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Leucine이 정상 또는 굶게 된 쥐의 골격근육의 단백질 생합성에 미치는 영향

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Effects of Leucine on *in Vivo* Protein Synthesis in Skeletal Muscles of Fed and Food-Deprived Rats

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Branched-chain 아미노산인 leucine이 골격근육의 단백질 합성을 촉진하는 요소로 보고되어 왔다. 이러한 역할은 다른 어떤 아미노산도 나타낼 수 없는 특이한 것이나 생체로부터 분리된 근육(isolated muscle system)에서 라는 제한된 실험조건하에 얻어진 결과에 바탕하므로, 본 연구는 생체내에서 leucine이 단백질의 생합성에 미치는 영향을 조사하고자 시도되었다. 실험동물은 정상으로 먹이를 먹인 또는 하루 굶게된 쥐로 leucine군과 비교군으로 나누었다. Leucine군은 80 또는 160μmoles의 leucine을 복강으로 한번 주사하여 주입받았고 비교군에는 생리적 식염수를 같은 방법으로 주입했다. 단백질 합성속도는 ^{14}C-tyrosine을 주사한 후 근육의 단백질에 혼입된 ^{14}C의 량으로 측정하였다. 본 연구에 이용된 근육은 두가지 형태의 뒷다리 근육 즉 oxidative형 soleus와 glycolytic형 extensor digitorium longus(EDL)와 plantaris근육이었다.

만 하루 굶게된 쥐의 EDL과 plantaris근육은 160μmoles의 leucine에 의해 단백질 합성 속도가 24%, 29%씩 각각 상승했다. 하루 굶게된 쥐의 soleus근육과 정상으로 먹인 쥐의 어느 근육도 첨가된 leucine에 대해 반응을 보이지 않았다. Leucine에 의해 단백질 합성 속도가 상승된 근육은 굶게되므로 합성속도가 정상군의 54%로 떨어졌고 soleus 근육은 정상군의 78%에 상당한 단백질 합성 능력을 가지고 있었다. 따라서 생체로부터 분리된 근육에서 동물의 영양상태나 홀몬 분비의 정상여부에 관계없이 leucine이 단백질 생합성 속도를 상승시키는 현상은 생체내에서 재현되지 않았다. 본 연구결과는 식이 제한등으로 인한 스트레스에 민감하게 반응하여 근육내 생성 활동이 저하된 특수한 상태에서만 leucine은 골격근육의 질소 보유능력을 상승시키리라는 것을 시사한다.

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INTRODUCTION

A number of studies have indicated that the branched-chain amino acid, leucine, has stimulatory effecs on protein synthesis of skeletal muscles[1-4]. *In vitro* experiments with isolated diaphragm demonstrated that leucine but no other amino acids including the other two branched-chain amino acids, valine and isoleucine, increased the rate of protein synthesis[3]. Studies with perfused heart[5], isolated arterial muscle[4], and perfused hemicorpus preparation[6] also showed similar stimulatory effects of leucine. The report by Chang Hong and Layman[7] clearly demonstrated that leucine can stimulate protein synthesis in isolated skeletal muscles. Further the stimulation was shown in a wide range of conditions such as fed, total food deprived, and diabetic animals thus suggesting an anabolic role of leucine in the regulation of skeletal muscle protein synthesis. The mechanism for the stimualtion of protein synthesis by leucine remains unknown. Several suggestions include that leucine may supply energy substrate, increase leucyl-tRNA which may be a rate limiting in the precursor pool[4], o stimulate the ribosomal aggregation to promote the initiation of protein synthesis[8].

Clinical studies have shown that the infusion of leucine into fasting or post-surgery patients improved nirogen balance[9-10]. Sherwin[9] suggested that reduced nirogen loss be obtained by stimulation on skeletal muscle protein synthesis since the concentration of 3-methylhistidine excretion was not changed. However, the studies with rats indicated that leucine increased the fractional synthesis rate of liver protein without any stimulation on skeletal muscle protein synthesis[11]. Further McNurlan *et al*[12]. recently reproted that there was no *in vivo* effects on the rate of skeletal muscle protein synthesis after intravenous(i.v.) injection of 100μmoles of leucine into fed, 2-day starved, or 9-day protein deprived rats. These findings appear contradictory to the observations in the isolated muscle systems. While Buse *et al*[8]. reported that ribosomal aggregation was stimulated by the injection of 200μmoles of leucine into starved rats. They also demonstrated that infusion of 300μmoles of leucine into starved rats during a 6-hour period increased the incorporation of tracer amino acid by various degrees, 0-100%, in different muscles[13].

Although possible nitrogen nitrogen sparing effects of leucine have been suggested presently it is controversial whether leucine directly stimulates *in vivo* skeletal muscle protein synthesis. Therefore this study was to examine the effect of leucine on *in vivo* protein synthesis in rat skeletal muscles. Instead of examining long-term infusion effects of leucine, experiments were designed to simulate the conditions of *in vitro* system by using a single injection of a large dose of leucine.

MATERIALS AND METHODS

Animals and Chemicals

Male Sprague-Dawley rats weighing 80-110 grams were used in all experiments. Weanling rats(55-60 grams) were purchased and housed individually. Rats were fed *ad libitum* a commercial pelleted diet(crude protein 23%) in a temperature and light (12/12 hour light-dark cycle) controlled room. Group assignment was made randomly by balancing mean body weights among groups. The 1-day fasted group animals were deprived of food for 24 hours. Water was on free access all the time. All the chemicals used were the highest quality obtainable and L-(U-^{14}C)-tyrosine(specific activity, 513mCi/mmol) was purchased from Amersham(Arlington Height, IL).

Supplementation of Leucine

For leucine treatment animals had a single intraperitoneal(i.p.) injection of either 80μmoles or 160 μmoles of leucine at five minutes prior to the injection of the radioactive amino acid. Control group animals while were sham injected with saline. An injection volume was 1㎖.

Determination of the Rate of in Vito Protein Synthesis

A single large dose injection method which was initially described by Henshaw *et al*[14]. was used. To minimize the diurnal variation, the rate of protein synthesis was measured between 8 : 30 and 11 in the morning. Animals were injected i.p. with 1.5㎖ of solution containing 10μCi L-(U-^{14}C) tyrosine per 100 gram body weight(B.W.) and 18μmoles of unlabeled tyrosine. After timed, 10, 20, 30, 40, 60 minutes incorporation animals were killed by decapitation. Soleus, EDL, and plantaris muscles from hind limb were rapidly removed and put into 6㎖ of ice cold 10% trichroloacetic acid(TCA). Total incorporation of ^{14}C-tyrosine and tissure free tyrosine specific activity were measured as described in the previous report[7]. In brief, muscles were homogenized in TCA with polytron homogenizer (Brinkman). The homogenates were centrifuged to precipitate protein and the acid soluble supernatant was decanted and aliquots were used to determine the tyrosine concentration by the method of Waalkes and Udenfriend[15] and radioactivity by liquid scintillation counting(Model LS 9000, Beckman Instrument). The protein pellet was washed two times with 5% TCA and once with ethanol-ether(1 : 1) centrifuging between each wash. The pellet was solubilized in 4㎖ of 0.5N NaOH by heating at 80℃ in a water bath. An aliquot of solubilized muscle protein was used to determine protein concentration[16] and specific activity of ^{14}C-tyrosine. For the determination of serum specific activity blood was collected into test tubes from the cervical trunk after decapitation. After clotting *in situ* the blood was pooled and centrifuged(Dynac$_{TM}$ Clay-Adams) at 1500×g for 15 minutes. Serum was removed and deproteinized in an equal volume of ice cold 20% TCA by centrifuging(Beckman LS 2000). Concentration and radioactivity of free tyrosine in the serum were analyzed in the same way as the acid soluble muscle samples. The rate of protein synthesis was determined from the incorporation of ^{14}C-tyrosine divided by total cellular specific activity of acid soluble tyrosine and expressed as nanomoles of tyrosine incorporated per milligram of tissue in 30 minutes.

Cellular Free Leucine Concentration

From one side of hind limb of sacrified animals, soleus muscles were isolated and rapidly frozen in liquid nitrogen. Muscles from 4 animals in each treatment were put in 1.5㎖ of ice cold 3.5% sulfsalycilic acid and then homogenized with a polytron for 20 seconds. The homogenate was kept on ice for 20 minutes and centrifuged for 5 minutes at 3000rpm at 4℃ (Beckman LS 2000). An aliquot(300 μl) of supernatant was diluted 1 : 1 with 0.3N lithium citrate buffer, pH 2.2. Leucine concentration in the sample was determined by an automatic amino acid analyzer(Beckman 121) using an external standard of aminobutylic acid.

Table 1. Body and muscle weights of fed and food-depreived rats[1]

	Fed	24hr Food-Deprived
Body Weight, g	106± 2.8	87± 3.1 (82%)[2]
Muscle Weight, mg		
Soleus	38± 0.6	36± 0.8 (95%)
EDL	44± 1.2	38± 1.0 (86%)
Plantaris	92± 1.6	80± 2.2 (87%)

[1]Data are presented as mean± SEM with n=12
[2]Values in parentheses indicate percentage relative tc fed group.

Statistical Analysis

Experimental results are presented as mean± standard error mean(SEM). Significant difference between means was determined by Student's t-test. Two tailed t-values were used to determine probability levels.

RESULTS AND DISCUSSION

Table 1 presents the body weights and the weights of soleus, extensor digitorium longus(EDL), and plantaris muscles of fed and 1-day food-deprived rats. Fast twitch, glycolytic muscles EDL and plan-

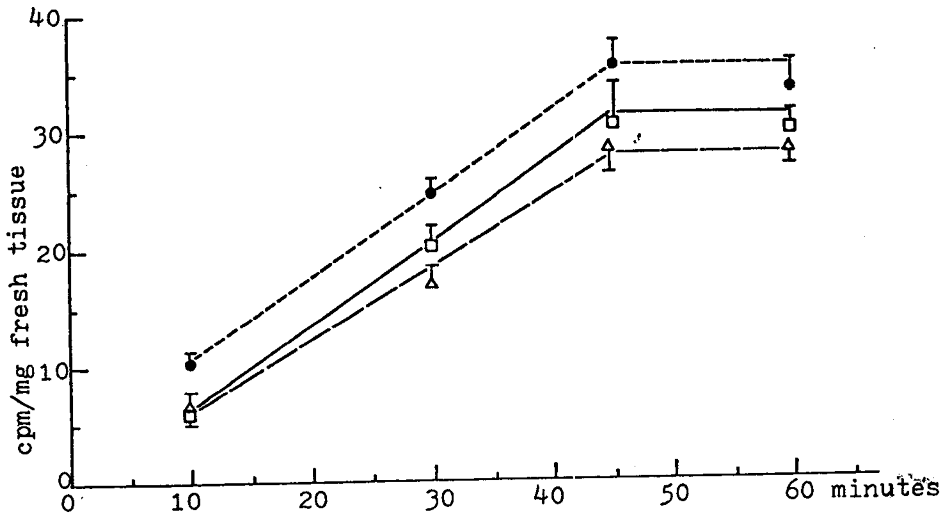

Fig. 1A. Incorporation of ^{14}C-tyrosine into muscle proteins

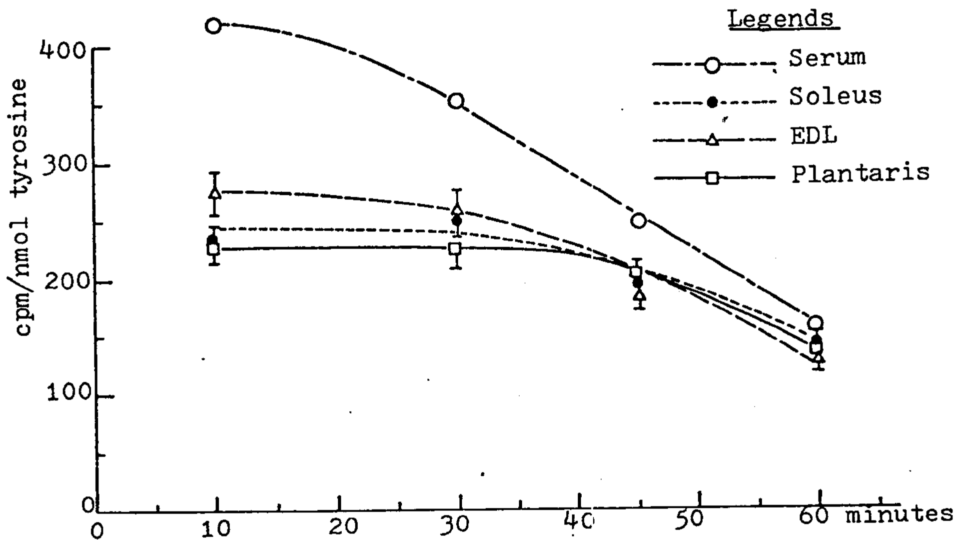

Fig. 1B. Changes in specific activities of acid-soluble ^{14}C-tyrosine in serum and muscle

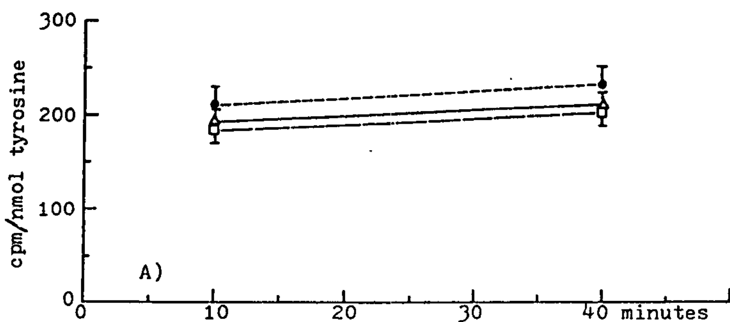

Fig. 2A. Rats weighing an average of 100 grams were injected i.p. with 18μmoles of unlabelled tyrosine and 10μCi(U-^{14}C)-tyrosine in 1.5mℓ of solution. Each point represents samples from 5-6 animals.

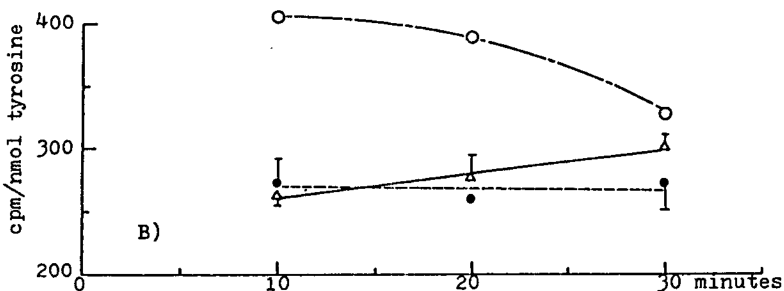

Fig. 2B. Rats weighing an average of 81 grams were injected i.p. with 60μmoles of unlabelled tyrosine and 9μCi(U-^{14}C)-tyrosine in 2mℓ of solution. Each point represents samples from four animals except for serum specific activity which was determined on a pooled sample from four rats.

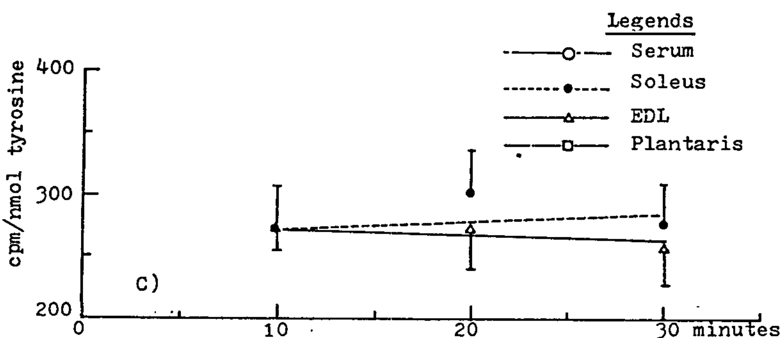

Fig. 2C. Rats weighing an average of 100 grams were injected i.p. with 24μmoles of unlabelled tyrosine and 10μCi(U-^{14}C)-tyrosine in 2mℓ of solution. Each point represents samples from four animals.

Fig. 2. Changes in specific activities of acid-soluble ^{14}C-tyrosine after different levels of unlabelled tyrosine injection

taris were more stressed than the soleus muscle which is slow twitch and oxidative one. This observation was confirmed in several experiments[4)13)18)].

Time-Course Changes in Specific Activities of ^{14}C- Tyrosine

The time-course changes in specific activity of ^{14}C-tyrosine in TCA soluble supernatant from muscle homogenate and in muscle proteins are presented in Figure(Fig.) 1A and Fig. 1B. A large dose of unlabeled tyrosine(18μmoles) was injected with the labeled tyrosine, thus the specific activity of the free amino acid pool would reach equilibrium rapidly and the precursor specific activity would remain relatively constant throughout the period of incorporation. As shown in Fig. 1A ^{14}C-tyrosine into tissue protein increased linearly between 10 and 45 minutes(mins) in all three muscles. Observation of tissue free tyrosine indicated that the specific activity reached its peak within 10 mins after injection and maintained this level for the following 20 mins(Fig. 1B). The specific activity started to drop between 30 and 45 mins after injection and declined continuously up to 60 mins. When higher concentration of unlabeled tyrosine 24 or 60μmoles per 100gram B.W. were administered, free tyrosine specific activity was relatively constant between 10 and 30 mins(Figs. 2B & 2C). However, the limited solubility of tyrosine created more individual variation and injection difficulties. In comparison to previous studies which used a massive dose of unlabeled amino acids 75μmoles of lysine[14)], 100μmoles of leucine[18)], or 267μmoles of valine[19)], a much lower amount of tyrosine, 18μmoles, appears to be adequate to stabilize the precursor pool(Fig. 1 & 2).

With these observations it appears proper that the rate of protein synthesies be determined between 10 to 40 mins after the injection of the radioisotope tracer. The nanomoles of tyrosine incorporated into tissue for 30 mins was determined by the incorporated ^{14}C-tyrosine between 10 to 40 mins divided by the average total cellular free tyrosine specific activity in the same period.

Serum specific activity declines rapidly after 20 mins, however, it remained higher than cellular specific activity(Figs. 1B & 2B). Gan and Jeffay[20)] reprorted that regardless of the route of radioisotope injection plasma specific activity remains higher than that in the tissue. Intracelluar dilution of isotope with unlabeled amino acids released from proteolysis within the muscle may attribute to that partly. In this case calculated precursor specific activity may be slightly higher than true one thus, the rate of protein synthesis might be slightly under-estimated.

Effects of Leucine on Muscle Protein Synthesis

In Fed Rats : The *in vivo* effects of leucine on skeletal muscle of fed rats were examined by a single large dose injection of leucine. The rates of protein synthesis determined were almost 3-fold higher than those with the isolated muscle technique(Table 2 & Chang Hong & Layman[7)], *in vitro* soleus, 0.036 and EDL, 0.026 *vs. in vivo* soleus, 0.102 and EDL, 0.076nmol/㎎ tissue-30 mins). The oxidative soleus muscle had higher synthesis rate than that in the glycolytic EDL muscle, which is in accord with the previous findings.

Fifteen minutes after injection of 80 or 160μmoles of leucine, the free leucine in the soleus muscle was increased by 4-and 6.8-fold, respectively(Table 2). Leucine, 160μmoles, elevated the rate of protein synthesis slightly in the EDL however this stimulation was not significant. Neither the soleus nor the EDL muscles responded to the administered leucine.

In this experiment no stimulatory effect of leucine was observed in muscles of fed rats. This observation is in contrast to the stimulatory effects demonstrated in isolated muscles from fed rats. Since

Table 2. *In vivo* effects of leucine on muscle protin synthesis of fed rats[1]

Treatment[2]	Total Cellular Free Leucine	^{14}C in Protein	Total Cellular Free Tyr Specific Activity	Protein Synthesis
	μmol/g tissue	*cpm/mg tissue*	*cpm/nmol tyr*	*nmol tyr / mg tissue-30min*
Soleus				
Control	0.308	20.6± 2.3	203± 22	0.102± 0.012
(+) 80Leu	1.245	22.8± 0.8	225± 24	0.101± 0.004
(+) 160Leu	2.091	25.6± 1.3[3]	232± 28	0.110± 0.006
EDL				
Control	–	17.0± 2.4	223± 24	0.076± 0.011
(+) 80Leu	–	18.1± 1.2	239± 20	0.076± 0.005
(+) 160Leu	–	21.1± 1.5	235± 20	0.089± 0.007

[1]Results are presented as mean± SEM with n=4. None of the results of treatment was statistically different from control by Student's t-test

[2]Control ; saline sham injection, (+) 80Leu ; 80μmoles of leucine injection, (+) 160Leu ; 160μmoles of leucine injection.

[3]$0.05<p<0.01$.

the isolated muscles are in catabolic states the stimulatory effects of leucine have been proposed to be most significant under catabolic conditions[3)7)], possible effects of leucine on protein synthesis of muscles cannot be ruled out.

In Fed and 1-Day Food-Deprived Rats : The effects of i.p. injection of 160μmoles of leucine on protein synthesis in skeletal muscles of fed and starved rats were examined. The results are presented in Table 3. Protein synthesis was expressed as nanomoles of tyrosine incorporated per milligram protein instead of per milligram fresh tissue. Since 16% of muscle is protein, the rates in Table 3 are approximately 6 times than those in Table 2.

The differences in the synthesis rates between red and white muscles in normal fed rats were not as obvious as expected. The red soleus had a higher rate than the pale EDL but the plantaris muscle which has similar fiber type composition as the EDL showed a higher rate than the soleus (Table 3). Previously it was reported that different muscles have different growth spurt[21)]. That may explain the result shown in this study.

Protein synthesis in skeletal muscles appears to be very sensitive to food availability. Food deprivation caused greater losses of muscel mass in the EDL and plantaris muscles than the soleus and this was reflected in the rates of protein synthesis (Tables 1 & 3). Fractional synthesis rates(FSR), 16.9~20.9%/day, in the muscles of fed rats(Table 3.) are in agreement with the weight matched FSR values in the literature utilizing either a single injection method or a continuous infusion technique (16.1~20.0%/day) in the gastrocnemius[22-24)].

A massive dose of leucine(160μmoles) stimulated protein synthesis in the EDL and plantaris muscles of 1-day food deprived rats. This dose of leucine is equivalent to one third of daily requirement for leucine[25)26)] and is sufficient to raise the muscle leucine concentration to at least 6x the normal level. The muscles from fed rats and soleus muscle from starved rats did not respond to leucine. The lack

Table 3. *In vivo* effects of leucine on protein synthesis in fed and 1-day food deprived rats[1]

Treatment[2]	^{14}C in Protein	Acid Soluble Tyr S.A.	Synthesis Rate	FSR[3]
	cpm / mg prot-30 min	cpm / nmol tyr	nmol tyr / mg prot-30min	%/day
FED				
Soleus				
Control	135 ± 3	221 ± 13	0.613 ± 0.013	19.0
(+) 160Leu	148 ± 9	228 ± 10	0.649 ± 0.055	
EDL				
Control	112 ± 5	205 ± 8	0.547 ± 0.023	16.9
(+) 160Leu	118 ± 10	209 ± 5	0.567 ± 0.050	
Plantaris				
Control	130 ± 8	192 ± 8	0.675 ± 0.041	20.9
(+) 160Leu	123 ± 11	200 ± 4	0.613 ± 0.053	
1-DAY DEPRIVED				
Soleus				
Control	118 ± 3	250 ± 19	0.476 ± 0.011	14.7
(+) 160Leu	136 ± 6*[4]	290 ± 19*	0.468 ± 0.021	
EDL				
Control	61 ± 4	211 ± 11	0.291 ± 0.018	9.0
(+) 160Leu	85 ± 3**	227 ± 12	0.376 ± 0.015**	
Plantaris				
Control	64 ± 6	189 ± 13	0.337 ± 0.003	11.5
(+) 160Leu	80 ± 7*	202 ± 7	0.420 ± 0.031*	

[1]Data are presented as mean± SEM with 6 observations in each group.
[2]Control ; saline sham injection, (+) 160Leu ; 160μmoles of leucine injection.
[3]Fractional Synthesis Rate(FSR) is calculated on the basis that muscle protein has 115μmol tyrosine/g protien[22)]
[4]Significance by Student's t-test ; $^{*}P<0.05$, $^{**}P<0.01$.

of a stimulatory effect of leucine on muscle protein synthesis in fed rats was not unexpected, since in the fed condition muscle protein synthesis may be in its maximum efficiency and would not respond to any additional anabolic factor. Injection of insulin into normal fed rats[27)] or feeding of excess protein above the requirement in growing rats[28)] did not further stimulate protein synthesis. On the other hand, anabolic effects of leucine were found in isolated muscles from normally well fed animals[1-7)]. These stimulations may be related to the catabolic conditions developed *in vitro* muscle system. Rates of protein synthesis measured *in vitro*[7)] and those *in vivo*(Table 2 & 3) indicate that the rate in the isolated muscles is suppressed regardless of the nutritional state. The red muscle soleus that maintains 78% of the normal level of protein synthesis after 1-day food deprivation failed to respond to leucine, while the pale EDL and plantaris muscles were reduced to almost one-half(54%) of the nor-

mal rate and responded to leucine. The catabolic states developed in EDL and plantaris muscles may have provided the conditions for leucine to exert its anabolic potential.

Since skeletal muscle accounts for over 40% of adult body mass and at least 45% of total body protein any alteration in the protein metabolism of this tissue would cause a significant impact on whole body nitrogen balance. Leucine has been proposed as a regulator for muscle protein turnover and its potential clinical application to conserve lean body mass in obese starving human subjects[9], post-surgery patients[10)11], and diabetic patients[3] has been indicated. However *in vivo* studies with leucine infusion or injecion in starved rats showed variable responses. Buse[13] reported that 300μmols of leucine infusion into 2-day starved rats(100g B. W.) failed to stimulate pretein synthesis in the gastrocnemius, while the soleus and diaphragm were stimulated. Injection of 100μmoles of leucine also failed to demonstrate any stimulation on the gastrocnemius muscle of 2-day starved or 9-day protein deprived rats[12]. The results of present and others suggest that the marked stimulation by leucine shown in isolated muscle system is rather due to the limited *in vitro* experimental condition than a general phenomenom. It appears that the development of specific catabolic states in the muscles of starved or stressed animals would be a preceeding condition for leucine to stimulate muscle protein synthesis.

While the results of this study could not define the specific conditions for leucine to activate synthesis, it appears that the primary conditions are the intial phases of the changes in protein synthesis that occur during the transition to a catabolic condition. Possible reasonings for this suggestion are that leucine stimulates the initiation which is inhibited during early starvation. Leucine may activate initiation factors[5], restrict inhibitory factors present [2)8], or provide an energy substrate[29] such as ketone bodies which may be an important energy source for skeletal muscle during short-term starvation but not in prolonged starvation[30]. Leucine is ketogenic and actively metabolized in skeletal muscles and its oxidation is increased during early starvation[31]. Therefore the fate of this amino acid may provide a unique role in the regulation of protein synthesis. Leucine may also improve the reutilization of amino acids released from proteolysis[4]. Further studies with leucine and its metabolites are necessary to clarify these speculations. In addition, it is essential to determine whether the short-term effects of leucine demonstrated in this study can be maintained for extended periods by means of infusion or feeding.

SUMMARY

In vivo effects of leucine on skeletal muscle protein synthesis in fed and 1-day food deprived young rats were examined. Animals assigned to leucine group were given a single i.p. injection of 80 or 160μmoles of leucine while control group animals were saline sham injected. The rate of protein synthesis was measured by the amount of ^{14}C incorporated into muscle protein after a single injection of ^{14}C-tyrosine, 10μCi/100g B.W. Examined muscles were two different types of hind limb muscles, the oxidative solues and the glycolytic EDL and plantaris.

Administered leucine elevated the concentration of free leucine in soleus muscles by 4-6.8 times the normal level. A massive dose of leucine, 160 μmoles, stimulated protein synthesis in the EDL and plantaris by 24%, 29% respectively of straved rats. The soleus of 1-day food deprived rats and both types of muscles in fed rats did not respond to the injected leucine. The synthesis rate of the EDL and plantaris was supressed to one-half of

the normal while the soleus that was not stimulated by leucine maintained a relatively normal rate, 78%, of protein synthesis after 1-day of food deprivation.

Thus, *in vivo* stimulatory effect of leucine appears to be not a general phenomenon but to be related to the degree of catabolic condition developed by stress such as food deprivation. Although anabolic effects of leucine observed in this study was limited, any applicability of this special property of leucine to human subjects for the purpose of protein sparing in skeletal muscles remains to be examined.

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