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# Isoleucine, a Blood Glucose-Lowering Amino Acid, Increases Glucose Uptake in Rat Skeletal Muscle in the Absence of Increases in AMP-Activated Protein Kinase Activity

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ABSTRACT Leucine and isoleucine were shown to stimulate insulin-independent glucose uptake in skeletal muscle cells in vitro. In this study, we examined the effects of leucine and isoleucine on blood glucose in food-deprived rats and on glucose metabolism in skeletal muscle in vivo. Furthermore, we investigated the possible involvement of the energy sensor, 5′-AMP-activated protein kinase (AMPK), in the modulation of glucose uptake in skeletal muscle, which is independent of insulin, and also in leucine- or isoleucine-stimulated glucose uptake. Oral administration of isoleucine, but not leucine, significantly decreased the plasma glucose concentration. An i.v. bolus of 2-[1,2-$^3$H]-deoxyglucose (2-[$^3$H]DG) was administered to calculate glucose uptake. Glucose uptake in the skeletal muscle did not differ after leucine administration, but glucose uptake in the muscles of rats administered isoleucine was 73% greater than in controls, suggesting that isoleucine increases skeletal muscle glucose uptake in vivo. On the contrary, in the skeletal muscles, administration of leucine but not isoleucine significantly increased [U-$^{14}$C]-glucose incorporation into glycogen compared with controls. AMPK $\alpha$1 activity in skeletal muscle was not affected by leucine or isoleucine administration. However, isoleucine, but not leucine, significantly decreased AMPK $\alpha$2 activity. The decrease in AMPK $\alpha$2 activity was thought to be due to decreases in AMP content and the AMP:ATP ratio, which were related to the isoleucine administration. This is the first report of isoleucine stimulating glucose uptake in rat skeletal muscle in vivo, and these results indicate that there might be a relation between the reduction in blood glucose and the increase in skeletal muscle glucose uptake that occur with isoleucine administration in rats. The alterations in glucose metabolism caused by isoleucine may result in an improvement of the availability of ATP in the absence of increases in AMP-activated protein kinase activity in skeletal muscle. J. Nutr. 135: 2103–2108, 2005.

KEY WORDS: • *isoleucine* • *leucine* • *glucose* • *muscle* • *AMP-activated protein kinase*

The BCAAs, leucine, isoleucine, and valine, are essential for normal growth and development. The importance of BCAAs as nutrient regulators of protein synthesis was recognized >20 years ago. Of the BCAAs, leucine appears to be the specific effector on protein synthesis in several tissues including skeletal muscle, liver, and adipose tissue (1–3). The stimulatory effect of leucine on protein synthesis occurs at the level of translation initiation and involves signaling through the mammalian target of rapamycin (mTOR).[2]

Of the BCAAs, only leucine is a key regulator of protein synthesis via the mTOR (2–6). Furthermore, leucine inhibits autophagic proteolysis via the mTOR-independent pathway (7). Recently, we reported that isoleucine, a positional isomer of leucine, prevents a rise in the plasma glucose concentration, and that the effect of isoleucine is greater than that of leucine or valine in an oral glucose tolerance test in normal rats (8). In $C_2C_{12}$ myotubes, leucine and isoleucine stimulate glucose uptake in an insulin-independent manner, and the effect of isoleucine is greater than that of leucine (8). In such cells, the signaling pathway analysis using a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), a protein kinase C (PKC) inhibitor (GF109203X), and an mTOR inhibitor (rapamycin) suggests that PI3K and PKC, but not mTOR, are involved in the enhancement of glucose uptake by isoleucine. These data suggest that isoleucine assumes the role of a signal for glucose metabolism, thereby stimulating insulin- and mTOR-independent glucose transport in cultured skeletal muscle cells. This may be part of the mechanism for the blood glucose-lowering effect of isoleucine in rats administered glucose. However, it remains unclear whether isoleucine decreases the plasma glucose concentration in food-deprived rats, or increases glucose uptake in the skeletal muscles. Furthermore, both the precise

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[2] Abbreviations used: AMPK, 5′-AMP-activated protein kinase; 2DG, 2-deoxy glucose; 2-[$^3$H]DG, 2-[1,2-$^3$H]-deoxyglucose; 2-[$^3$H]DGP, 2-[1,2-$^3$H]-deoxyglucose-6-phosphate; mTOR, mammalian target of rapamycin; NEFA, nonesterified fatty acid; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; wt, weight.


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mechanism and the signaling pathway associated with this isoleucine-stimulated glucose lowering remain unknown.

Recent studies showed that 5′-AMP-activated protein kinase (AMPK) is a key regulator of cellular pathways that consume and generate cellular energy (9). AMPK is activated by elevation of the cellular AMP content, either by interfering with ATP production or by increasing ATP consumption (10). Hence, AMPK acts as an energy sensor that leads to contraction-stimulated glucose transport in skeletal muscle (11,12). AMPK is a serine/threonine kinase consisting of a catalytic subunit ($\alpha$) and 2 regulatory subunits ($\beta$ and $\gamma$). The catalytic $\alpha$ subunit occurs in 2 distinct isoforms in mammals. AMPK $\alpha$1 is widely expressed, whereas the $\alpha$2 isoform is expressed predominantly in the liver, heart, and skeletal muscle (13). The AMPK $\alpha$2 activity is much more dependent on the cellular AMP content than AMPK $\alpha$1 activity (14). AMPK mediates an increase in glucose uptake by stimulating glucose transporter 4 translocation to the plasma membrane (15) and by a similar insulin-independent signaling mechanism (16,17). At present, the role of the isoleucine-signaling pathway in the mediation of the AMPK activity in skeletal muscle is unknown.

This study was designed to examine whether leucine or isoleucine increased glucose uptake in rat skeletal muscle in vivo. We used 2-[1,2-$^3$H]-deoxyglucose (2-[$^3$H]DG) and [U-$^{14}$C]-glucose to examine the effects of leucine and isoleucine on glucose metabolism in skeletal muscle in vivo. To determine whether the glucose uptake occurred via an AMPK-mediated mechanism, we also measured the activity of AMPK $\alpha$1 and $\alpha$2, and the contents of adenine nucleotides (ATP, ADP, and AMP) in skeletal muscle from rats administered leucine or isoleucine.

## MATERIALS AND METHODS

***Animals and experimental design.*** Male Wistar rats (3 wk old; Clea Japan) were maintained under conditions of constant humidity and temperature (22 ± 2°C) and a 12-h light:dark cycle (0700–1900 h; lights on at 0700 h). They were fed a standard AIN-93G diet (18) (Nosan) for 10 d, and water was freely available. The 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, and the Guidelines for Animal Experiments of Utsunomiya University, Utsunomiya University Animal Research Committee. In all experiments, rats were food deprived for 18 h and then administered saline (0.155 mol/L), 1.35 g L-leucine/kg body weight (wt) (prepared as 54.0 g/L of the L-amino acid in distilled water) or 1.35 g L-isoleucine/kg body wt by oral gavage, as previously described (2). The volume of saline, leucine, or isoleucine administered was 2.5 mL/100 g body wt.

***Sample collection.*** All sampling for the measurement of the integral of the plasma 2-[$^3$H]DG and [U-$^{14}$C]-glucose concentrations was performed by tail bleeding at 5, 10, 20, 30, and 40 min after the tracer boluses, which corresponded to the disappearance period from the plasma of 2-[$^3$H]DG and [U-$^{14}$C]-glucose. Because of the marked initial decay of the 2-[$^3$H]DG and [U-$^{14}$C]-glucose in the blood, the bulk of 2-[$^3$H]DGP and [U-$^{14}$C]-glycogen are synthesized in the muscles during the 40-min labeling period (19,20). Exactly 1 h after oral administration, the rats were anesthetized with sodium pentobarbital (40 mg/kg body wt), and blood was collected via the abdominal aorta for measurement of the plasma concentrations of glucose, insulin, nonesterified fatty acid (NEFA), leucine, isoleucine, and valine. The gastrocnemius muscles as a unit were rapidly excised. For measurement of the AMP:ATP ratio, 1 portion of the muscle was frozen in liquid nitrogen, and then stored at −80°C until the assay was performed. The remaining portion of the muscle was weighed and homogenized in 7 volumes of ice-cold buffer A consisting of 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 50 mmol/L NaF, 5mmol/L sodium pyrophosphate, 1 mmol/L EDTA, 5 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.1 mmol/L benzamidine, 0.1 mmol/L phenylmethylsulfonyl fluoride, 4 mg/L leupeptin, 1 $\mu$mol/L microcystin, 1% Triton X-100, and 5 mg/L soybean trypsin inhibitor. Homogenates were centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was used for measurement of AMPK activity as described below.

***Plasma measurement.*** The blood samples for the measurements of the plasma concentrations of glucose, insulin, and NEFA were collected 1 h after administration. Plasma glucose concentration was measured using the glucose oxidase method (Glucose CII Test-Wako; Wako Pure Chemical Industries), and plasma insulin concentration was measured by a double antibody enzyme immunoassay, which used rat insulin as a standard (Rat Insulin ELISA Kit; Shibayagi). Plasma NEFA concentration was measured using the acyl-CoA oxidase method (NEFA C Test-Wako; Wako Pure Chemical Industries). An automatic amino acid analyzer (JLC-300; JEOL) was used to measure the plasma concentrations of the amino acids after sulfosalicylic acid treatment (final concentration 1.5%).

***Glucose uptake in gastrocnemius muscle.*** In all experiments, 2-[1,2-$^3$H]-deoxyglucose-6-phosphate (2-[$^3$H]DGP) accumulation in the tissue was measured as described previously (21). Exactly 20 min after oral administration of saline, leucine or isoleucine, a bolus i.v. injection of 30 $\mu$Ci/kg body wt of 2-[$^3$H]DG (Amersham Bioscience) was given to rats. The rats were anesthetized with sodium pentobarbital 40 min after the tracer injection (1 h after saline, leucine, or isoleucine administration), and the gastrocnemius muscles were excised for measurement of 2-[$^3$H]DGP in the muscles. Tissues were analyzed for accumulation of 2-[$^3$H]DG and 2-[$^3$H]DGP content, as described previously (19). Briefly, muscle 2-[$^3$H]DG concentrations were determined in 500 mg of gastrocnemius muscle. The tissue was dissolved in 2 mL of 1 mol/L NaOH at 55°C for 60–120 min and then neutralized with 2 mL of 1 mol/L hydrochloric acid. Neutralized tissue extract (400 $\mu$L) was deproteinized in 800 $\mu$L of 6% perchloric acid and centrifuged at 5000 × *g* for 20 min. The supernatant (900 $\mu$L) count gave the total radioactivity for 2-[$^3$H]DG + 2-[$^3$H]DGP. In addition, 400 $\mu$L of the neutralized tissue extract was treated with 400 $\mu$L of 0.15 mol/L $ZnSO_4$ and 400 $\mu$L of 0.15 mol/L $Ba(OH)_2$ and centrifuged at 5000 × *g* for 20 min. Because the treatment with $ZnSO_4$ and $Ba(OH)_2$ removed everything except 2-[$^3$H]DG, the count of this supernatant (900 $\mu$L) gave the radioactivity of 2-[$^3$H]DG. Accumulation of 2-[$^3$H]DGP in gastrocnemius muscle was then calculated as the difference between the total (2-[$^3$H]DG + 2-[$^3$H]DGP) and the 2-[$^3$H]DG radioactivity.

***[U-$^{14}$C]-glucose incorporation into glycogen in gastrocnemius muscle.*** [U-$^{14}$C]-glycogen in tissue was measured, as previously described (20,22). Exactly 20 min after oral administration of saline, leucine, or isoleucine, an i.v. injection of 50 $\mu$Ci/kg body wt of [U-$^{14}$C]-glucose (Amersham Bioscience) was given to rats as a bolus. The rats were anesthetized with sodium pentobarbital 40 min after the tracer injection, and the gastrocnemius muscles were excised for measurement of [U-$^{14}$C]-glucose incorporation into glycogen in the muscles. Muscles were dissolved by heating for 1 h at 100°C in 30% KOH before glycogen (final concentration 0.5%) and then ethanol (final concentration 70%) were added. After 8 h at −20°C, the samples were centrifuged at 5000 × *g* for 30 min. The glycogen pellets were washed 4 times with 70% ethanol and dissolved with water. Samples were spotted on filter paper (Whatman 31ET), which were then washed 5 times with 70% ethanol. The papers were dried before the amount of [U-$^{14}$C]-glycogen was determined by scintillation counting.

***HPLC analysis of adenine nucleotide contents in skeletal muscle.*** Frozen tissues were broken into pieces on a cold plate at −80°C, and 0.5 g of tissues pieces was transferred to a 15-mL tube, to which 5 mL of ice-cold 0.5 mol/L perchloric acid/g wet wt was added. The tissues were immediately homogenized with a homogenizer (Polytron; Kinematica AG). The homogenates were centrifuged at 1500 × *g* at 4°C for 20 min. The supernatants were neutralized with 140 $\mu$L of neutralizing agent (0.5 mol/L triethanolamine, 2.0 mol/L $K_2CO_3$) added to 1 mL of the supernatant, kept on ice for 10 min, and then centrifuged at 1500 × *g* at 4°C for 20 min. Supernatants were stored at −80°C until HPLC was performed using a Waters Alliance 2695

system (2487 Dual λ absorbance detector; Empower chromatographic manager; Waters). Measurements of ATP, ADP, and AMP in gastrocnemius muscle were performed as described previously (23). An aliquot (100 μL) of each sample was passed through a Unison C18 column (150 × 4.6, 3-μm particle size; Imtakt). Detection of absorbance occurred at 260 nm, and the flow rate was set at 0.7 mL/min. A gradient was initiated using 2 buffers, in which buffer B consisted of 25 mmol/L $NaH_2PO_4$, and PIC-A reagent (Waters) at pH 5, and buffer C consisted of a mixture of 10% (v:v) acetonitrile, 200 mmol/L $NaH_2PO_4$, and PIC-A reagent (Waters) at pH 4. The buffers were filtered through a 0.45-μm filter (Pall) and degassed in a flask linked to a vacuum pipe. The gradient used in the HPLC assay was changed from 100% buffer B to 100% buffer C from 0 to 30 min, and then 100% buffer B from 31 to 40 min for column reequilibration. Peaks were identified from their retention times and by chromatography of standards.

***AMPK assay.*** Isoform-specific AMPK α1 and α2 activities were measured. Supernatants (150 μg) of muscle homogenate were subjected to immunoprecipitation with specific antibodies to the α1 or α2 catalytic subunits of AMPK (Bethyl Laboratories) and BioMag goat anti-rabbit beads (Qiagen). Immunoprecipitates were washed twice in buffer A (plus 1 mol/L NaCl and 1% Triton X-100) and then twice in buffer A alone. Kinase reactions were performed as described previously (24).

***Calculations.*** Glucose uptake ($R_g$) was calculated from the muscle accumulation of 2-[$^3$H]DGP, the integral of the plasma 2-[$^3$H]DG concentration after a 2-[$^3$H]DG bolus, and the plasma glucose concentration. The relation was defined as follows:

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

where $t$ = 20–60 min after the administration of the amino acids, $(G)_p$ was the average arterial plasma glucose concentration (mmoL/L) from $t$ = 20 to 60 min, and $(2\text{-}[^3H]DGP)_m$ was the accumulation of 2-[$^3$H]DGP in the muscles at $t$ = 40 min (Bq/mg wet wt). $(2\text{-}[^3H]DG)_p$ represents the plasma 2-[$^3$H]DG concentration (Bq/mL); at the point at which the tracer bolus was administered, $t$ was set to $t$ = 0. The measurement of $R_g$ was described previously (21,25,26). Incorporation of [U-$^{14}$C]-glucose into glycogen was calculated essentially as described for $R_g$ except that $(2\text{-}[^3H]DGP)_m$ represented [U-$^{14}$C]-glycogen in muscle (Bq/mg wet wt) 40 min after the administration of the tracer, and $(2\text{-}[^3H]DG)_p$ represented the plasma [U-$^{14}$C]-glucose concentration (Bq/mL) (20).

***Statistical analysis.*** Tests for statistical significance were performed using SAS software (version 7.0; SAS Institute). Significant differences between the control, leucine, and isoleucine treatment groups were assessed by 1-way ANOVA followed by the Tukey-Kramer multiple comparisons test. The accepted level of significance was a $P$-value $< 0.05$. Values are given as means ± SEM.

## RESULTS

***Animal characteristics.*** Food-deprived male Wistar rats weighing 103–127 g were used in the study. The body weights of the control (112 ± 2 g), leucine (110 ± 2 g), and isoleucine groups (112 ± 2 g) did not differ.

***Plasma concentrations of glucose, insulin, and NEFA.*** The administration of leucine did not alter plasma glucose concentration compared with controls. In contrast, isoleucine decreased the plasma glucose concentration ($P < 0.05$; **Table 1**) compared with controls. Oral administration of leucine or isoleucine did not alter the plasma insulin concentrations at 1 h after administration (Table 1). Although plasma insulin concentrations in rats administered leucine temporarily increased at 30 min after administration, the plasma glucose concentration was not altered at 30 min (data not shown). On the other hand, isoleucine administration in the rats did not affect plasma glucose or insulin concentrations at 30 min (data not shown). Plasma NEFA concentrations were not affected by the administration of leucine or isoleucine at 1 h after administration (Table 1).

**TABLE 1**

*Plasma concentrations of glucose, insulin and NEFA in rats 1 h after administration of saline, leucine, or isoleucine*[1]

| Treatment group | Glucose | Insulin | NEFA |
|---|---|---|---|
| | *mmol/L* | *pmol/L* | *mmol/L* |
| Control | 6.18 ± 0.16[a] | 91.0 ± 20.8 | 0.879 ± 0.051 |
| Leucine | 5.67 ± 0.36[ab] | 96.2 ± 12.6 | 0.756 ± 0.060 |
| Isoleucine | 4.69 ± 0.27[b] | 100.1 ± 10.1 | 0.710 ± 0.037 |

[1] Value are means ± SEM, $n$ = 5. Means in a column with superscripts without a common letter differ, $P < 0.05$.

***Plasma concentration of leucine, isoleucine, and valine in normal rats.*** One hour after oral administration of leucine, the plasma leucine concentration was significantly increased to 1844 ± 228 μmol/L. In rats administered isoleucine at the same dose, the plasma isoleucine concentration was significantly elevated to 4352 ± 160 μmol/L, more than twice the increase in leucine in the leucine-treated rats (**Table 2**). Additionally, leucine administration reduced plasma concentrations of isoleucine and valine to less than half of that in control rats. In contrast, administration of isoleucine did not affect the plasma concentrations of leucine and valine. These changes can be explained by 2 different mechanisms. First, leucine is unique among the BCAAs in its ability to stimulate protein synthesis in vivo (3). Provision of leucine stimulates protein synthesis in skeletal muscle. In contrast, the administration of isoleucine is ineffective at stimulating protein synthesis. The reduction of plasma concentrations of isoleucine and valine after leucine administration may reflect an increase in the uptake of these amino acids to support the enhanced rates of protein synthesis. Second, leucine has a specific stimulating effect on BCAA catabolism in rats (27). The unique effects of leucine compared with isoleucine and valine on BCAA catabolism (28) could explain the decrease in plasma isoleucine and valine concentrations produced by excess leucine intake.

***Effects of leucine and isoleucine on glucose uptake in skeletal muscles in rats.*** Measurement of glucose uptake in vivo using 2-[$^3$H]DG relies upon the assumption that the phosphorylated form, 2-[$^3$H]DGP, cannot be further metabolized and cannot be countertransported to the extracellular space. The $R_g$ in the skeletal muscle of rats administered leucine did not differ from that in control rats (**Table 3**). However, the $R_g$ in the skeletal muscle of rats administered isoleucine was significantly greater than that in the control rats (Table 3). Also, the $R_g$ values tended to be increased compared with that in rats administered leucine ($P$ = 0.10). These data indicate that isoleucine increases skeletal muscle glucose uptake in vivo.

***Effects of leucine and isoleucine on [U-$^{14}$C]-glucose incorporation into glycogen in the skeletal muscles in rats.*** The administration of leucine increased [U-$^{14}$C]-glucose incorporation into glycogen ($P < 0.05$; Table 3) compared with control rats. On the other hand, the [U-$^{14}$C]-glucose incorporation into glycogen in the skeletal muscle did not differ between rats administered isoleucine (Table 3) and control rats.

***Effects of leucine or isoleucine on AMPK activity in skeletal muscles.*** The activation of AMPK leads to insulin-

**TABLE 2**

*Plasma concentrations of leucine, isoleucine, and valine in rats 1 h after administration of saline, leucine, or isoleucine*[1]

| Treatment group | Leucine | Isoleucine | Valine |
|---|---|---|---|
| | | *μmol/L* | |
| Control | 144 ± 14[b] | 108 ± 5[b] | 161 ± 11[a] |
| Leucine | 1844 ± 228[a] | 51 ± 4[b] | 66 ± 8[b] |
| Isoleucine | 140 ± 9[b] | 4352 ± 160[a] | 192 ± 9[a] |

[1] Value are means ± SEM, $n = 6$. Means in a column with superscripts without a common letter differ, $P < 0.001$.

independent glucose transport in the skeletal muscle (16,17). In this study, we examined whether isoleucine increases glucose uptake via an AMPK-mediated mechanism. The activity of the AMPK α1 isoform was unchanged in rats administered leucine and isoleucine compared with control rats (**Table 4**). The activity of the AMPK α2 isoform was also unchanged in rats administered leucine compared with control rats (Table 4). In contrast, AMPK α2 activity was decreased in rats administered isoleucine ($P < 0.05$; Table 4) compared with control rats.

***Effects of leucine or isoleucine on adenine nucleotide contents and the AMP/ATP ratio in skeletal muscle.*** After the discovery that isoleucine decreases AMPK α2 activity in skeletal muscle, we measured the contents of high-energy phosphate metabolites (AMP, ADP, and ATP) in the skeletal muscles of rats administered leucine and isoleucine to evaluate the cellular energy state. Oral administration of leucine or isoleucine did not alter the ADP or ATP contents in the skeletal muscle compared with control rats (**Table 5**). However, isoleucine caused a 21% decrease in AMP content in skeletal muscle compared with the control ($P < 0.05$) and leucine ($P < 0.05$) groups; however, the AMP content was not affected after administration of leucine compared with the control group. Furthermore, although leucine did not change the AMP:ATP ratio, isoleucine caused a significant 20% decrease in this ratio ($P < 0.05$) in the skeletal muscle compared with control groups. The decrease in the AMP concentration and the AMP:ATP ratio in the skeletal muscle of rats administered isoleucine corresponded to the decrease in activity for AMPK α2 after the administration of isoleucine.

**TABLE 3**

*Effect of leucine or isoleucine on glucose uptake ($R_g$) and [U-$^{14}$C]-glucose incorporation into glycogen in gastrocnemius muscles in rats 1 h after administration of saline, leucine, or isoleucine*[1]

| Treatment group | Glucose uptake ($R_g$) | [U-$^{14}$C]-Glucose incorporation into glycogen |
|---|---|---|
| | *μmol/(100 g · min)* | |
| Control | 21.7 ± 4.1[b] | 2.59 ± 0.43[b] |
| Leucine | 25.7 ± 3.7[ab] | 4.52 ± 0.22[a] |
| Isoleucine | 37.5 ± 3.4[a] | 3.09 ± 0.27[ab] |

[1] Value are means ± SEM, $n = 6$ for $R_g$; $n = 4$ for [U-$^{14}$C]-glucose. Means in a column with superscripts without a common letter differ, $P < 0.05$.

**TABLE 4**

*Activity of the AMPK α1 and α2 isoform of the gastrocnemius muscles in rats 1 h after administration of saline, leucine, or isoleucine*[1]

| Treatment group | AMPK α1 | AMPK α2 |
|---|---|---|
| | *% of control* | |
| Control | 100.0 ± 10.3 | 100.0 ± 9.5[a] |
| Leucine | 97.4 ± 2.5 | 97.0 ± 7.6[ab] |
| Isoleucine | 91.3 ± 5.1 | 71.9 ± 5.1[b] |

[1] Value are means ± SEM, $n = 6$. Means in a column with superscripts without a common letter differ, $P < 0.05$.

## DISCUSSION

As previously reported, isoleucine increased glucose uptake both in the absence and presence of insulin on cultured muscle cells (8). The previous study also showed that leucine and valine did not decrease blood glucose during an oral glucose tolerance test in vivo, in contrast to isoleucine, which significantly decreased blood glucose (8). Our data demonstrated that isoleucine stimulated glucose uptake in the skeletal muscle in vivo (Table 3) and lowered blood glucose in rats without increasing plasma insulin (Table 1). However, leucine did not affect the glucose uptake and blood glucose despite the increase in plasma insulin at 30 min. This is the first report of isoleucine stimulating glucose uptake in rat skeletal muscle in vivo.

Various studies showed that elevations of plasma amino acids after administration of an amino acid mixture induce insulin resistance in skeletal muscle, the so-called amino acid-induced insulin resistance, by inhibiting glucose transport and glucose phosphorylation in humans (29,30). In this study, although isoleucine administration led to a high plasma concentration (Table 2), isoleucine lowered blood glucose and stimulated glucose uptake in rat skeletal muscle in vivo without increasing plasma insulin. These data indicate that isoleucine, compared with an amino acid mixture, has a different effect on glucose metabolism in vivo.

Previous reports suggested that increased AMPK activity induces glucose uptake independently of insulin, leading to decreased plasma glucose concentrations (31). Activation of AMPK inactivates protein kinase B/mTOR signaling, resulting in decreased p70S6k phosphorylation and protein synthesis in skeletal muscle (32). Furthermore, AMPK blocks amino acid–induced activation of acetyl-CoA carboxylase and p70S6k (33). Therefore, to examine the correlation between AMPK activity and isoleucine-stimulated glucose uptake, the effects of isoleucine on AMPK α1 and α2 activities in skeletal muscle were investigated in food-deprived rats administered oral isoleucine. Contrary to our expectations, AMPK α1 activity was unchanged in rats administered leucine and isoleucine compared with control rats, and AMPK α2 activity was significantly decreased in rats administered isoleucine compared with control rats. These results indicate that isoleucine-stimulated glucose uptake is increased in the absence of increases in AMPK α1 and α2 activities in skeletal muscle in food-deprived rats. The decreases in the AMP concentration and the AMP:ATP ratio in the skeletal muscle of the rats administered isoleucine corresponded to the decrease in activity for AMPK α2 after the administration of isoleucine. We assume that a depletion of cellular AMP would result in a

**TABLE 5**

*Effects of leucine or isoleucine on adenine nucleotide contents and the AMP:ATP ratio in gastrocnemius muscles of rats 1 h after administration of saline, leucine, or isoleucine*[1]

| Treatment group | AMP | ADP | ATP | AMP:ATP |
|---|---|---|---|---|
| | | *μmol/g* | | |
| Control | 0.107 ± 0.004[a] | 0.99 ± 0.03 | 8.05 ± 0.21 | 0.0133 ± 0.0005[a] |
| Leucine | 0.107 ± 0.007[a] | 1.05 ± 0.03 | 8.77 ± 0.26 | 0.0121 ± 0.0006[ab] |
| Isoleucine | 0.084 ± 0.005[b] | 0.95 ± 0.03 | 8.00 ± 0.27 | 0.0106 ± 0.0008[b] |

[1] Value are means ± SEM, $n = 5$. Means in a column with superscripts without a common letter differ, $P < 0.05$.

decrease in the AMP:ATP ratio and improve availability of ATP in the skeletal muscle without any remarkable increase in the ATP concentration, thereby resulting in an improvement in the cellular energy state. Hence, the results of our study suggest that isoleucine administration increased glucose uptake in the muscle and, as a result, the AMP concentration decreased, leading to a decrease in the AMP:ATP ratio and AMPK activity.

Leucine stimulates glycogen synthesis as a result of the inactivation of glycogen synthase kinase-3 in L6 muscle cells in a manner that is dependent on mTOR and independent of insulin (34). We reported previously that leucine causes a significant increase of D-[U-$^{14}$C]-glucose incorporation into the intracellular glycogen in the myotube cells in vitro, whereas isoleucine does not affect glycogen synthesis compared with that in controls (8). Furthermore, the present study demonstrates that muscle glycogen synthesis, as determined by [U-$^{14}$C]-glucose incorporation into glycogen, was significantly increased by administration of leucine but not isoleucine in the rat skeletal muscles in vivo compared with control rats. Although leucine has less effect on glucose uptake in skeletal muscle, it stimulates protein synthesis (2) and glycogen synthesis without affecting the AMP concentration, the AMP:ATP ratio, or AMPK activity in skeletal muscle. In contrast, isoleucine stimulates glucose uptake and improves the cellular energy state in skeletal muscle, although it has less effect on protein synthesis and glycogen synthesis (2,8). For fatty acid metabolism, plasma NEFA concentration was not affected in control rats, or rats administered isoleucine or leucine (Table 1). Because the source of this increased glycogen by leucine is still unknown, further investigations are required to elucidate the reason behind this change. On the basis of these results, we speculate that leucine signaling promotes anabolism of protein and glycogen in skeletal muscle. In contrast, isoleucine signaling accelerates catabolism of incorporated glucose for energy production and consumption via insulin-, mTOR-, and AMPK-independent signaling pathways without affecting the net glycogen synthesis. Therefore, we conclude that not all amino acids negatively modulate glucose utilization in vivo, and that individual amino acids have differing effects on glucose uptake due to their effect on different signal pathways within the glucose metabolic pathways.

In examining the signaling pathway for isoleucine-stimulated glucose uptake, our results suggest that the signal for activation and inactivation of AMPK is not upstream of the signal for glucose uptake. Therefore, the signal downstream of PI3K in the isoleucine-stimulated glucose uptake remains unknown. On the other hand, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid, a system L-specific amino acid transport inhibitor (35), does not affect isoleucine-stimulated glucose uptake in skeletal muscle cells (M. Doi, unpublished data). Thus, we presume that an as yet unknown signaling pathway exists and that may include the presence of isoleucine-binding proteins or receptors through which signaling for insulin-independent glucose uptake can occur.

In conclusion, administration of isoleucine leads to an increase in glucose uptake in skeletal muscle in vivo. This effect, however, does not occur via an AMPK-mediated mechanism. Although further studies are required to determine the mechanism of enhanced glucose uptake with isoleucine, isoleucine signaling is more effective than leucine signaling for glucose uptake in vivo, and occurs through an unknown signaling pathway that is independent of insulin.

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