Selective Use of L-Valine and L-Isoleucine for the Biosynthesis of Branched-chain Fatty Acids in Rat Skin

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Iso- and anteiso-fatty acids are detected in more than trace amounts in rat skin surface lipid. The terminal portion of even carbon number iso- and anteiso-fatty acids are synthesized respectively from valine (Val) and isoleucine (Ile) by essentially the same reaction sequences established for straight chain fatty acids. This paper describes the stereospecific biosynthesis of these branched chain fatty acids (BCFAs) and alcohols (BCFALs) in rat skin. Dependence of the concentration of these BCFAs on dietary L-Val and L-Ile was studied. Concentrations of even carbon number iso- and anteiso-fatty acid increased respectively with dietary L-Val and L-Ile. The saturation dose appeared to be 2% for L-Val and 1% for L-Ile. Supplementation of the diet with 2% D-Val, however, did not affect the concentration of even carbon number iso-fatty acid in rat skin surface lipid despite a comparable serum Val level to that of the 2% L-Val group. A similar experiment using 1% DL-Ile found that I-isomer, but not D-isomer, in the circulation was used for the biosynthesis of anteiso-fatty acids. This view was applicable to the incorporation of D-Val and DL-Ile into related BCFALs.

Branched chain fatty acids (BCFAs) are unique constituents of the skin surface lipid of mammals. Our recent study demonstrated the precursor role of branched chain amino acids (BCAAs) for the biosynthesis of BCFAs in rat skin.1 Valine (Val) and isoleucine (Ile) in the circulation are taken up by rat skin tissue and metabolized toward biosynthesis of BCFA and branched chain fatty alcohols (BCFALs).

Extensive studies with the bacterial cells found that the pathway for BCFA biosynthesis was essentially identical to that established for straight chain fatty acids.2,3) BCAAs are deaminated to give rise to respective x-keto acids, which then undergo oxidative decarboxylation to give the short branched-chain acyl-CoAs.4,5 These acyl CoA substitute for acetyl-CoA, which is the primer for straight chain fatty acids, and the chains are elongated by successive condensation with malonyl CoA to produce related BCFAs.4,5 The overall reaction for biosynthesis of fatty acids is strikingly uniform throughout the system of microorganisms, plants and mammals. Val and Ile are therefore used as a precursor of even carbon number iso-fatty acids and anteiso-fatty acids in rat skin, respectively.

Stereoselectivity is a distinctive feature of biological systems. Several lines of studies have been done to measure the bioefficacy of D-amino acids in experimental animals.6,7,8) However, no detailed evidence has been presented on the stereoselective use of BCAAs for mammalian BCFA biosynthesis. Dietary supplements of l-isomers of BCAAs (Val and Ile) increased the level of the related BCFAs, and consequently elevated the concentration of related BCFALs in the skin surface lipid.9,10) This study aimed to address the stereoselective use by rat skin of BCAAs for BCFA synthesis in vivo. Thus the incorporation of stereoisomers of BCAAs into both BCFAs and BCFALs was studied to discover the efficacy of D-BCAAs for the biosynthesis of mammalian BCFAs.

Materials and Methods

Chemicals. D-Val was generous gift by Ajinomoto Co. (Tokyo, Japan), and its optical purity was 93.2%. L-Val and L-Ile were purchased from Nakalai Tesque (Kyoto, Japan), and their optical purity was 99.1 and 99.5%, respectively. D-Val purchased from Wako Pure Chemicals (Tokyo, Japan) was found to contain 50% l- and 50% D-isomer. D-Ile, D- and l-alle-Ile were purchased from Sigma Chemical Company (St. Louis, MO).

Animals and diets. Male Wistar rats weighing about 150 g were supplied by Kyudo (Kumamoto, Japan) or Japan-SLC (Shizuoka, Japan). Animals were maintained on a commercial diet up to 200 g of body weight, and then fed the test diet ad libitum for 1 week. Animals were housed individually in stainless steel cages under controlled room temperature (25 C) and lighting cycle (light was on from 0800 to 2000 h). Composition of the test diet (weight %) was as follows: casein 20; corn oil 10; mineral mixture 4; vitamin mixture 1 (both mixture according to Harper,10) and purchased from Oriental Yeast (Tokyo, Japan); cellulose powder 2; BCAAs 0 to 8%; corn starch to 100.

The skin surface of the animal was washed with ethanol before test diet feeding to remove the pre-existing lipid. On completion of the feeding of the test diet, the skin surface lipid was extracted with ethanol, and analyzed for BCFA and BCFAL concentrations.

Extraction of the skin surface lipids. Extraction of the skin surface lipids was done as previously described.11,12) Extra pure ethanol (100 ml) was poured on the back of the animals. Care was taken to minimize the contamination by non-skin derived lipid i.e. from the mouth and anal region. The back was repeatedly washed with the ethanol collected in petri dishes. The lipid extract was filtered, evaporated to dryness, re-dissolved in chloroform/methanol (2:1, by vol), and stored at −20 C.

Analysis of fatty acids and fatty alcohols. The monooester fraction (wax plus cholesterol ester) separated by thin layer chromatography was saponified with 0.5 M potassium hydroxide in 94% ethanol. Fatty acids and fatty alcohols of the monooester fraction were prepared as described previously.11,12) Fatty acid methyl ester and trimethylsilyl derivatives of fatty alcohol were analyzed by gas chromatography equipped with a flame

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ionization detector. The column was a fused silica capillary column of chemically bound non-polar liquid phase (CBP1-M50-025, Shimadzu Kyoto).

**Analysis of amino acids.** Blood (about 0.2 ml) was withdrawn at 1400 h from the tail vein on days 0, 3, and 7 of the feeding period. Serum was obtained from blood after centrifugation at 3000 × g for 20 min. Serum protein was precipitated in 2% TCA, and was removed by centrifugation. Amino acid concentrations were analyzed by high pressure liquid chromatography (HPLC) system (LC-6A, Shimadzu Kyoto). A detailed procedure for amino acid analysis was described previously.

**Polarimetry of amino acids.** Polarimetry of amino acids in 5 N HCl was done on Nihon Bunko DIP-180 polarimeter using a 5-cm sample cell with a sample volume of 4 ml. Readings were taken at 25°C using a wavelength of 589 nm.

**Statistical analysis.** Data were treated by one-way analysis of variance followed by inspection of differences between pairs of means.

**Results**

Our previous study showed that a dietary supplement of L-Val or L-Ile increased the concentrations of related BCFA and BCFAL in the monoester fraction of rat skin surface lipid. Thus, the stereoselectivity can be studied by examining the incorporation of D-isomers of these BCAAs into BCFA with LC-MS. D-Amino acids are converted to L-isomers by a two-step reaction of deamination and reamination. The first step involves oxidative deamination catalyzed by D-amino acid oxidase, primarily in the liver and kidney. The next step is a transamination reaction producing the l-amino acid isomer. It is, therefore, essential to discover the dose dependence of BCFA concentration on dietary supplements of BCAAs. Efficacy of D-isomers as a precursor of BCFA biosynthesis should be studied in the range of dietary supplements where the L-isomers are incorporated into BCFA in a dose-dependent fashion. Our previous work also showed that Val and Ile, but not leucine, were preferentially used for BCFA production in rat skin. Thus, incorporation of stereoisomer of Val and Ile into BCFA of monoester fraction was studied in this study.

Figure 1 depicts the dose-dependent incorporation of L-Val into related BCFA (even carbon number iso-fatty acids), and into related BCFAL (even carbon number iso-fatty alcohol) in the monoester fraction of the skin surface lipid. Concentrations of both BCFA and BCFAL increased proportionally with the dietary Val up to 2%, and thereafter appeared to reach the saturation level. This is particularly true for incorporation into BCFA (Fig. 1A). Similar trends were reproduced in the case of isoleucine (Fig. 2). In this case, the concentrations of BCFA and BCFAL appeared to reach the plateau at a rather lower level of dietary supplements compared with the case of Val. We therefore chose a dose of 2% for Val and 1% for Ile to study the efficiency of D-isomers as precursors of BCFA biosynthesis.

It has been shown that the absorption rate of D-isomers of BCFA from the gut was 2 to 3 times slower than that of L-isomers. It is the pre-requisite that the D-isomers of BCAAs are absorbed similarly to L-isomers to evaluate their biological significance. We therefore first examined the changes in serum BCAA levels following dietary administration of Val (Fig. 3). The HPLC column used for analysis of amino acids did not distinguish D- and L-isomers.

![Fig. 1. Effects of Dietary l-Val on the Concentrations of Even Carbon Number Iso-fatty Acid (A) and Iso-fatty Alcohol (B) in the Monoester Fraction of Rat Skin Surface Lipid. Data are % of total fatty acids, and expressed as mean±SE of 4 rats. Values not sharing a common letter are significantly different at p<0.05.](image1)

![Fig. 2. Effects of Dietary l-Ile on the Concentrations of Anteiso-fatty Acid (A) and Anteiso-fatty Alcohol (B) in the Monoester Fraction of Rat Skin Surface Lipid. Data are % of total fatty acids, and expressed as mean±SE of 4 rats. Values not sharing a common letter are significantly different at p<0.05.](image2)
The value therefore represents the sum of \( \alpha \)- and \( \beta \)-isomer. Data were drawn from an assay of pooled samples of 5 rats. Values for pooled samples closely approximated the average of values for individual samples.\(^{16}\)

As shown in Fig. 3, serum concentrations of Val in animals fed \( \beta \)-isomer compare favorably with that of animals fed the \( \alpha \)-isomer. This data were good enough to warrant the suggestion that \( \beta \)-Val was sufficiently absorbed from gut as was the case for \( \alpha \)-isomer despite its lower absorption rate.

There was no significant difference in food intake and body weight gain between animal groups. Intake of \( \beta \)-Val was 48.8 ± 1.5 mg/day, and was comparable to that for \( \alpha \)-isomer, 45.8 ± 1.1.

Figure 4 shows the concentration of even carbon number iso-fatty acids and alcohols of the monoester fraction in rats fed either 2% \( \beta \)- or \( \alpha \)-Val diet. Supplementation of the diet with \( \alpha \)-Val significantly elevated the concentration of even carbon number iso-fatty acids and alcohols. Addition of \( \beta \)-Val to the test diet, however, did not affect the concentration of either iso-fatty acids or alcohols. The concentration of even carbon number iso-fatty acid in the \( \beta \)-Val group was comparable to that of a control group that received no dietary Val. This finding unequivocally shows that the \( \beta \)-isomer of Val is poorly used by rat skin for biosynthesis of iso-fatty acids and alcohols.

Similar experiments were done with Ile. In the case of Ile, \( \alpha \)-Ile for \( \beta \)-Ile was used in this study for economic reasons. With respect to synthetic \( \alpha \)-Ile, four diastereomers are possible (\( \alpha \)- and \( \beta \)-Ile; \( \alpha \)- and \( \beta \)-allo-Ile) due to two asymmetric carbon atoms in this compound. In general, synthetic \( \beta \)-Ile is considered to be a mixture of 25% \( \alpha \)-Ile, 25% \( \beta \)-Ile, 25% \( \beta \)-allo-Ile, and 25% \( \alpha \)-allo-Ile. Analysis of amino acids found that the specimen used currently consisted of 50% Ile and 50% allo-Ile. This HPLC system, however, did not distinguish between \( \beta \)- and \( \alpha \)-isomers. Thus another HPLC system (JLC-300, Jeol Tokyo), which distinguishes between \( \beta \)- and \( \alpha \)-isomers but not Ile and allo-Ile, was introduced. This analysis showed that the specimen contain equal proportions (50%) of \( \beta \)- and \( \alpha \)-isomers.

It therefore seems to be quite all right to consider that our specimen of \( \beta \)-Ile consists of four equal proportions (25%) of diastereomer (\( \beta \)- and \( \alpha \)-Ile, and \( \beta \)- and \( \alpha \)-allo-Ile).

Figure 5 shows the changes in serum Ile concentration in rats fed the diet containing either one of 1% \( \beta \)-Ile, 1% \( \beta \)-Ile, or 0.5% \( \alpha \)-Ile. Values for 1% \( \beta \)-Ile represent the sum of Ile and allo-Ile. A trace of \( \alpha \)-allo-Ile was detectable in the serum of other animal groups (control, 1% \( \beta \)-Ile, and 0.5% \( \alpha \)-Ile). The concentration of serum Ile was comparable between 1% \( \beta \)-Ile, 1% \( \beta \)-Ile, and 0.5% \( \alpha \)-Ile at day 7 of the feeding period, suggesting sufficient absorption of \( \beta \)-isomer of Ile from the gut as was the case for Val (Fig. 5).

No significant differences were observed in food intake and body weight gain between the animal groups. Intakes of added Ile were 25.1 ± 1.2 mg/day for the 1% \( \beta \)-Ile group, 25.1 ± 0.2 for 1% \( \beta \)-Ile, and 12.8 ± 0.1 for 0.5% \( \alpha \)-Ile.

Figure 6 illustrates the incorporation of \( \beta \)-Ile into antiso-fatty acids and alcohols of the monoester fraction. Con-

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**Figure 3.** Changes in Serum Val Concentration in Rats Fed a Diet Containing Either 2% \( \alpha \)-Val, 2% \( \beta \)-Val, or 0% Val (Control).

Data were drawn from an assay of pooled samples of 5 rats.

- ○ 2% \( \alpha \)-Val
- ● 2% \( \beta \)-Val
- □ control

**Figure 4.** Concentration of Even Carbon Number Iso-fatty Acids (A) and Iso-fatty Alcohol (B) in the Monoester Fraction of Rats Fed a Diet Containing Either 2% \( \alpha \)-Val, 2% \( \beta \)-Val, or 0% Val (Control).

Data are % of total fatty acids, and expressed as mean ± SE of 5 rats. Values not sharing a common letter are significantly different at \( p < 0.05 \).

**Figure 5.** Changes in Serum Ile Concentration in Rats Fed a Diet Containing Either 1% \( \beta \)-Ile, 1% \( \beta \)-Ile, 0.5% \( \alpha \)-Ile, or 0% Ile (Control).

Data were drawn from an assay of pooled samples of 5 rats.

- ○ 1% \( \beta \)-Ile
- ● 1% \( \beta \)-Ile
- □ 0.5% \( \alpha \)-Ile
- □ control
centration of anteiso-fatty acids in the 1% DL-Ile group was significantly lower than that for 1% L-Ile, and compared well with the value for 0.5% L-Ile. The most probable explanation for this finding is that only L-isomers of DL-Ile were used for biosynthesis of anteiso-fatty acids in rat skin. Similar result was reproduced with the incorporation of DL-Ile into anteiso-fatty alcohol (Fig. 6B).

Discussion
This study described stereoselective biosynthesis of BCFAs and BCFALs in rat skin. D-Isomers of both Val and Ile were poorly used for biosynthesis of BCFAs and BCFALs. It is unlikely that the lower absorption rate from gut is responsible for the poor use of D-isomers of Val and Ile. Analysis of serum Val and Ile suggested that D-isomers of both Val and Ile were absorbed sufficiently despite their lower absorption rate.

This study did not address which step is responsible for the stereospecific biosynthesis of BCFAs and BCFALs. As mentioned above, the lower absorption rate may not be critical. It is probable that stereospecific discrimination took place at the initial step of BCFA biosynthesis where BCAAs are converted to active intermediates. This reaction is catalyzed by BCAA transaminase and branched chain \( \alpha \)-keto acid (BCKA) dehydrogenase: BCAAs are transaminated to give rise to BCKA, which then undergo oxidative decarboxylation to produce branched short chain acyl-CoA for further reaction with BCFA synthetate. Despite the wealth of papers on the metabolism of BCAAs, no information is available on the stereoselectivity of BCAA transaminase. It is plausible that BCAA transaminase is more active toward L-isomers than D-isomer because the former is the naturally occurring form.\(^1\)\(^7\) Direct evidence for this postulate is, however, yet to come. The asymmetric center of Val at the \( \alpha \)-carbon is destroyed by the transamination reaction. Thus deamination of both D- and L-Val will give rise to identical non-asymmetric compounds of \( \alpha \)-keto acids. It is therefore apparent that beyond this reaction step no stereospecific discrimination takes place. Thus the transamination reaction appeared to be primarily involved in the stereospecific biosynthesis of even-carbon number iso-fatty acids and alcohols.

Grigor et al.\(^1\)\(^8\) first described the dietary protein or BCAA dependent changes in the BCAA concentrations of rat skin surface lipid. They reported that feeding D-Val as well as L-Val elevated the concentration of even-carbon number iso-fatty acids in rat skin surface lipids. Although the reason for the conflicting results between the present and other data remains obscure, the difference in the dose of dietary supplements may explain in part the discrepancy. On the basis of our observations (Fig. 1), 5% of their dose appears to far exceed the saturation level of dietary supplements. Under these conditions, it can be anticipated that excess D-Val undergo oxidative deamination by D-amino acids oxidase \textit{in situ} and/or in kidney and liver to produce \( \alpha \)-keto acid. This \( \alpha \)-keto acid is reaminated by transaminase to produce \( \alpha \)-isomer. It is therefore possible that conversion of D-Val to L-isomer is accelerated in rats fed excessive D-Val. Elevated serum level of L-Val by this mechanism may account for the increased proportion of even carbon number iso-fatty acids.

This paper is the first report to show that mammalian tissue specifically uses L-isomers of Val and Ile for the biosynthesis of iso- and anteiso-fatty acids. The reaction step responsible for the stereospecific biosynthesis, however, remains to be identified. Studies on individual enzymes involved in BCFA biosynthesis will shed light on this question. The low enzyme activity is the major obstacle to a detailed studies to add insight of BCFA metabolism. Epidermis or sebaceous gland are the potential contributors of BCFAs to skin surface lipids. Establishment of culture method of these cells therefore may provide an alternative method for more detailed study of BCFA metabolism. Further work along this line is in progress.

References
Stereoselective Biosynthesis of Branched-chain Fatty Acids
