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ORIGINAL ARTICLE

Combination of BCAAs and glutamine enhances dermal collagen protein synthesis in protein-malnourished rats

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Abstract Skin collagen decreases in protein-malnourished states. Amino acids regulate protein metabolism, glutamine stimulates collagen synthesis through the conversion process to proline and provides 75 % of the intracellular free proline in fibroblasts. However, the impact of these amino acids on collagen synthesis under malnutrition has not been examined. We investigated the effect of amino acids on dermal tropocollagen synthesis in protein-malnourished rats. The fractional synthesis rate (FSR, %/h) of dermal tropocollagen was evaluated by the incorporation of L-[ring-$^2H_5$]-phenylalanine after 4 h infusion of each amino acid and the stable isotope. None of the infused 12 single amino acids (glutamine, proline, alanine, arginine, glutamate, glycine, aspartate, serine, histidine, lysine, phenylalanine and threonine) significantly increased the FSR ($P = 0.343$, one-way ANOVA). In contrast, amino acid mixtures of essential amino acids + glutamine + arginine (EAARQ) and branched-chain amino acids + glutamine (BCAAQ) significantly increased the FSR compared to saline, but the branched-chain amino acids (BCAAs) and amino acid mixture of collagen protein (AAC) did not alter the FSR (saline, 0.96 ± 0.24 %/h; EAARQ, 1.76 ± 0.89 %/h; BCAAQ 1.71 ± 0.36 %/h; BCAAs, 1.08 ± 0.20 %/h and AAC 1.39 ± 0.35 %/h, $P < 0.05$, Tukey's test). Proline conversion from glutamine represented only 3.9 % of the free proline in skin, as evaluated by the primed-constant infusion of L-d7-proline and L-α-15N-glutamine in rats. These results suggested that the combination of BCAAQ is a key factor for the enhancement of skin collagen synthesis in protein-malnourished rats. The contribution of extracellular free glutamine on de novo proline synthesis and collagen synthesis is very low in vivo compared to the contribution in vitro.

Keywords Amino acids · Skin collagen synthesis · Glutamine and proline metabolism · Protein-malnourished rat

Abbreviations

FSR	Fractional synthesis rate
mTOR	Mammalian target of rapamycin
S6K	Ribosomal protein S6 kinase
BCAAs	Branched-chain amino acids
Leu	Leucine
Ile	Isoleucine
Val	Valine
Gln	Glutamine
Arg	Arginine
Pro	Proline
Ala	Alanine
Glu	Glutamate
Gly	Glycine
Asp	Aspartate
Ser	Serine
His	Histidine
Lys	Lysine
Phe	Phenylalanine
Thr	Threonine
EAARQ	Essential amino acids + arginine + glutamine
BCAAQ	BCAAs + glutamine
AAC	Amino acid mixture composed of collagen protein
mTORC	Mammalian target of rapamycin complex

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SLC7A5	Solute carrier family 7 member 5
SLC3A2	Solute carrier family 3 member 2
GCN2	General controller non-derepressible 2 kinase
MMPs	Matrix metalloproteases

Introduction

Skin aging, especially wrinkling and sagging, is induced by several factors including malnutrition, ultraviolet irradiation, dryness, chemical stimulation and exposure to activated oxygen species (Rittie and Fisher 2002). Dermal collagen is a major component of the skin dermis, and it is necessary for the maintenance of skin structure. Protein malnutrition affects the decrease in skin collagen under malnourished conditions. Dietary protein deprivation blunts collagen synthesis and degradation, such as protein expression and the mRNA levels of type I and III collagen, collagenase and metalloproteinases in rats (Oishi et al. 2002). Protein malnutrition also decreases skin collagen content during wound healing in rats (Mistry et al. 1994). Dietary low protein decreases the turnover of soluble acetic acids and insoluble collagen proteins (Molnar et al. 1988). Improvements in the protein turnover rate during protein malnutrition are important for the maintenance of dermal collagen levels.

Amino acids are protein substrates and regulators of protein metabolism. Amino acids are highly safe for humans. Bellon et al. demonstrated that glutamine increases procollagen mRNA levels and collagen content in human fibroblast cells in vitro, and suggested that de novo proline synthesis from glutamine is important for collagen synthesis (Bellon et al. 1987, 1995). One possible mechanism of glutamine-induced collagen biosynthesis in cultured human skin fibroblasts is pyrroline-5-carboxylate, which is an intermediate of proline and glutamine increases during collagen synthesis (Karna et al. 2001). The inhibition of glutamine uptake into cells diminishes the intracellular conversion of glutamine to proline via glutamate, which reduces pro-α1 collagen gene transcription (Bellon et al. 1995). Furthermore, some amino acids, such as arginine (Shi et al. 2003; Stechmiller et al. 2005), ornithine (Shi et al. 2002) and amino acid mixtures (Badiu et al. 2010; Corsetti et al. 2010) enhance wound healing in rats in vivo. Leucine supplementation exerts an anabolic effect on protein metabolism in skin wounds in rabbits (Zhang et al. 2004). A leucine-rich diet increases the hydroxyproline content in the tension region in protein-malnourished rats (Barbosa et al. 2012). Specific amino acid mixtures, including BCAA and glutamine or proline, stimulate the fractional synthesis rate (FSR) of skin tropocollagen in rats irradiated with ultraviolet B radiation, whereas single amino acids, including glutamine and proline, do not (Murakami et al. 2012). These previous studies focus on collagen metabolism under a condition where protein turnover is high, such as inflammation in wound healing, UV irradiation and fibroblast. However, few studies have focused on the capacity of amino acids to recover dermal collagen synthesis under a low protein turnover condition such as protein malnourishment.

Consequently, this study investigated the effect of amino acids on skin tropocollagen, which is newly collagen, synthesis using a stable isotope technique in rats that were fed a protein-free diet for 1 week.

Methods

Animals

The Institutional Animal Care and Use Committee of Ajinomoto Co., Inc., approved this study. Nine-week-old male Sprague-Dawley rats (Charles River Laboratories Japan, Inc.) were housed in a temperature controlled room on a 12-h light–dark cycle. The animals were 10 weeks old at the end of the experiment. The rats were provided a standard commercial chow (CR-F1; Charles River Laboratories Japan, Inc., Japan) and water ad libitum.

Experimental design

Experiment 1: The effect of dietary protein level on the FSR of skin tropocollagen and mixed skin proteins were measured using an isotope incorporation technique in rats. Dietary composition was based on AIN93G, which replaces protein with starch as described in Table 1. Each diet was supplied to rats for 1 week. The mean daily dietary consumption was 24.2 ± 2.4 g in normal protein diet group (Normal), 27.6 ± 3.6 g in low protein diet group (Low) and 18.0 ± 2.0 g in protein-free diet group (Free), and the mean body weight after treatment was 415.0 ± 23.7, 402.6 ± 10.0 and 355.4 ± 7.7 g, respectively. Catheters were implanted in the carotid artery and the jugular vein under anesthesia (0.0576 g kg^{-1} ketamine and 0.0037 g kg^{-1} xylazine) in rats ($n = 4$ in each group). A primed continuous infusion of L-[ring-$^2H_5$]-phenylalanine (prime, 14.40 μmol kg^{-1} h^{-1}; infusion rate, 28.79 μmol kg^{-1} h^{-1}) was performed for 4 h under anesthesia following the collection of 200 μl of blood to measure the background enrichment of phenylalanine. 200 μl of blood were collected from the artery at 60, 120, 180, 210, 225 and 240 min, and subcutaneous skin samples (1 g) were taken at 60 and 240 min during the infusion. The fat tissue of the skin was immediately removed, and

the dermis was frozen in liquid nitrogen and stored at −80 °C. The plasma was separated by centrifugation at 3,000×*g* for 15 min at +4 °C and stored at −80 °C.

Experiment 2: The effects of amino acid infusion on the FSR of skin tropocollagen and mixed skin proteins were evaluated using an isotope incorporation technique in rats that were fed a protein-free diet for 1 week. L-[ring-$^2H_5$]-phenylalanine was continuously infused to rats using the same method as in Experiment 1. Each single amino acid [glutamine ($n = 5$), proline ($n = 5$), alanine ($n = 5$), arginine ($n = 4$), glutamate ($n = 4$), glycine ($n = 5$), aspartate ($n = 4$), serine ($n = 5$), histidine ($n = 4$), lysine ($n = 4$), phenylalanine ($n = 4$) and threonine ($n = 4$)] and amino acid mixtures [essential amino acids + arginine + glutamine (EAARQ, $n = 4$), branched-chain amino acids + glutamine (BCAAQ, $n = 6$), BCAAs ($n = 5$), or an amino acid mixture composed of collagen protein (AAC, $n = 4$)] were infused for 4 h. Composition and infusion rate of amino acids are described in Table 2. Skin and blood were collected in the same manner as in Experiment 1.

Experiment 3: The interaction between free glutamine and proline metabolism in the dermal tissue was investigated in rats that were fed a standard commercial chow. The catheters were implanted in rats using the same method that is described in Experiment 1 ($n = 4$ in each group). Primed continuous infusions of L-d7-proline (prime, 1.5 μmol kg^{-1}; infusion rate, 3.0 μmol kg^{-1} h^{-1}) and L-α-15N-glutamine (prime, 90 μmol kg^{-1}; infusion rate, 180 μmol kg^{-1} h^{-1}) were performed for 4 h under anesthesia (0.0576 g kg^{-1} ketamine and 0.0037 g kg^{-1} xylazine), following the collection of 200 μl of arterial blood to measure the background enrichment of each amino acid. The skin and blood were collected and stored as described in Experiment 1.

Table 1 Composition of experimental diet

%	Normal protein	Low protein	Protein-free
Casein	20	5	0
L-Cystine	0.3	0.3	0.3
Corn starch	39.7	54.7	59.7
Alpha-starch	13.2	13.2	13.2
Sucrose	10	10	10
Corn oil	7	7	7
Cellulose	5	5	5
Mineral mixture (AIN93G)	3.5	3.5	3.5
Vitamin mixture (AIN93)	1	1	1
Choline bitartrate	0.25	0.25	0.25
t-Butylhydroquinone	0.0014	0.0014	0.0014
Total	100	100	100

Dietary composition was based on AIN-93G, replacing protein with starch

All amino acids were purchased from Ajinomoto Co., Ltd. (Japan). All stable isotopes were purchased from Cambridge Isotopes (Cambridge, MA, USA). Dietary ingredients were purchased following manufactures: casein, alpha-starch, mineral and vitamin mixtures (Oriental Yeast Co., Japan); corn starch (Nihon Syokuhin Kako, Co. Ltd., Japan); sucrose (Uehara Co., Japan); cellulose (ADVANTEC Toyo Roshi Ltd. Japan) and choline bitartrate and t-butylhydroquinone (Wako Pure Chemical Industries Ltd., Japan).

Sample preparation

Approximately 0.04 g of dermal skin was homogenized in 15 % sulfosalicylic acid, and the homogenate was centrifuged. The supernatant was used for free amino acid measurements in dermal tissue. The precipitate was hydrolyzed in 2 ml of 6 N hydrochloric acid at 90 °C for 16 h for mixed skin protein analysis.

Tropocollagen was extracted using a previously described method (Murakami et al. 2012). In brief, approximately 1 g of dorsal skin was homogenized in the homogenate buffer (10 ml). The homogenized sample was shaken overnight at 4 °C and centrifuged. The supernatant was filtered, adjusted to 4.5 M with NaCl and shaken for 5 h. The solution was centrifuged, and the precipitate was dissolved in the extraction buffer. The solution was dialyzed in the extraction buffer for 2 h. This dialyzed sample, which included large amounts of skin tropocollagen protein, was hydrolyzed in 6 N hydrochloric acid at 90 °C for 16 h. The hydrolysate was used to measure the isotope incorporation rates in the skin tropocollagen.

Amino acids in the hydrolysate and supernatant were purified using cation exchange chromatography (Dowex 50 W 8X; Bio-Rad Laboratories, CA, USA), and dried in a rotary evaporator (Nakajima Corp., Japan).

Analysis

Enrichments of free phenylalanine ($E_{\text{phe (skin free)}}$), proline ($E_{\text{pro (m+7, skin free)}}$, $E_{\text{pro (m+1, skin free)}}$) and glutamine ($E_{\text{gln (skin free)}}$) in the dermal tissue, and the enrichments of free phenylalanine ($E_{\text{phe (plasma)}}$), proline ($E_{\text{pro (m+7, plasma)}}$, $E_{\text{pro (m+1, plasma)}}$) and glutamine ($E_{\text{gln (plasma)}}$) in plasma were determined using *tert*-butyldimethylsilyl (*t*-BDMS, Thermo Fisher, IL, USA) derivatization. Gas chromatography–mass spectrometry monitored ions *m/z* 336 and *m/z* 341 for natural and [ring-$^2H_5$]-phenylalanine, respectively; for proline, *m/z* 286, *m/z* 287 and *m/z* 293 for natural, biosynthetic 15N-proline metabolized from 15N-glutamine

Table 2 Compostions and infusion rate of amino acid solutions

	Gln	Pro	Ala	Arg	Glu	Gly	Asp	Ser	His	Lys	Phe	Thr	EAARQ	BCAAQ	BCAAs	AAC
Isoleucine													0.072	0.072	0.072	0.010
Leucine													0.090	0.090	0.090	0.023
Valine													0.060	0.060	0.060	0.011
Histidine									0.602				0.030			0.006
Lysine										0.592			0.030			0.055
Glutamine	0.420												0.108	0.108		
Phenylalanine											0.316		0.003			0.014
Proline		0.33											0.030			0.118
Threonine												0.376	0.042			0.014
Tryptophan													0.003			
Methionine													0.030			0.004
Alanine			0.256													0.044
Aspartate							0.497									0.034
Glutamate					0.537											0.055
Glycine						0.215										0.145
Serine								0.302								0.027
Arginine				0.667									0.102			0.041
Infusion rate (g kg^{-1} h^{-1})	0.420	0.330	0.256	0.667	0.537	0.215	0.497	0.302	0.602	0.592	0.316	0.376	0.600	0.330	0.222	0.600

Infusion rate of each single amino acid was also matched as 2.87 mol kg^{-1} h^{-1}

and d7-proline, respectively; for glutamine, *m/z* 431 and *m/z* 432 for natural and α-15N-glutamine, respectively, in the electron impact mode (GC–MS; 6890 GC system and 5473 Network mass selective detector, Agilent Technology, CA, USA). Phenylalanine enrichment in the tropocollagen and mixed skin protein samples ($E_{(\text{tropocollagen})}$, $E_{(\text{mixed skin})}$) was determined by measuring AQC-detergent (Waters Co., MA, USA) derivatization using liquid chromatography–mass spectrometry to monitor ions *m/z* 336 and *m/z* 341 for natural and [ring-$^2H_5$]-phenylalanine, respectively in the first MS, and ion *m/z* 171 in the second MS (LC–MS/MS; Prominence HPLC system, Shimazu, Japan and API 3200, Applied Biosystems, CA, USA).

Calculation and statistics

The FSRs of the skin tropocollagen and mixed skin proteins were calculated using the precursor-product model. The increments of enrichment in the product [i.e., the difference of the enrichment of amino acids within proteins between the first (1 h) and the second (4 h) skin biopsies ($\Delta E_{(\text{tropocollagen or mixed skin})}$)] were divided by the difference of free tissue amino acid tracer-to-tracee ratios between the first and second skin biopsies ($\Delta E_{\text{phe (skin free)}}$). The FSR was calculated as FSR (%/h) = $\Delta E_{(\text{tropocollagen or mixed skin})}$/($\Delta E_{\text{phe (skin free)}} \times t$) × 100, where *t* represents the time interval between the first and the second skin biopsies.

The contribution of glutamine and proline in blood to dermal tissue and the conversion from glutamine to proline in skin tissue were calculated by the enrichment of stable isotopes of free amino acids in plasma ($E_{(\text{plasma})}$) and skin tissue ($E_{(\text{skin})}$) in the steady state, where the enrichment of free glutamine and proline in plasma represents as $E_{\text{gln (plasma)}}$, $E_{\text{pro (m+7, plasma)}}$, respectively. Free glutamine and proline in the dermal tissue supplied from blood were calculated as T_{gln} (%) = $E_{\text{gln (skin)}}/E_{\text{gln (plasma)}} \times 100$, T_{pro} (%) = $E_{\text{pro (m+7, skin)}}/E_{\text{pro (m+7, plasma)}} \times 100$. *Pro (m+1)* represents biosynthetic proline metabolized form glutamine. Thus, the conversion from glutamine to proline in the dermal tissue was calculated as C (%) = ($E_{\text{pro (m+1, skin)}} - E_{\text{pro (m+1, plasma)}} \times T_{\text{pro}}$)/$E_{\text{gln (skin)}} \times 100$.

The values are presented as the mean ± SD. The statistical significance in each diet group was evaluated using Tukey's test, following a one-way ANOVA for multiple comparisons (GraphPad Prism, GraphPad Software, Inc., CA, USA). Values of $P < 0.05$ were considered significant.

Results

The FSR of skin tropocollagen significantly decreased in rats that were fed a protein-free diet compared to rats that received a normal protein diet [Fig. 1a; $P < 0.05$; effect size (ES) = 0.54]. However, the FSR of mixed skin proteins tended to decrease a protein-free diet group but was not significant (Fig. 1b; $P = 0.0664$; ES = 0.43).

The effects of single amino acid infusions on the FSR of skin tropocollagen and mixed skin proteins are shown in Table 3. No significant differences between groups were

observed (Table 3; tropocollagen $P = 0.3431$, ES = 0.30; mixed skin protein $P = 0.6676$; ES = 0.25). The effect of amino acid mixtures infusion on dermal tropocollagen is shown in Fig. 2a. EAARQ and BCAAQ significantly increased the FSR of tropocollagen compared to saline, but BCAAs and AAC did not affect the FSR (saline 0.96 ± 0.24 %/h, EAARQ 1.76 ± 0.89 %/h, BCAAQ 1.71 ± 0.36 %/h, BCAAs 1.08 ± 0.20 %/h and AAC 1.39 ± 0.35 %/h; $P < 0.05$; ES = 0.72). None of the amino acid mixtures investigated significantly increased the FSR of mixed skin proteins (Fig. 2b; $P = 0.1422$; ES = 0.57).

Change in the enrichment levels of proline and glutamine during the primed-constant infusion is shown in Table 4. The enrichment levels of these amino acids confirmed a steady state after 2 h starting the infusion. Totals of 11.6 % of free proline and 51.7 % of free glutamine in the dermal tissue were derived from blood. The conversion of glutamine to proline in the dermal tissue was only 3.9 %, and the conversion in the body was 5.2 %. Therefore, most of the free proline (84.5 %) in the dermal tissue was derived from skin protein degradation and/or the de novo synthesis from other amino acids (Fig. 3).

Table 3 Effect of single amino acid infusions on the FSR of tropocollagen and mixed skin proteins

FSR (%/h)	Tropocollagen Mean ± SD	Mixed skin proteins Mean ± SD
Saline ($n = 6$)	1.029 ± 0.308	0.309 ± 0.226
Gln ($n = 5$)	0.903 ± 0.685	0.390 ± 0.196
Pro ($n = 5$)	1.114 ± 0.515	0.284 ± 0.272
Ala ($n = 5$)	1.440 ± 0.877	0.624 ± 0.533
Arg ($n = 4$)	1.194 ± 0.440	0.660 ± 0.456
Glu ($n = 4$)	1.527 ± 0.332	0.460 ± 0.229
Gly ($n = 5$)	1.350 ± 0.587	0.467 ± 0.411
Asp ($n = 4$)	1.519 ± 0.425	0.465 ± 0.106
Ser ($n = 5$)	1.488 ± 0.411	0.473 ± 0.167
His ($n = 4$)	1.544 ± 0.811	0.343 ± 0.274
Lys ($n = 4$)	1.287 ± 0.281	0.398 ± 0.202
Phe ($n = 4$)	0.773 ± 0.052	0.207 ± 0.074
Thr ($n = 4$)	1.532 ± 0.361	0.405 ± 0.222

There were no significant differences between any amino acid and saline control

Values are given as mean ± SD

Discussion

This study investigated the effect of amino acids on dermal tropocollagen synthesis in protein-malnourished rats. The FSR of tropocollagen decreased in rats that were fed a protein-free diet. The diminished FSR was improved by specific amino acid mixtures such as EAARQ and BCAAQ, but BCAAs, AAC and all single amino acids (including glutamine and proline) did not affect the FSR. These results suggested that the combination of amino acids, such as BCAAs and glutamine, is a key factor for increasing skin collagen synthesis in protein-malnourished rats.

The amount of dietary protein affects the collagen protein metabolism. Protein expression and the mRNA levels of skin collagen are blunted during protein malnutrition (Mistry et al. 1994; Molnar et al. 1988; Oishi et al. 2002). This study confirmed the reduction in collagen synthesis in protein-malnourished rats (Fig. 1a). Also, it was indicated that the FSR of dermal tropocollagen was more affected by dietary protein malnutrition than were mixed skin proteins (Fig. 1a, b). The change of protein turnover of collagen will induce thin skin.

The same molar concentration (2.87 mol kg^{-1} h^{-1}) of single amino acid was infused to the rats to reduce the impact of concentration on the uptake of amino acid in tissues. Also, the same amount of BCAAs was infused to the rats from the groups of amino acids mixture to understand effect of additional amino acids on BCAAs. All single amino acids, except low-soluble amino acids and BCAAs, were evaluated in this study to investigate the

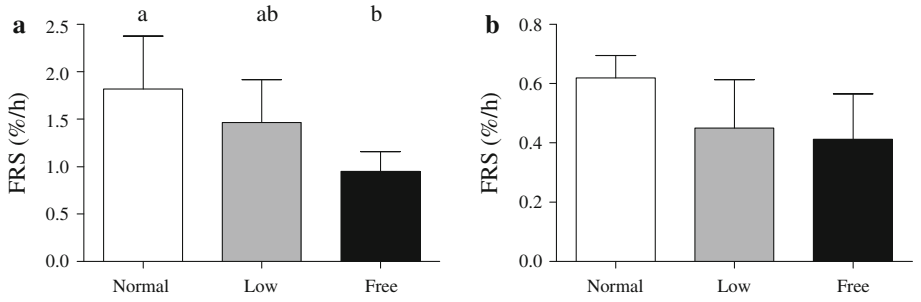

Fig. 1 Effect of dietary protein levels on the FSR of skin tropocollagen (**a**) and mixed skin proteins (**b**) in rats. The FSR of skin tropocollagen significantly decreased in rats that were fed a protein-free diet compared to rats that received a normal protein diet. However, the FSR of mixed skin proteins was not affected by dietary protein level ($n = 4$ in each group). The values are presented as mean ± SD. The values with *different superscripts* are significantly different ($P < 0.05$)

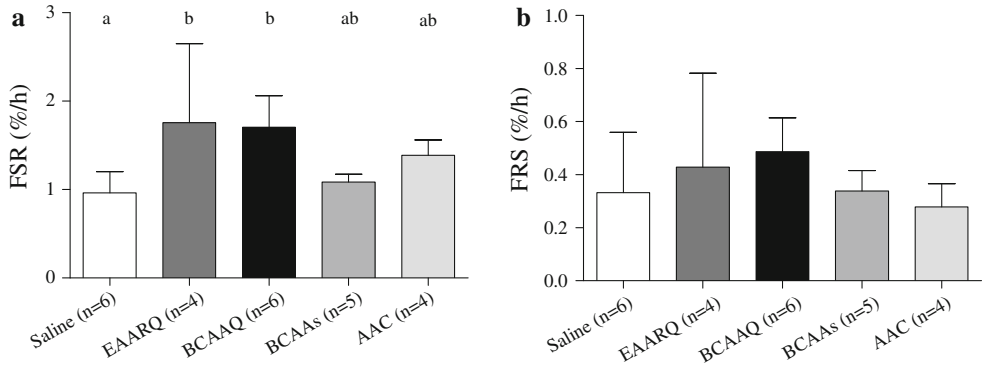

Fig. 2 Effect of amino acid mixtures on the FSR of tropocollagen (**a**) and mixed skin proteins (**b**) in protein-malnourished rats. EAARQ and BCAAQ significantly increased the FSR of tropocollagen, but BCAAs and AAC did not affect the FSR (**a**). These amino acid mixtures did not affect the FSR of mixed skin proteins (**b**). The values with different superscript are significantly different ($P < 0.05$)

Table 4 Changes in enrichment of proline and glutamine in blood and dermal tissue

Enrichment (%)	1 h	2 h	3 h	4 h
Blood				
15N-proline	0.18 ± 0.24	0.29 ± 0.24	0.30 ± 0.48	0.50 ± 0.40
d7-Proline	9.15 ± 0.98	11.18 ± 1.10	11.42 ± 1.52	10.83 ± 1.36
15N-glutamine	8.50 ± 3.78	9.91 ± 4.86	10.10 ± 4.78	9.66 ± 4.19
Skin				
15N-proline	0.10 ± 0.19			0.25 ± 0.17
d7-Proline	1.86 ± 0.54			1.25 ± 0.60
15N-glutamine	3.86 ± 2.23			4.99 ± 3.04

Blood proline and glutamine enrichments were at steady state after 2 h of isotope infusion

Values are given as mean ± SD

impact of single amino acids on skin tropocollagen synthesis None of the single amino acids that we studied, including glutamine, proline and arginine, significantly increased the FSR of tropocollagen and mixed skin proteins (Table 3). Glutamine, proline and arginine did not increase skin tropocollagen synthesis in UV-irradiated mice (Murakami et al. 2012), which is similar to what was observed in this study. On the contrary, Bellon et al. indicated that glutamine and de novo synthesized proline are the key factors of collagen protein synthesis in vitro. They demonstrated that glutamine provides 75 % of the intracellular free proline and 85 % of procollagen-bound proline (Bellon et al. 1987). Procollagen synthesis is not altered over a wide range of high proline concentrations in fibroblasts (Bellon et al. 1987). The de novo synthesis of proline is required for the glutamine-dependent induction of collagen gene expression in fibroblast cells (Bellon et al. 1995). Pyrroline-5-carboxylate, which is the intermediate of proline, may contribute to the stimulation of collagen synthesis in human fibroblast cells (Karna et al. 2001). However, few studies have focused on the interaction between proline and glutamine metabolism in dermal tissue in vivo. Amino acid metabolism associated with the interaction among tissues through blood and skin tissue is the complex of several types of cells such as fibroblast, keratinocyte. Therefore, we have to consider the interaction between glutamine and proline metabolism in whole body study in vivo. Proline conversion from glutamine was 5.2 % in the entire rat body and only 3.9 % in dermal tissue (Fig. 3). This result demonstrated that glutamine contributed less to de novo proline synthesis in dermal tissue in vivo compared to a previous in vitro study (Bellon et al. 1987). In addition, 11.6 % of free proline in the dermal tissue was supplied from the blood (Fig. 3). These results suggested that 84.5 % of free proline in the dermal tissue is supplied from within the dermal tissue in vivo. Therefore, the difference in contribution of glutamine metabolism to free protein in the dermal tissue between in vivo and in vitro studies is one of the reasons why glutamine did not increase collagen synthesis in this study.

The combination of BCAAQ stimulated dermal collagen synthesis in our previous study (Murakami et al. 2012). Essential amino acids stimulate protein synthesis (Katsanos et al. 2006; Murakami et al. 2012; Volpi et al. 2003).

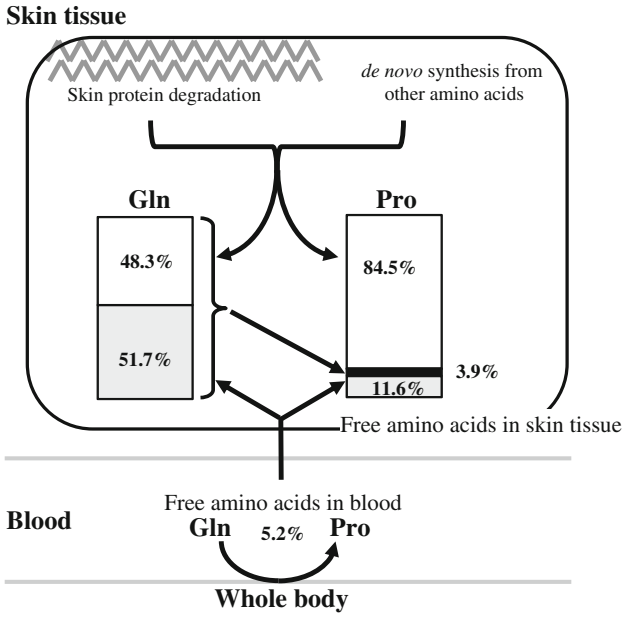

Fig. 3 Interaction between proline and glutamine metabolism in blood and dermal tissue. In total, 3.9 and 11.6 % of free proline in the skin tissue were derived from de novo synthesis from glutamine in the dermal tissue and blood, respectively. Most (84.5 %) of the free proline in the dermal tissue was derived from skin protein degradation and/or de novo synthesis from other amino acid ($n = 4$)

BCAAs also stimulate protein synthesis in several tissues such as skeletal muscle (Kimball and Jefferson 2004). Amino acid mixtures composed of collagen protein may stimulate collagen synthesis. Thus, these amino acid mixtures were evaluated in the present study. The amount and composition of branched-chain amino acids between EAARQ, BCAAQ and BCAAs groups were the same. EAARQ and BCAAQ significantly increased the FSR of tropocollagen, but BCAAs and AAC did not affect the FSR (Fig. 2a). This result indicated no-impact of arginine supplementation and the synergistic effect of glutamine supplementation in BCAAs on the FSR of tropocollagen. Leucine and glutamine stimulate the synthesis of proteins that regulates the mTOR signaling pathway in skeletal muscle (Anthony et al. 2000; Kimball and Jefferson 2004; Meijer 2003; Proud 2004; Xu et al. 2001). Xu et al. demonstrated that the combination of leucine and glutamine synergistically stimulates the activity of S6K in pancreatic beta cells (Xu et al. 2001). In addition, supplemented glutamine affects BCAAs metabolism. A part of supplemented BCAAs is metabolized to glutamine because BCAAs are essential donors of nitrogen in synthesis of glutamine in skeletal muscle (Holecek 2002). Nicklin et al. showed that glutamine is used to import leucine via the antiporter SLC7A5-SLC3A2 in the cell, leading to mTORC1 activation (Nicklin et al. 2009). In the present study, each plasma BCAAs concentration after 4 h infusion was significantly higher in BCAAQ than that in BCAAs (leucine 1.3 ± 0.18^{a}, 13.8 ± 1.1^{b}, 10.2 ± 0.1^{c} mmol/l; isoleucine 0.3 ± 0.08^{a}, 9.6 ± 0.9^{b}, 7.4 ± 0.1 mmol/l^{c}; valine 0.9 ± 0.16^{a} 14.9 ± 0.2^{b}, 12.4 ± 0.4^{c} mmol/l in saline, BCAAQ and BCAAs, respectively; $P < 0.05$; Tukey's test), despite infused BCAAs concentration was same between groups. The increase of FSR is depending on plasma leucine concentration in rats (Crozier et al. 2005). This may be one of the mechanisms how BCAAQ stimulated the FSR of dermal tropocollagen stronger than BCAAs in protein-malnourished rats.

In conclusion, protein malnourishment decreased the FSR of tropocollagen, but single amino acids, such as glutamine and proline, did not stimulate the FSR. However, combinations of amino acids, such as BCAAs and glutamine, increased the FSR of tropocollagen. This combination is a key factor to stimulate the FSR of tropocollagen. The contribution of extracellular free glutamine on de novo proline synthesis and collagen synthesis is very low in vivo compared to the contribution in vitro. Therefore, further investigations of collagen protein metabolism should be performed in vivo. Protein turnover and signaling pathway of collagen protein metabolism such as mTOR, GCN2 (general controller non-derepressible 2 kinase) and MMPs (matrix metalloproteases) were not measured in the present study. Further study to investigate the mechanism of BCAAQ on stimulated collagen protein turnover is required.

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Conflict of interest The authors declare no conflicts of interest.

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