Adverse Effects of Excessive Leucine Intake Depend on Dietary Protein Intake: A Transcriptomic Analysis to Identify Useful Biomarkers

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Summary The present study was conducted to identify reliable gene biomarkers for the adverse effects of excessive leucine (Leu) in Sprague-Dawley rats by DNA microarray. It has long been known that the adverse effects of excessive amino acid intake depend on dietary protein levels. Male rats were divided into 12 groups (n = 6) and fed for 1 wk a diet containing low (6%), moderate (12%) or high (40%) protein. Different levels of Leu (0, 2, 4, and 8%) were added to the diets. Consumption of diets containing more than 4% Leu in 6% protein resulted in growth retardation and reduced liver weight, whereas the administration of the same dose of Leu with 12% or 40% protein did not affect them. By a process of systematic data extraction, 6 candidate gene markers were identified. The liver gene expression data obtained from another experiment with 0, 2, 3, 4, and 8% Leu in a low-protein diet was used to examine the validity of these biomarker candidates with receiver operating characteristic (ROC) curve analysis. All of AUC values of the biomarker candidates were more than 0.700, suggesting the effectiveness of the marker candidates as the indices of Leu excess. The cut-off value for the ROC curve of the gene-marker panel, which was obtained by multiple regression analysis of gene markers, indicated that Leu levels higher than 3% have adverse effects. In conclusion, the gene-marker panel suggested that for male rats dietary Leu supplementation of 2% is the NOAEL dose in low-protein (6%) diets.

Key Words leucine, transcriptome, biomarker, DNA microarray, dietary protein

Amino acids have recently gained popularity as dietary supplements since the functions of each amino acid have been revealed, which include the promotion of health and the enhancement of sport performance (1). In this trend, the possibility of excessive intakes of a single amino acid has become an issue to be considered. At least in experimental animals, it is well known that the excessive intake of a single amino acid leads to adverse outcomes such as growth inhibition (2–4). It is therefore important to elucidate the mechanisms underlying the toxic effects of amino acid excess, which should provide bases for the identification of an upper limit (UL) intake level of each amino acid.

Studies addressing beneficial or adverse effects of nutrients and other food components often rely on the use of biomarkers that reflect biological responses. For example, the change of Gpx1 (glutathione peroxidase 1) mRNA expression in liver and blood is an efficient biomarker reflecting selenium deficiency; therefore Gpx1 mRNA is used to determine dietary selenium requirements (5, 6). Since changes of gene expression are usually early biological events manifested by dietary stimulation, mRNA biomarkers (gene biomarkers) are likely to be effective tools to assess adverse effects caused by excessive intakes of nutrients including amino acids. Gene markers can then be used to determine the NOAEL and UL. Owing to recent advancement of DNA microarray technology, the expression of whole sets of mRNA in target organs or cells can be simultaneously determined. Thus, DNA microarray is a very efficient tool for the discovery of marker genes.

Leu is one of branched chain amino acids and it has been reported to have a role as a signal factor to activate protein synthesis and inhibit protein degradation in ani-
With the expectation of such effects, Leu, as well as other branched chain amino acids (Ile and Val), is taken by athletes in relatively high amounts. Although supplemental intake of amino acids is considered to be safe without any side effects, excessive Leu has an adverse effect on growth performance or the immune system in rats, chicks, pigs, and lambs (2, 9–11). However, the mechanism underlying the toxicities remains to be elucidated. The present study was conducted to identify gene expression markers reflecting excessive intake of Leu and to gain insights into the safe and toxic doses of Leu supplementation.

It has long been known that the occurrence of adverse effects of excessive single amino acid intake depends on dietary protein levels (2, 4). Namely, adverse effects of an excessive amino acid tend to be more prominent under dietary conditions of lower protein content than those of sufficient protein content. For these reasons, the estimation of the UL of each single amino acid remains difficult. The object of this study was using DNA microarray analysis to search for effective biomarkers related to the adverse effects caused by excessive Leu, which would provide a basis for the estimation of UL.

**METHODS**

**Animals and diets.**

Experiment 1: Seventy-two 10-wk-old male Sprague-Dawley rats (Japan SLC, Inc., Japan) were individually

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**Table 1. Composition of diets.**

<table>
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<tr>
<th>LP groups (6% casein)</th>
<th>Leucine (%)</th>
<th>0 (LP0)</th>
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<th>4 (LP4)</th>
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<tr>
<td>Mineral mix (AIN-93G) (g)</td>
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<td>Total (g)</td>
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<td>1,000</td>
<td>1,000</td>
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</table>

1 The diets are generally based on the AIN-93G formulation.
2 l-Leucine was generously provided by Ajinomoto Co., Inc., Japan.
3 Cysteine concentrations were adjusted for protein concentration.
Gene Markers for Leucine Excess

housed in stainless-steel cages and maintained on a 12-h light: dark cycle at 23±2°C, and at 55±10% humidity. Rats were acclimated for 3 or 4 d and provided ad libitum access to a 20% casein diet based on the AIN-93G diet. Water was provided for 1 wk. The composition of the experimental diets is shown in Table 1. After a week of feeding, the rats were anesthetized by intraperitoneal administration of pentobarbital (50 mg/mL, 100 μL/100 g BW) after 4 h of fasting, and killed. Fresh livers were weighed and then portions of the liver were soaked in RNAlater (Ambion Inc., Austin, TX) for collecting total RNA. The protocol of experiments received prior approval by the animal experiment committees of Kyoto Prefectural University and the University of Tokyo.

Experiment 2: Thirty 10-wk-old male Sprague-Dawley rats (Japan SLC, Inc.) were acclimatized to the same conditions as described above and were assigned to 5 groups (n=6). They were then fed the diet containing 6% casein supplemented with five levels of Leu (0, 2, 3, 4, and 8%). Experimental conditions except for the inclusion of an additional Leu level (3%) were the same as in Experiment 1.

Isolation of liver RNA and DNA microarray analysis. Liver total RNA was extracted from the homogenized liver samples using TRIzol Reagent (Invitrogen, USA) and the amounts and purity were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The quality of total RNA was confirmed by visualization of 18 s and 28 s rRNAs after agarose gel electrophoresis analysis.

Equal amounts of total RNA were obtained from the livers of the rats of LP0, LP2, LP4, and LP8 groups. The same amount of total RNA from five rats (the individual with largest body weight in each group was omitted in DNA microarray analysis) of each group was pooled and subjected to cDNA synthesis. The cRNAs were reverse-transcribed with T7 oligo (dT) primer and copied to ds cDNAs. Then biotin-labeled cRNAs were synthesized (Affymetrix GeneChip 3′ IVT Expression Kit, Affymetrix, USA) and fragmented by heating at 94°C for 35 min. cRNAs were hybridized to the Affymetrix GeneChip Rat Genome 230 2.0 array (Affymetrix) at 45°C for 16 h. Chips were washed and then scanned using the Affymetrix Scanner 3000 7G. Microarray Suite 5.0 (MAS5) was used to correct for background, normalize, and generate expression data with Affymetrix GeneChip Command Console Software (AgCC, Affymetrix). Expression data of the microarray were analyzed through the use of Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). On microarray data analysis, more than 0.5 or less than −0.5 of the log ratio {log2 (signal value in experimental group/signal value in control group)} value was considered as the differentially expressed genes.

Transcript filtering for identification of biomarkers. To filter the genes having the Leu dose-dependency and high sensitivity for excessive Leu intake, genes exhibiting changes at log ratio of more than 0.5 or less than −0.5 in LP4, and more than 1.0 or less than −1.0 in LP8, were selected. Then gene sets of commonly detectable molecules in human blood were selected using a IPA-Biomarker Filter, which provides ways to select biomarker candidates from the information of the specific biological characteristics in the IPA-Knowledge Base. Then gene sets were compared with the probe list of Rat Tox FX array 1.0 to select the genes related to the generation of biological toxicity and with the liver microarray data of rats subjected to 1-wk caloric restriction (13) to exclude the genes changed by reduced food intake. The relationship among liver gene-marker candidates was examined by IPA-Knowledge Base.

Quantitative RT-PCR. qRT-PCR was conducted by using The SYBR Green EX (Takara Bio, Madison, WI, USA) and a Thermal Cycler Dice Real-Time PCR System TP8000 (Takara, Japan). The primers used for the analysis of respective mRNAs were as follows:

**actin, beta (Actb)**
- Forward: 5′-TTGTCTGACAGATGACGAGA-3′
- Reverse: 5′-TGTGTTTCTCAGTTGCTG-3′

**insulin-like growth factor binding protein 1 (Igfbp1)**
- Forward: 5′-GACGTTCGTTCCGTCTCA-3′
- Reverse: 5′-CCTTGTCTACCTGCGGTCTTC-3′

**fibroblast growth factor 21 (Fgf21)**
- Forward: 5′-AGATCGAGGAGGAGGAGGACA-3′
- Reverse: 5′-AGAGGCTTTTGACACCCAGGA-3′

**inhibit (Inhbe)**
- Forward: 5′-GCCGTACATGGTGCTGCTTA-3′
- Reverse: 5′-CCAGCCTGCTTCTGACGTCTT-3′

**cyclin-dependent kinase inhibitor 1A (Cdkn1a)**
- Forward: 5′-GGCTGCCCAAGATCTACCTC-3′
- Reverse: 5′-AGTCGAGAGCGAGCAGAAG-3′

**Cyclin B2 (Ccnb2)**
- Forward: 5′-CTGCTGTCCTGCTCTC-3′
- Reverse: 5′-CATGTGCTGCTATGCTTCCA-3′

**Cyclin D1 (Cnd1)**
- Forward: 5′-AAGAAGCTGTTCTGGCTGTCG-3′
- Reverse: 5′-CTTGGAGGTGCACTACTTTG-3′

**insulin-like growth factor 1 (Igf1)**
- Forward: 5′-GACGTTCGTTCCAGGATGACGAGA-3′
- Reverse: 5′-AGATCGAGGAGGAGGAGGACA-3′

**insulin-like growth factor binding protein 3 (Igfbp3)**
- Forward: 5′-GACTCCATCCCAA-ACTGTGACG-3′
- Reverse: 5′-GCCGTATCTACTGCTGGCT-3′

**insulin-like growth factor binding protein 5 (Igfbp5)**
- Forward: 5′-TGACATGGTACAGGACAGAAGG-3′
- Reverse: 5′-TGTCGACACCATACCGAGATG-3′

**insulin-like growth factor binding protein, acid labile subunit (Igflas)**
- Forward: 5′-GGCTGGCAACAAACTGACTT-3′
- Reverse: 5′-AGATCGAGGAGGAGGAGGACA-3′

**signal transducer and activator of transcription 5A (Stat5a)**
- Forward: 5′-AGATCGAGGAGGAGGAGGACA-3′
- Reverse: 5′-GCCCTAGGTCTCTACATCC-3′
The amount of each mRNA was normalized relative to that of Actb.

Data analysis and statistics. Multiple regression analyses against body weight gain were performed for the results of qRT-PCR of the 6 liver biomarkers identified by DNA microarray and the amounts of Leu intake. The measurements of liver Igflp1, Cdkn1a, Ccnb2, Inhbe, Fgf21, Ccnd1 mRNA as well as the Leu intake index obtained by the following formula in LP and NP groups were used as an explanatory variable.

Leu intake index
=Leu content in diet (%)×food intake (g/d)/[casein content in diet (%)−casein-derived Leu content in diet (%)]

Leu content of diet
=Sum of added Leu and casein-derived Leu

Multiple regression analysis was performed by Excel-Toukei 2010 (Social Survey Research Information Co., Ltd., Japan). The Tukey-Kramer method was used for multiple comparisons to determine significant differences. Multiple comparisons were conducted within each protein level (LP, NP, and HP). The differences of means were considered significant at p<0.05. All results are expressed as the means±SE for each group.

Receiver operating characteristic (ROC) curve analysis. ROC curve analysis was used to validate the effectiveness of biomarker candidates using the data obtained in Experiment 2. More than 0.900 of AUC-ROC value was considered as high accuracy and 0.900–0.700 as moderate accuracy for growth retardation (14, 15). The cut-off point was determined by the Youden index, i.e. the point with the value of (sensitivity+specificity−1) being maximal was adopted as the cut-off point of the ROC curve (16, 17). In other words, the cut-off value is the point where the distance between the AUC curve and the putative line with AUC-ROC value of 0.500 was maximal. ROC curve analysis was performed by Excel-Toukei 2010.

RESULTS

Growth performance and liver weight

Mean body weight gain during the experimental period (final body weight−initial body weight) of each group is shown in Fig. 1. The liver weight is shown in Fig. 2. Consumption of a diet containing 6% casein and more than 4% Leu resulted in significantly lower body weight gain (vs LP0, p<0.05; Fig. 1A), whereas the administration of the same dose of Leu with 12% or 40% protein did not affect body weight gain (Fig. 1B, C). Significant decreases in total food intake were also
observed in LP4 and LP8 groups as compared with other groups (data not shown). To examine if the growth retardation was solely attributable to the reduction in food intake, a pair-feeding experiment was conducted, in which the amount of diet consumed by the LP8 group was given to the LP0 group. As the result, pair-feeding also resulted in growth retardation, although the body weight loss of LP8 was significantly greater than that of the pair-fed group (Supplemental Fig. 1A). In addition, the rate of body weight loss of the group that was pair-fed a LP8 diet supplemented with high isoleucine and valine was similar to that of the pair-fed LP0 group (Supplemental Fig. 1A, LP8IV). The difference between the pair-fed groups and LP8 group was augmented by extending the feeding period to 49 d (Supplemental Fig. 1B). A significant decrease of liver weight/body weight (%) was also observed in the LP8 (p<0.05, vs LP0; Fig. 2A), and the rats of the LP4 group showed a similar tendency (vs LP0, p=0.080). On the other hand, liver weight was not affected by Leu supplementation in NP or HP groups (Fig. 2B, C).

The screening of biomarker candidates from liver through DNA microarray analysis and multiple regression analysis

The total RNA samples obtained from the livers of LP group rats were subjected to DNA microarray analysis. The expression profile data was used to select possible biomarker genes as described in “Methods.” Briefly, genes were filtered in light of the relevance to toxicological events, changes of expression with Leu dose dependency and high sensitivity, and expression in human blood in consideration of future use as biomarkers in humans. As the result, the transcript filtering identified 6 genes; two were up-regulated (Igfbp1, Cdkn1a) and four were down-regulated (Ccnb2, Inhbe, Fgf21, Ccnd1) in Leu dose-dependent manners (Table 2). Relationship analysis using IPA-Knowledge Base identified close connection between Igfbp1 and Fgf21 and among Cdkn1a, Ccnb2, and Ccnd1 (Supplemental Fig. 2). The expression levels of these genes were then examined by qRT-PCR analyses, the results of which were highly consistent with that of DNA microarray (Fig. 3) whereas no significant differences were found among NP groups (data are not shown). As the expression level of liver Igfbp1 and Fgf21 was drastically affected by excessive Leu intake, mRNA levels of their related factors were also examined by qRT-PCR. No significant change was observed in the expression of Igf1, Igfbp3, Igf5, Igfals, Stat5a, or Stat5b by Leu supplementation (Supplemental Table 1). Functional annotation of altered genes and Fisher’s exact test indicated that the pathway relating to the metabolism of Gly, Ser and Thr and the functional group of amino acid metabolism were most significantly affected in the liver of LP-fed rats by 8% Leu (Supplemental Table 2).

Table 2. Changes of liver gene expression of biomarker candidates1 compared with rats fed 6% casein+0% Leu diet.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Affimetrix ID</th>
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<td>Ccnd1</td>
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1 Biomarker candidates were identified by IPA-Biomarker Filter analysis and the comparison of the LP8 dataset with the liver microarray data of calorie restricted rat (13), and the probe list of Rat ToxFX 1.0 Array. The analytical procedure is described in detail in the text.

2 Changes of gene expression are shown as log2 ratio, i.e. log2(signal value of experimental group/signal value of LP0).
Fig. 4. Relationship between individual biomarker panel (BP) and body weight gain. BP was obtained by multiple regression analysis with use of values of the expression of 6 genes and Leu intake index described in the text in LP and NP groups. The variance inflation factor (VIF) of all explanatory variables was less than 3.00. The contribution ratio is shown in the right bottom of the figure. The numbers following LP, NP, and HP indicate the amount of Leu supplemented to the diets (LP, 6% casein diet; NP, 12% casein diet).

Fig. 5. Body weight gain (A) and liver weight (g)/100 g body weight (B) in Experiment 2. Columns with different letters are significantly different (p<0.05). Data are presented as means±SE (n=6). NP0, NP2, NP3, NP4, and NP8 are 12% casein diets supplemented with 0, 2, 3, 4, and 8% Leu.

Fig. 6. Receiver operating characteristic (ROC) curves against growth retardation for (A) Igfbp1, (B) Cdkn1a, (C) Ccnb2, (D) Inhbe, (E) Fgf21, and (F) Ccnd1. The expression values of gene-marker candidates identified by liver transcriptome analysis in Experiment 1 and body weight gain were used for the analysis. Area under the curve (AUC-ROC) values are shown in the right bottom of each graph. Rats that lost weight during the experimental period were considered to be individuals with growth retardation when the ROC curves were drawn.
Gene Markers for Leucine Excess

To obtain a more effective marker to predict the toxicity of excessive Leu intake, multiple regression analysis using the qPCR measurements of 6 liver genes was performed. The resulting equation shown below was named the biomarker panel (BP).

\[ BP = -1.31 \times Fgf21 + 11.52 \times Ccnb2 + 13.22 \times Ccnd1 - 17.8 \times Cdkn1a - 8.84 \times Inhb - 10.9 \times Igfbp1 - 0.39 \times \text{Leu intake index} + 25.1 \]

The contribution ratio \( R^2 \) of the values of the equation generated by multiple regression analysis against body weight gain was 0.728 in Experiment 1 (Fig. 4).

Validation experiment for biomarker candidates

In Experiment 2, growth retardation and decreased liver weight per body weight were also observed (Fig. 5). Both were significantly lower in LP3, LP4, and LP8 as compared with LP0 \((p<0.05)\).

Next, hepatic mRNA levels of the 6 marker candidate genes that were identified by microarray analysis of Experiment 1 were quantified. Their responses to Leu supplementation were similar to the result of Experiment 1 and highly dose-dependent (data not shown). Their validity as biomarkers was examined by receiver operating characteristic (ROC) curve analysis against growth retardation caused by excessive Leu intake. As a result, area under the curve (AUC)-ROC values for all gene markers were more than 0.700 (Fig. 6).

BP values were calculated by applying the results of qRT-PCR of 6 gene markers of each rat in Experiment 2 to the equation obtained in Experiment 1. \( R^2 \) value between the body weight gain and the BP values was 0.742 (Fig. 7A). The AUC-ROC value of BP was very high (0.904) (Fig. 7B). The cut-off point based on the Youden index in the ROC curve of BP was determined to be \(-15.85\), and all rats in the LP8 group, and 3 and 2 rats out of 6 in LP4 and LP3, respectively, were considered to be positive (BP values were less than the cut-off value), whereas all BP values of rats fed LP0 and LP2 were negative (Fig. 7B).

**DISCUSSION**

The results of Experiment 1 and Experiment 2 showed that excessive Leu intake causes growth retardation and a decrease in relative liver weight of rats when included in a relatively low-protein diet, but not a higher-protein diet. This is consistent with research of others reporting that 7% Leu in a 4% casein diet causes growth retardation (18), and 5% Leu in a 6% casein diet, but not in a 34% casein diet, causes growth retardation (2). In Experiment 2, growth retardation was observed in rats fed 3% Leu. To our knowledge, this dose is the minimum...
Leu amount causing growth retardation in rats. So our results newly suggest that more than 3% Leu can cause growth retardation in male SD rats fed a 6% casein diet. Results of the pair-feeding experiment suggest that growth retardation caused by excessive Leu intake is not solely attributable to decreased food intake. Moreover, growth retardation caused by excessive Leu was suppressed by adding comparable amounts of Val and Ile during pair-feeding. Similarly, other studies reported the alleviation of growth retardation by adding Val and Ile to excessive Leu diets in rats (18) and chicks (19). All together, previous reports and our results suggest that the relative amount of BCAAs in the diet is the primary determinant of the adverse effects of excessive Leu. In addition, decreases in liver weight were observed in rats fed not less than 3% Leu. Similarly to other amino acids, those changes caused by excessive Leu intake were only observed under conditions of relatively low protein amounts in foods (2). Thus when considering the safe intake level of Leu, one must also consider the protein content of the diet.

In the present study, no obvious adverse effects was seen in NP or HP groups, suggesting that up to 8% Leu intake is essentially safe under conditions of moderate to high protein intakes. However, it should be noted that we have previously observed that higher supplementation of Leu (15%) to a 20% casein diet caused growth retardation and other adverse effects (20). Thus, Leu supplementation of around 8% may have a potential risk of adverse effects under moderate protein intake conditions. Hence, the use of sensitive biomarkers relating to the adverse effects caused by excessive Leu intake can be effective for more precise assessment of the safe intake level of Leu. Aiming at effective and broad screening of gene biomarkers, the expression changes by Leu under low protein conditions in which growth response was highly dependent on Leu dose was applied in this study. As the result, 6 genes which are known to be regulators of growth or the cell cycle were selected by a systematic analysis method.

IGFBP1 (insulin-like growth factor binding protein 1), known as a regulator of growth, binds to IGF-1 and inhibits IGF activity (21). It has been reported that growth retardation is observed in mice overexpressing Igfbp1 in liver (22). The result of this research revealed that the hepatic expression of Igfbp1 was higher in LP group rats fed excessive Leu, whereas the expression levels of the genes for Igf1, Igfbp3, Igfbp5, and Igfals were not changed by excess intake of Leu. IPA-core analysis revealed the amino acid metabolism to be the most significantly affected function in the livers of the LP8 group. Additionally, the aggravation of protein nutrition is known to up-regulate the expression of Igfbp1 in liver, which is thought to be a cause of growth retardation (23, 24). We have observed in hepatoma cells that addition of a disproportionate amount of a single amino acid to the culture media up-regulates Igfbp1 expression (data not shown), suggesting that the amino acid directly regulates the Igfbp1 gene. On the other hand, the expression of the IGF-1 gene is also up-regulated by deficiency of a single amino acid (25). Taken together, it could be hypothesized that the disturbance of amino acid metabolism in the liver of Leu excess may up-regulate the expression of Igfbp1, leading to growth retardation. FGF21 is a member of the FGF family, and its gene is abundantly expressed in liver (26). It has been reported that Fgfg21-transgenic mice show growth retardation with increased expression of liver Igfbp1 (27). Increased expression of Igfbp1 is also observed in mice intravenously administered FGF-21 protein (28). FGF-21 is therefore considered to be an important positive regulator of the expression of Igfbp1 in liver. However, the present study revealed that the intake of excessive Leu down-regulated the liver Fgfg21 gene. The down-regulation of Fgfg21 by excessive Leu intake is contradictory to previous observations that FGF21 is an activating factor of Igfbp1 expression and that Fgfg21 expression is up-regulated by fasting. Thus, a specific mechanism to regulate Fgfg21 expression by an amino acid may exist. Moreover, decreased expression of Fgfg21 was also observed in rats fed the 2% Leu diet during which no growth retardation was observed. These results suggest the decreased Fgfg21 expression is a response independent of the growth retardation or the increase of Igfbp1 expression. The qRT-PCR results that Igf1, Igfals, Stat5a, and Stat5b, genes known to be regulated by FGF21 (27), were not affected by Leu supplementation support this idea. Regulation of Fgfg21 gene expression by carbohydrate metabolism or lipid metabolism in liver (29–31) has been reported, while information on the regulation by amino acid metabolism is limited (32). The result of the gene expression of Fgfg21 in this study raises the possibility that protein nutritional status and amino acid metabolism in liver are novel regulators of Fgfg21 activity. Although further study is needed to elucidate the biological significance of the change of Fgfg21 by excessive Leu, the result suggests that Fgfg21 is a very sensitive and specific biomarker for amino acid excess.

Cyclin B and Cyclin D are proteins which have key functions in the cell cycle (33). It has been reported that Cyclin B binds to cyclin-dependent kinase 1 (CDK1) and acts to proceed to M-phase in the cell cycle, and that Cyclin D binds to CDK4 and activates the cells to transit from G1-phase to S-phase. The liver gene expression of Ccnb2 and Ccn1d was down-regulated in LP4 and LP8, respectively, suggesting the possibility of inhibition of the cell division procedure under the situation of excessive Leu intake. Moreover, the gene expression of liver Cdkn1a (cyclin-dependent kinase inhibitor 1A) showed significant up-regulation in rats fed the excessive-Leu diet accompanied by a decrease in the liver weight. It has been reported that CDKN1A competitively binds to CDK4 and acts as an inhibitor of Cyclin D (34). This report is consistent with our hypothesis that excessive Leu intake can cause negative effects on the stable cell division procedure. Taken together, the decrease of the liver weights by high Leu may be at least in part attributable to the regulation of gene expressions of the regulators of the cell division procedure.

Based on the DNA microarray analysis data, several
genes related to growth and the cell cycle have been identified as candidates for biomarkers responding to the adverse effects caused by excessive Leu intake. Furthermore, multiple regression analysis provided the biomarker panel, which is expected to have the capability of more accurate assessment for the safe amount of Leu intake. Using the dataset of Experiment 2, the effectiveness of biomarker candidates including the biomarker panel were validated with ROC curve analysis. ROC curve analysis revealed all biomarker candidates have good accuracy of prediction for growth retardation caused by excessive Leu. As the value of the AUC-ROC of the biomarker panel was highest (0.904), the cut-off point of the curve was deduced by the Youden index. The cut-off value of the biomarker panel indicated that Leu levels no more than 2% with 6% protein have no adverse effects but those higher than 3% have hazardous potential, which is consistent with the results of body weight gain or liver weight. The result suggests that dietary Leu supplementation of 2% is the NOAEL dose, and a higher dose may cause adverse effects in rats. Further studies including additional doses of Leu and the different amounts and sources of protein are needed to obtain a more conclusive value for the safe level of Leu intake.

The study showed that exhaustive analysis of gene expression is effective for the discovery of biomarkers. In addition, using the same animals as in the present study, we have obtained a gene expression profile of blood cells, which provided many biomarker candidates (unpublished data). Metabolome analyses have also been carried out and some metabolites in liver and plasma have been identified as effective biomarkers (unpublished data). The data also suggested a Leu dose over 2% could cause adverse effects under 6% protein conditions. The combination of different omics disciplines, i.e. integrated omics, is likely a promising strategy in addressing the issue of safety of food.

CONCLUSION

In conclusion, DNA microarray analysis has identified several genes whose expression in the liver have the potential as biomarkers for excessive Leu intake. Dietary Leu supplementation of 2% may be the NOAEL dose in a 6% casein diet for male SD rats.

Acknowledgments

W.I., R.K. and H.K. designed the research; W.I., R.Y., M.T. and R.K. conducted the research; W.I., J.Y. and H.K. analyzed the data; W.I., J.Y. and H.K. wrote the paper; and H.K. had primary responsibility for the final content. All authors read and approved the final manuscript. The authors are grateful to Prof. Yuji Nakai and Ms. Kyoko Nakazawa (The University of Tokyo) for their technical assistance and Mr. K. Saito for his help in data analysis. We thank Drs. H. Jia and L. Otani for their advice during the preparation of the manuscript. We are obliged to Ajinomoto Co., Inc. for providing leucine. This research was supported by International Council on Amino Acid Science (ICAAS). Dr Kato received an honorarium and travel expenses to attend the 8AAA W and present a lecture based on this paper.

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18) Aschner A. 1979. Prevention of the immunodepressive effects of excess dietary leucine by isoleucine and


Supplemental Fig. 1. Body weight gain of rats in the pair-feeding experiment during 10 d (A) and 49 d (B). The average amount of diet consumed by the LP8 rats was given to pair-fed groups. The composition of the pair-fed diet was same as the LP0 diet in Experiment 1. The LP8IV diet was LP8 supplemented with 4.42% Ile and 5.19% Val, the ratio of Leu/Val and Leu/Ile being equal to the AIN-93G diet. Multiple comparison testing was done by the Tukey-Kramer test. Columns which have different letters are significantly different (p<0.05). Data are shown as means±SE.

Supplemental Fig. 2. Direct connections among the biomarker candidates identified by transcriptome analysis based on IPA-knowledge. Genes are represented as nodes, and the biological relationship between two nodes is represented as a line. Red nodes mean up-regulated genes, and green nodes mean down-regulated genes in DNA microarray. Nodes are connected by canonical information stored in the IPA-knowledge base. Edges are displayed with labels that describe the relationship between the nodes. E, expression; I, inhibition; PP, protein-protein interaction; RB, regulation of binding; LO, localization.
Supplemental Table 1. Changes of liver gene expression of *Igf-1*, *Igfbp3*, *Igfbp5*, *Igfals* by Leu in rats fed the 6% casein diet.\

<table>
<thead>
<tr>
<th>mRNA</th>
<th>LP0</th>
<th>LP2</th>
<th>LP4</th>
<th>LP8</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Igf1</em></td>
<td>1.00±0.075</td>
<td>1.28±0.166</td>
<td>1.21±0.095</td>
<td>1.16±0.141</td>
</tr>
<tr>
<td><em>Igfbp3</em></td>
<td>1.00±0.092</td>
<td>0.88±0.064</td>
<td>0.99±0.139</td>
<td>1.11±0.124</td>
</tr>
<tr>
<td><em>Igfbp5</em> A</td>
<td>1.00±0.223</td>
<td>0.99±0.221</td>
<td>0.92±0.232</td>
<td>1.7±0.227</td>
</tr>
<tr>
<td><em>Igfals</em></td>
<td>1.00±0.072</td>
<td>1.05±0.142</td>
<td>1.08±0.153</td>
<td>0.63±0.086</td>
</tr>
<tr>
<td><em>Stat5a</em></td>
<td>1.00±0.144</td>
<td>0.85±0.96</td>
<td>0.81±0.063</td>
<td>0.91±0.137</td>
</tr>
<tr>
<td><em>Stat5b</em></td>
<td>1.00±0.168</td>
<td>1.03±0.134</td>
<td>1.02±0.124</td>
<td>0.74±0.078</td>
</tr>
</tbody>
</table>

Results of qPCR of *Igf1*, *Igfbp3*, *Igfbp5*, and *Igfals* in LP0, LP2, LP4, and LP8 group in Experiment 1 are shown. Actb was used as an internal standard for each measurement. Values are presented as the ratio to the average value of LP0. Multiple comparison testing was done by the Tukey-Kramer test. Data are shown as means±SE.

Supplemental Table 2. Groups of genes exhibiting highly significant changes in the liver of rats fed 6% casein+8% Leu diet compared with rats fed the 6% casein diet.\

<table>
<thead>
<tr>
<th>Pathway name</th>
<th>−Log (p-value)$^{2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine, serine and threonine metabolism</td>
<td>2.10E−05</td>
</tr>
<tr>
<td>Alanine and aspartate metabolism</td>
<td>6.25E−05</td>
</tr>
<tr>
<td>Type II diabetes mellitus signaling</td>
<td>1.14E−04</td>
</tr>
<tr>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>1.49E−04</td>
</tr>
<tr>
<td>Glycerophospholipid metabolism</td>
<td>1.76E−04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Functional category name</th>
<th>−Log (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid metabolism</td>
<td>2.51E−04</td>
</tr>
<tr>
<td>Small molecule biochemistry</td>
<td>2.51E−04</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>2.61E−04</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>6.52E−04</td>
</tr>
<tr>
<td>Cellular movement</td>
<td>2.11E−03</td>
</tr>
</tbody>
</table>

$^{1}$ The result of IPA-core analysis is shown. The top 5 significantly changed canonical pathways or biological functions in LP8 liver are shown in the table in descending order.

$^{2}$ Fisher’s exact test was used to calculate a p-value determining the probability that the association between the changed genes (more than log2 ratio 0.5 or less than −0.5) in LP8 microarray data and each biological functions or canonical pathway is explained by chance alone.