes incubated with Ala, Ala + Leu, or Ala + Ile were prepared as stated in the legend to Fig. 6. Real-time quantitative RT-PCR was performed as described in the legend to Fig. 5. G6Pase activity was measured as the production of phosphate from glucose-6-phosphate as detailed in MATERIALS AND METHODS. Data are expressed as means $\pm$ SE ($n = 6$). [a,b] $P < 0.05$, means in a column not sharing a superscript are different; determined using Tukey-Kramer test.

muscle cells reached an initial peak with the use of 2 mM isoleucine in an insulin-free medium (8). These results indicate that there is an initial peak for the glucose-lowering effect at similar concentrations of isoleucine for both in vivo and in vitro conditions.

In this study, R_g was significantly increased in the muscle of isoleucine-administered rats compared with that in controls at the most effective dose of isoleucine (Fig. 2*B*). By contrast, there was no significant difference in the R_g in liver (Fig. 2*C*) or adipose tissue of isoleucine-administered rats compared with controls. These results suggest that the skeletal muscle is the major organ contributing to the hypoglycemic effect of isoleucine on glucose uptake. Leucine, an isomer of isoleucine, has a stimulatory effect in insulin secretion (11). Since a temporal increase in the plasma insulin level after an oral administration of leucine was observed in this study, the effect of leucine on glucose metabolism may be mainly insulin dependent. On the other hand, the hypoglycemic effect of isoleucine was more potent than that of leucine, although

Table 3. *Effect of leucine or isoleucine on proteolysis and residual rate of alanine in culture medium of isolated hepatocytes*

Treatment Group	Proteolysis, nmol valine·ml⁻¹·h⁻¹	Residual Alanine in Medium, % of added alanine
Ala	88.2 ± 2.4[a]	30.4 ± 0.4[b]
Ala+Leu	73.0 ± 0.9[c]	31.5 ± 0.6[a,b]
Ala+Ile	81.3 ± 1.1[b]	32.3 ± 0.3[a]

Experimental details are the same as those presented in the legends to Figs. 6 and 7. Net valine release from hepatocytes, used as an indicator of proteolysis, was measured as the production of valine. At the end of the 60-min incubations, aliquots of the medium were taken, and measurement of alanine and valine concentrations in the medium was performed as described in MATERIALS AND METHODS. Data are means $\pm$ SE ($n = 6$). [a,b,c] $P < 0.05$, means in columns not sharing a superscript are different; determined using Tukey-Kramer test.

significant changes in plasma insulin and glucagon levels were not observed after administration of isoleucine (Fig. 3, *A* and *B*). Furthermore, isoleucine had an additive increased effect on insulin-stimulated glucose uptake via PI3 kinase (8), in contrast to leucine, which inhibited insulin-stimulated glucose uptake in skeletal muscle cells (34). Although the molecular basis of increased glucose uptake by isoleucine remains unclear, a previous study by another group revealed that glucose uptake by isoleucine is involved in increased glucose transporter GLUT1 and GLUT4 translocation in the skeletal muscle of rats with liver cirrhosis (27). These data suggest that isoleucine improves insulin sensitivity in skeletal muscle via an intracellular signal pathway.

We showed that isoleucine administration increased whole body glucose oxidation (Fig. 4), unlike that which has been reported in previous studies using amino acids mixtures (28). On the basis of the above results, the muscle glucose uptake in isoleucine-administered rats was increased at 60 min, which was followed by significant increases in expiratory excretion of $^{14}CO_2$ from [U-^{14}C]glucose compared with the controls between 60 and 90 min. Since the time to achieve maximum

plasma isoleucine level was at 60 min (8, 9), this seems to indicate there is a strong correlation among the increase in muscle glucose uptake, the decrease in blood glucose, and the subsequent increase in glucose oxidation. By contrast, it has been reported that isoleucine suppressed $^{14}CO_2$ production from [1-^{14}C]pyruvate in isolated skeletal muscle (7). As a potential explanation, the suppression might have been caused by an increased total pyruvate content in the skeletal muscle (13) that was due to an increased glucose uptake by isoleucine (8, 9), which thereby resulted in the diluents effect of [1-^{14}C]pyruvate and an increase in total CO_2 production. Meanwhile, glycogen synthesis in isoleucine-administered rats was not altered in the skeletal muscle (9), suggesting that when there is a lowering of the blood glucose level, isoleucine increases glucose uptake in the skeletal muscle with the incorporated glucose mainly oxidized in the muscle immediately after the uptake.

Hepatic gluconeogenesis, as well as glucose utilization by the peripheral and hepatic tissues, may be a possible mechanism by which amino acids lower blood glucose levels. During the fasting state, the glucose production is largely a result of gluconeogenesis as opposed to hepatic glycogenolysis (22). Therefore, we examined the effect of isoleucine on the gluconeogenic rate-limiting enzymes in vivo. The mRNA levels of PEPCK, which is a well-researched key gluconeogenic enzyme, parallel both PEPCK activity and the rate of gluconeogenesis (16, 36). The data showed that expression levels of hepatic PEPCK mRNA were lower in isoleucine-administered rats than in controls (Fig. 5), suggesting that PEPCK activity was lower in isoleucine-administered rats and that the inhibitory effects of isoleucine on PEPCK are regulated at the transcriptional level. Furthermore, we demonstrated that expression levels of hepatic G6Pase mRNA and G6Pase activity were also lower in isoleucine-administered rats (Fig. 5). Enzyme activity is regulated by regulation of both protein expression and existing enzyme. Although whether isoleucine regulates existing enzyme is unknown, we considered that isoleucine inhibited G6Pase activity by decreasing mRNA expression of G6Pase. These findings imply that isoleucine also downregulates G6Pase activity and associated mRNA, in addition to inhibiting gluconeogenesis in the liver in vivo.

Since gluconeogenesis is a process requiring energy consumption, we theorized that the energy status would be altered in the liver of rats administered isoleucine. Isoleucine decreases the AMP level and tends to improve the energy state in the liver compared with that seen in controls (Table 2). Similar results have recently been observed in the skeletal muscle (9). In addition, glycogen synthesis in the liver did not differ among the control, leucine, and isoleucine groups (data not shown). Our data support the theory that the inhibitory effect of isoleucine on gluconeogenesis is accompanied by an increase in energy conservation and an improvement in the energy state in both the liver and muscle tissues.

Although we can calculate the R_g and endogenous glucose production (EGP) values at the same time through the use of a tracer, the value of EGP may be underestimated in experiments in which the R_g value greatly increases. Therefore, we employed a measurement of glucose production by using isolated hepatocytes to determine the mechanism underlying the hypoglycemic effect of isoleucine. As a glucogenic substrate, we examined alanine, because plasma alanine levels were found to be significantly higher in the 0.45 g/kg body wt isoleucine group (Table 1) and for which the most effective dose for decreasing plasma glucose was indicated compared with controls. Isoleucine significantly inhibited glucose production when alanine was used as a glucogenic substrate in isolated hepatocytes (Fig. 6). In addition, phenylalanine, which is a neutral amino acid that is transported via by the same neutral amino acid transport system as alanine, leucine, and isoleucine (4, 5), also significantly reduced glucose production. These results indicate that the inhibitory effect of isoleucine on glucose production with alanine may be due to a competitive inhibitory effect with alanine for transport via the neutral amino acid transporter. The residual alanine in the isoleucine-added medium reflects a balance between the alanine uptake to the hepatocytes and the alanine release from the hepatocytes. The alanine release from the hepatocytes in the isoleucine-added medium could be decreased, because the net proteolysis of the hepatocytes was lower than in controls (Table. 3). However, the residual alanine in the isoleucine-added medium was higher than in controls (Table 3). Therefore, the actual reduced amount of alanine uptake by isoleucine is more than
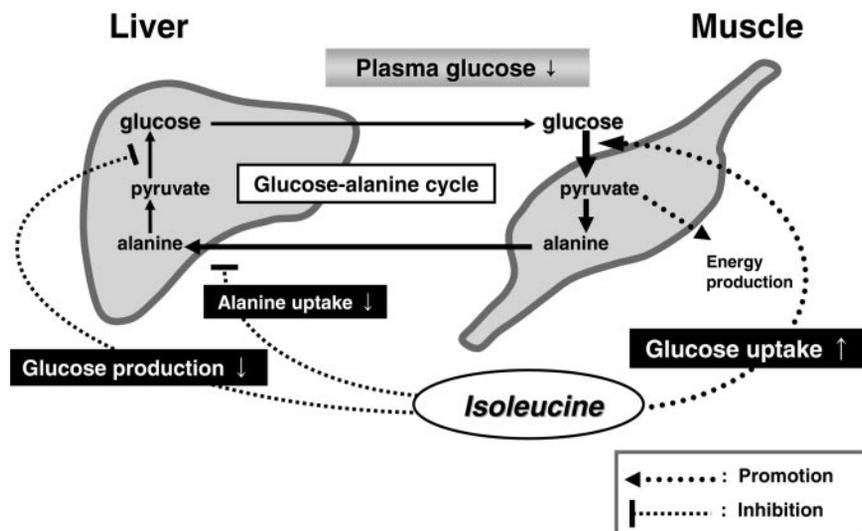

Fig. 8. Schematic diagram of the effect of Ile on glucose metabolism. Ile, a potent hypoglycemic amino acid, stimulates glucose uptake in skeletal muscle, and the incorporated glucose is oxidized without significant elevation of the plasma insulin level. In liver, Ile decreased the hepatic gluconeogenic enzyme activity and glucose production. These mechanisms are responsible for the hypoglycemic effect of Ile that improves the energy state of the muscle and liver and that may improve insulin resistance in vivo.

the increment of residual alanine, which is involved in the decrease of glucose production in the hepatocytes. The reason for the lower inhibitory effect of leucine on glucose production compared with that seen for isoleucine at the same concentration is unclear. It is possible that there may be a difference in the competitive inhibitory effect with alanine due to structural differences between leucine and isoleucine.

In the liver, a competitive effect of isoleucine with alanine for transport might elevate concentrations of alanine in the blood, so plasma alanine levels should increase in response to isoleucine in a dose-dependent manner (12). However, the present results were not consistent with these expected reactions at isoleucine doses over 0.45 g/kg body wt. On the other hand, isoleucine ingestion has been demonstrated to decrease plasma concentrations of other amino acids in a dose-dependent manner (Table 1). The mechanism of this effect is unknown but may involve specific stimulation of total amino acid oxidation to consume excess isoleucine. A plausible mechanism underlying the peaked change in plasma alanine levels is thus that the decrease in plasma alanine levels following stimulation of total amino acid oxidation in tissues to consume excess isoleucine exceeds the increase in plasma alanine levels due to competition at isoleucine doses over 0.45 g/kg body wt. The reduced hypoglycemic effect at higher isoleucine doses might occur as a result of increased amino acid oxidation as fuel in tissues.

Under in vitro conditions, there is an inhibitory effect of isoleucine on the expression of PEPCK and G6Pase in the isolated hepatocytes (Fig. 7A). Also, it was demonstrated that the G6Pase activity was lower in isoleucine-added cells compared with that in the controls (Fig. 7B). These findings imply that isoleucine downregulates the transcription of gluconeogenic enzymes and inhibits glucose production in the liver under insulin-free conditions. This suggests that the inhibitory effect of gluconeogenesis by isoleucine involves an insulin-independent signal pathway, as well as a competitive effect and an insulinotropic effect of isoleucine in vivo.

Recent investigations have revealed that the energy excess derived from amino acids activates mTOR and/or S6K1, which leads to insulin resistance (20, 21, 35). However, the single amino acid isoleucine decreases blood glucose through a simultaneous increase in glucose utilization that actually improves glucose metabolism and energy states of both the muscle and liver (Fig. 8). Although the molecular basis of these findings remains to be determined, elucidation of the associations related to these factors when using isoleucine may provide new insights into the role of amino acids on metabolic disorders related to insulin resistance.

In conclusion, our study revealed that isoleucine lowers blood glucose levels by both stimulation of the muscle glucose uptake and an increase in glucose oxidation within the whole body without significant elevation of plasma insulin levels. Our study also showed that isoleucine reduces hepatic glucose production in vitro and the expression and activity of hepatic gluconeogenic enzymes both in vitro and in vivo. By achieving a better understanding of the mechanism of isoleucine's activity, this may potentially provide important information relevant to the effects of amino acids on glucose metabolism.

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