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Research Article

EFFECT OF SHORT AND MEDIUM TERM CALORIE RESTRICTION ON SKELETAL MUSCLE PROTEIN SYNTHESIS AND MITOCHONDRIAL ENZYME ACTIVITIES IN RATS

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A B S T R A C T

The aim of the present study was to investigate the effect of different duration of moderate (40%) calorie restriction on skeletal muscle fractional protein synthesis rate and mitochondrial enzyme activities in rats. Sixty rats were randomly divided into five groups i.e. with *Ad libitum* Food intake as controls (AL), 5 days calorie restricted (CR 5), 10 days calorie restricted (CR 10), 15 days calorie restricted (CR 15), and 30 days calorie restricted (CR 30). Fractional protein synthesis rate, ATP production rate, electron transport chain activity and plasma amino acids levels, Insulin, IGF-1 levels were measured. Muscle protein fractional synthesis rate was decreased ($P<0.05$) in CR 10, CR 15 and CR 30 groups in comparison to AL. Fractional synthesis rate was unaltered in CR 5 group in comparison to other CR groups. Levels of essential amino acids (EAA) and branched chain amino acids (BCAA) decreased ($P<0.05$) in CR 10, CR 15 and CR 30 groups. ATP production rate was not significantly affected in CR groups compared to AL group. Citrate synthase activity was reduced ($P<0.01$) in all CR groups and Complex I activity significantly reduced in CR 10, CR 15 and CR 30 groups. No significant change was observed in Complex II, III and IV enzyme activities CR10 and CR15 groups compared to AL group. Complex IV activity was increased ($P<0.05$) in CR 5 group. Insulin levels were unaltered in other CR groups; IGF-1 levels were decreased in all CR groups. In conclusion, result suggests that the skeletal muscle fractional protein synthesis rate decreased in 10, 15 and 30 days calorie restricted groups and it was maintained in CR 5 group, along with mitochondrial enzyme activities.

INTRODUCTION

Mountaineers, elite athletes competing in ultraendurance events, and deployed military personnel may be subjected to prolonged periods when caloric intake is limited and energy expenditure is high. The resultant negative energy and nitrogen balance can lead to physical (Johnson *et al*., 1994, Nindl *et al*., 2002) and cognitive (Gomez-Merino *et al.,* 2004) 3) performance decrements as well as losses in muscle mass (Friedl *et al*., 2000, Nindl *et al*., 2003). For example, during a chronic energy restriction (62 days) there was a 20 % decrease in body mass with 6 % of the decrease from fat-free mass with an associated decrease in maximal lifting strength (Nindl *et al*., 2007). Significant losses in body mass, fat free mass and lower-body physical performance can occur as early as three days (Gomez-Merino *et al.,* 2004). Reports on military field training suggest that energy deficits can be as high as 1400 kcal/day (Johnson *et al*., 1994). Under such conditions, loss of lean mass could be detrimental to physical performance, resulting in increased risk of injuries for the individual or group.

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Information on protein metabolism with different duration of calorie restriction could elucidate the importance of adequate caloric intake and it can be used to design interventions to minimize protein losses, thus potentially reducing susceptibility to injury. To our knowledge, no studies have yet looked at the effects of short (from 5 days to 30 days) to prolonged negative energy balance on protein metabolism along with mitochondrial adaptation in young rats.

Classical studies observing young, normal-weight men undergoing prolonged semi starvation indicate that the body preserves lean mass over a period of time during caloric restriction (Keys *et al.,* 1950). Initial rapid losses in body weight tend to slowdown as there are compensatory reductions in basal and voluntary energy expenditure occur (Keys *et al.,* 1950; Henry *et al*.,; 1988; Heyman *et al*., 1992). Whether the slowed rate of weight loss is accompanied by changes in protein turnover and oxidation in lean subjects is not known. Studies of short-term fasting (4 days) show increases in leucine flux and oxidation during both rest and exercise in non obese subjects (Jensen *et al*., 1988; Knapik *et al*., 1991; Nair *et al*., 1987), but few studies are available on the effects of longer duration fasting or energy restriction. Data from Yang *et al*. (13) demonstrated that 13 days of caloric restriction without a

reduction in protein intake did not affect leucine flux in nonobese males at rest. Skeletal muscle constitutes the largest single component of the body and serves as the major repository of protein (close to 50 percent of total body protein) and free amino acids pool in the body. Besides its locomotive functions, skeletal muscle is also an important metabolic organ. Muscle mass constitutes 40 to 45 percent of body weight and accounts for approximately 70 percent of body cell mass. Specifically, the net loss of muscle mass is the result of a chronic imbalance between muscle protein synthesis and breakdown. It has been suggested that a decrease in basal muscle protein synthesis may contribute to the development of sarcopenia. Although a number of mechanisms have been proposed as the underlying causes of sarcopenia, including loss of motor units, fiber atrophy, and alteration of anabolic response to hormones and nutrients (Guillet *et al.,* 2004; Mosoni *et al.,* 1995), mitochondrial abnormalities have been suggested as the key factors in muscle alterations (Sohal *et al.,* 1996; Reid and Li, 2001). Research on the mitochondrial electron transport chain (ETC) in skeletal muscle has clearly demonstrated deficient ETC activity in muscles exhibiting the greatest loss of muscle mass with age (Bua *et al*., 2002).

Therefore, present study was designed to investigate the effect of different days of calorie restriction on skeletal muscle protein synthesis rate and mitochondrial function in young rats.

MATERIALS AND METHODS

Animals and diet

All procedures and protocols used in the present study were approved by the Animal Care and Use Committee of the Institute and followed the guidelines documented in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Male albino Sprague-Dawly rats (n=48), weighing 240-280g were housed in cages (46×24×20 cm) with two animals per cage in a temperature (22±1^{o}C), humidity and light control room. Animals were provided with standard rat chow diet and water *ad libitum*. The rats were randomly divided into eight groups [7 animals in each group] as control (without calorie restriction) and calorie restricted (CR) treatment groups. The control rats (AL group) were allowed *ad libitum* access to a control pellet diet consisting of 17% protein (casein), 14% fat, and 69% carbohydrate. The CR rats were given 60% of the energy intake of the ad libitum-fed control rats. The calorie restriction was conducted for 5, 10, 15 and 30 days.

Grip strength

Forelimb grip strength was determined using an automated grip strength meter (Linton Instruments). The animal was grasped by the tail and suspended above a grip ring. After about 3 seconds, the animal was gently lowered toward the grip ring and allowed to grasp the ring with its forepaws. The animal's body was quickly lowered to a horizontal position and the animal was pulled with tail until its grasp of the ring was broken. The mean for Comparisons were performed between the amplitude of the forces exerted by control and experimental animals in N or N/g of body weight.

Sample collection

At the end of the study period, animals were fasted overnight before they were killed by decapitation. Blood was collected in the heparinised tubes and plasma was separated and stored in aliquots at -80°C until analysis. Gastrocnemius (mixed fibers) muscles were removed and immediately stored at -80°C for posterior analyses.

Isolation of Mitochondria from liver and skeletal muscle

Mitochondria were isolated from liver and muscles by modified method (Bhattacharya *et al.,* 1991). The gastrocnemius muscles of each hind limb and liver were quickly removed and placed in ice-cold isolation medium (250 mM sucrose, 10 mM Tris HCl pH 7.4, 1 mM EGTA, 0.1% defatted BSA). The tissues were finely chopped and homogenates (10 % w/v) prepared in the isolation medium using glass-Teflon homogenizer at 4°C. The homogenate were centrifuged at 4000× *g* for 15 min at 4°C, and the resulting supernatant was decanted into clean centrifuge tubes and centrifuged at 12000× *g* for 30 min at 4°C. The supernatant was discarded, and the resultant pellet was resuspended in ice-cold isolation buffer and again centrifuged at 12000 ×*g*. The final pellet was resuspended in ice-cold suspension buffer (120 mM KCl, 20 mM Sucrose, 20 mM glucose, 10 mM KH_2P0_4, 5.0 mM HEPES, 2.0 mM $MgCl_2$, 1.0 mM EDTA, pH 7.2). Protein concentrations were determined using Folin's phenol reagent (20) with BSA as the standard.

Protein synthesis rate

Fractional synthetic rate of proteins and degradation rate [Protein turnover] were measured according to the reported method of Vary *et al*. 1998 (Vary 21). Muscles were incubated at 37 °C in a standard Krebs-Henseleit medium : 120 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM $CaCl_2$, (pH 7.4) containing 5 mM glucose, 5mM HEPES, 0.1% BSA, 0.17mM Leucine. After 30 min of pre-incubation, the muscles were transferred to a fresh medium (2 ml) and incubated for a further 2 h, with a change of medium every 60 min. During the final 60min of the incubation period, 2ml of KH buffer was supplemented with 0.5mM L-[^{14}C] Leucine (BARC, India). At the end of the incubation, muscles were removed from the incubation buffer, trimmed of connective tissue, immersed into 2ml of ice cold 10% (w/v) TCA and weighed. The rate of protein synthesis was estimated by the incorporation of radioactive leucine into muscle protein. Muscles were homogenized in 2ml of 10% TCA using polytron homogenizer. The homogenate was centrifuged at 10,000g for 10 min at 4^{o}C. The supernatant was decanted and the pellet was washed 3 times with additional 10% TCA to remove any acid soluble radioactivity. The resulting pellet was dissolved in 1N NaoH and incubated at 37^{o} C for 30 min. Aliquots were assayed for protein using BSA as a standard (Lowry *et al.,* 1951). Another aliquot was assayed for radioactivity by liquid scintillation counter using corrections for quenching [Disintegrations/min]. Rates of protein synthesis expressed as µmole of leucine incorporated per hour per milligram of protein, were calculated by dividing the amount of radioactivity incorporated into muscle protein over a 1-h period by the specific radioactivity of the leucine in the incubation medium.

Blood glucose, plasma insulin, IGF-1 and amino acids

Blood glucose was estimated by glucose oxidase method (Randox Ltd.) and determined immediately after sample collection. Plasma concentrations of insulin were determined using EIA/ELISA kit (Mercodia AB, Uppsala, Sweden).

Individual plasma amino acid concentrations were measured with an HPLC method (PICO-TAG reverse-phase column; Waters, Milford, MA) as previously described (Heinrikson and Meredith 1984).

Mitochondrial ATP production rate

ATP production was determined using a firefly luciferase-based ATP Bioluminescence Assay (HSII Kit, Boehringer Mannheim). ATP was quantified using a luminometer. A solution with known concentrations of ATP was used to establish a standard curve.

Mitochondrial enzyme activities

Citrate synthase (CS) and the activities of complexes I to IV were assayed spectrophotometrically in the mitochondrial suspension as described previously (Malgat, *et al.,* 1999). The activity of citrate synthase was measured in a reaction medium consisting of 100 mM Tris-HCl, 40 µg/ml 5, 5-dithio-bis (2-nitrobenzoic acid), 1 mM oxaloacetate, 0.3 mM acetyl CoA and 4% of Triton X 100, pH 8.1. After 3 min of incubation, the reaction was initiated by adding the homogenate (20 to 50 Ag proteins) and the change in optical density at 412 nm was recorded for 3 min.

The activity of complex I was determined by monitoring the oxidation of NADH at 340 nm. Homogenate (100 µg proteins) was preincubated for 3 min in 820 µl of distilled water. Following this, 10 mM KH_2PO_4, 2 mM KCN, 5 µg/ml antimycin A, 100 µM decylubiquinone, 1.3 mg/ml BSA, 5 mM $MgCl_2$, pH 7.5 were added. The reaction was initiated by adding 200 µM NADH and the change in the optical density was recorded for 3 min. In parallel, the NADH decylubiquinone reductase activity was also measured in the presence of 12.7 µM rotenone. The specific activity of complex I was the difference between NADH oxidation activity with and without the rotenone.

The activity of succinate dehydrogenase was measured by following the reduction of 2, 6-dichlorophenolindophenol (DCPIP), in the presence of phenazine methosulfate (PMS) at 600 nm. Homogenate (50 µg proteins) was preincubated in a buffer containing 50 mM KH_2PO_4, 16 mM succinate, 1.5 mM KCN, 100 µM PMS, pH 7.5 for 5 min. The reaction was initiated by the addition of 103 µM DCPIP and the optical density was recorded at 600 nm for 3 min.

The activity of complex III was determined by monitoring the reduction of cytochrome c at 550 nm. Homogenate (50 µg proteins) was incubated, for 30 s, in a reaction medium consisting of 35 mM KH_2PO_4, 5 mM $MgCl_2$, 2.5 mg/ml BSA, 1.8 mM KCN, 125 µM oxidized cytochrome c, 12.5 µM rotenone and 62.5 mM EDTA, pH 7.5. The reaction was initiated by adding 80 µM decylubiquinol and the optical density was measured for 3 min. The nonenzymatic reduction of cytochrome c was measured under the same conditions after the addition of 10 µg/ml antimycin A. The specific activity of complex III was calculated by subtracting the activity of the nonenzymatic reaction from that of the total activity of complex III. The activity of complex IV was measured by monitoring the oxidation of reduced cytochrome c at 550 nm. A 50 µM solution of reduced cytochrome c (97% reduced using dithionite) in 10 mM KH_2PO_4, pH 7.0 was preincubated for 5 min. The reaction was initiated by adding the homogenate (20 µg proteins) and the change in optical density was measured for 1.5 min.

Statistical analysis

Results were expressed as mean ±SEM. analysis of variance (ANOVA) was performed with commercially available software (SPSS version 15.0). A P value of <0.05 was considered significant change.

RESULTS

Body weight and grip strength

The mean weight gain of the AL group during the experiment was 17.4 ±3.7%, while the CR rats lost body weight with increase in the duration of CR and respective decreases were 6.1 ± 2%, 9.2% ± 2.6% and 10.4 ±3.9%, 17.0±6.4% of basal. Although CR, as expected led to a significant decrease in body weight ($P<0.05$ *vs.* AL) the gastrocnemius muscle weights remained unaltered (Table 1). No significance change was observed in grip strength of CR rats in comparison to ad libitum rats (Table 1).

Protein synthesis rate

Skeletal muscle fractional protein synthesis rate was decreased ($P<0.01$) significantly in CR 10, CR 15 and CR 30 groups in comparison to AL group. No significant difference was observed in skeletal muscle protein synthesis rate of CR 5 group in comparison to AL (Fig. 1).

Table 1 Body mass and muscle mass in AL, CR 5, CR 10, CR 15 and CR 30 groups

Variables	5 days		10 days		15 days		30 days	
	AL	CR	AL	CR	AL	CR	AL	CR
Body mass (g)								
Initial	212±12	210±8	208±10	210± 12	212±10	210± 12	208±10	214 ± 18
Final	220 ± 4	195±10	230±12	185±16*	235±12**	180±16	245±12**	175 ± 12
Muscle (g)	1.64 ±1.4	1.4 ± 1.9	1.51 ± 2.4	1.62 ± 1.2	1.51 ± 2.4	1.32 ± 1.2	1.51 ± 2.4	1.29 ± 10
Grip strength (Force/BW)	0.068±0.004	0.055±0.002	0.076±0.002	0.066±0.003	0.071±0.001	0.064±0.009	0.068±0.002	0.059±0.001

Values are mean± SEM. AL, ad libitum rats; CR, calorie restricted (n=12). *P<0.05, **P<0.01 in comparison with initial recording.

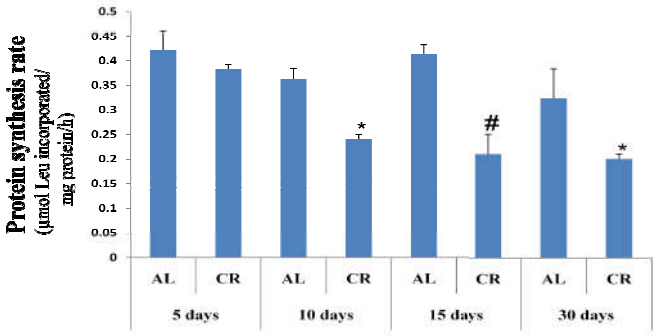

Fig 1 Muscles from all experimental groups were incubated with L-[^{14}C] Leucine for 2h and protein synthesis was then assessed based on the incorporation of radiolabelled amino acid. Results are expressed as means ± SEM (n=7).*P<0.05, #P<0.01 compared with AL group.

Rate of ATP production

No significant change was observed in mitochondrial ATP production rate in CR 5, 10 and 15 groups whereas, ATP production rate significantly decreased (P<0.05) in CR 30 group comparison to AL group (Fig.4).

Table 2 Plasma amino acids content of AL, CR groups

Variables	5 days		10 days		15 days		30 days	
	AL	CR	AL	CR	AL	CR	AL	CR
NEAA	1436±51	1288±55	1240±76	1212±56	1512±91	1034±56 #	1347±63	1216±56
EAA	716±32	686±28	804±34	604±30*	754±42	406±30 *	612±25	364±48##
BCAA	364±12	292±8	356±58	219±31*	402±21	225±18#	312±41	203±34*
Plasma glucose (mg/dl)	91.4±10.6	86.6±8.2	90.9±6.4	84±7.2	91±8.9	74.6±10.2*	92.1±12	76±9.2*

Values are represented as Mean ±SEM (n=7). Units are µM. NEAA, nonessential amino acids; EAA, essential amino acids; BCAA, branched chain amino acids. *P<0.05, #P<0.01, ## P<0.001 vs. control.

Blood glucose, plasma insulin, IGF-1 and amino acids

Plasma glucose levels were significantly decreased (P<0.05) in CR 15 and CR 30 groups compared to AL and other CR groups (Table 2). No remarkable change were observed in plasma insulin levels of CR 5, CR 10 groups in comparison to AL group, whereas plasma insulin levels decreased (P<0.05) in CR 15 and CR 30 groups (Fig.2). Plasma IGF-1 level was significantly decreased (P<0.05) in all CR groups in comparison to AL group (Fig.3). The essential amino acids (EAA) and branched chain amino acids (BCAA) levels significantly decreased in CR 10, CR 15 and CR 30 groups compared to AL group. No significant difference was observed in EAA and BCAA levels in CR 5 group compared to AL group (Table 2).

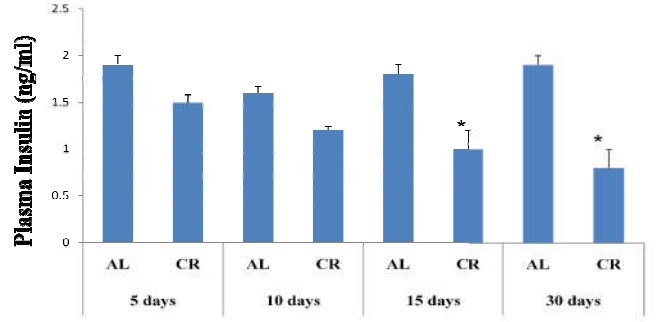

Fig 2 Plasma Insulin levels of AL and CR groups. Results are expressed as means ± SEM (n=7).

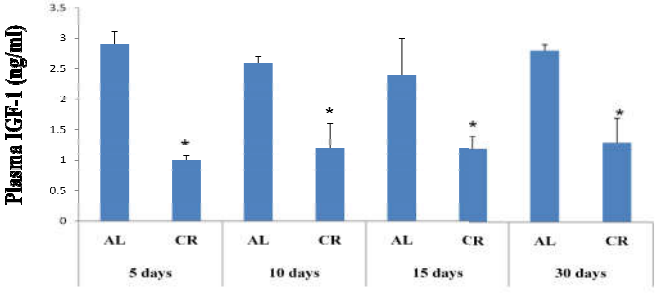

Fig.3 Plasma IGF-1 levels of AL and CR rats. Results are expressed as means ± SEM (n=7). *P<0.05 compared with AL group.

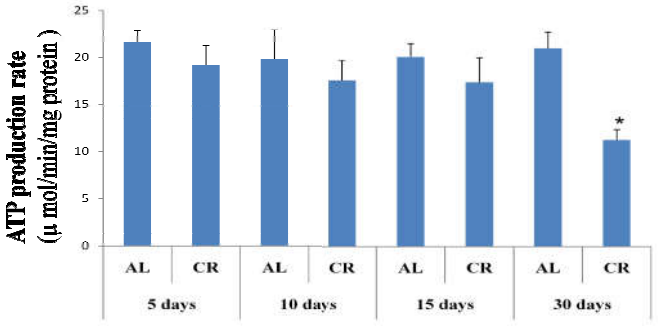

Fig 4 The mitochondrial ATP production rate in skeletal muscle of AL and CR rats. Results are expressed as means ± SEM (n=7). *P<0.05 compared with AL group.

Enzyme activities

Citrate synthase activity decreased (P<0.01) in CR 10, CR 15 and CR 30 groups in comparison to AL group, whereas enzyme activity was unaltered in CR 5 group. No significant difference was observed in CR 5 group in comparison to AL group. Given that a balanced proportion among complex activities is required for normal ETC function (Table 3). Complex I and the other complex activities (II, III and IV) were markedly reduced in the CR 15 and CR 30 groups compared to AL. No remarkable change was observed in CR 5 and CR 10 groups in comparison to AL group. Complex IV activity was significantly increased (P<0.01) in CR 5 group in comparison to AL group (Table 3).

Table 3 Mitochondrial enzyme activities in muscle of AL and CR rats

Enzyme activities	5 days		10 days		15 days		30 days	
	AL	CR	AL	CR	AL	CR	AL	CR
Citrate Synthase	4.6 ± 0.2	3.8 ± 0.1	4.4 ± 0.06	2.1 ± 0.1*	3.6 ±0.2	1.42± 0.2*	4.1± 1.2	1.4 ±0.2*
Complex I	0.98±0.04	0.84±0.06	1.2±0.08	0.87±0.02*	2.3±0.04	0.89±0.06#	1.8±0.04	0.76±0.04#
Complex II	0.36±0.02	0.29±0.06	0.46±0.01	0.16±0.04*	0.41±0.02	0.22±0.04*	0.46±0.06	0.17±0.02*
Complex III	1.34±0.3	1.1±0.2	1.46±0.2	1.01±0.1	2.14±0.4	1.1±0.1*	1.98±0.4	1.0±0.1*
Complex IV	6.7±1.1	10.6±2*	6.6±1.2	4.92±1.0	6.4±2	4.1±0.9*	7.1±1.4	3.2±1.1*

Enzyme activities are expressed in μ mol/min/mg protein. Values are represented as Mean ±SEM (n=7). AL; Ad Libitum, CR; Calorie restricted. *P<0.05, #P<0.01 vs. control.

DISCUSSION

The major finding of the present study is that 5 days moderate CR without exercise was able to maintain the skeletal muscle protein synthesis, whereas 10-30 days of moderate CR decreases the fractional protein synthesis rate in skeletal muscles, and it was associated with reduced mitochondrial enzymes adaptation along with maintained ATP production rate in young rats.

There was 28% weight gain in control animals over a period of 30 days in comparison to their initial weights. The CR rats progressively lost body weight as the duration of CR increased and no remarkable change was observed in muscle weight of CR rats. Our result correlates with the some of the published reports (Bevilacqua *et al.,* 2004; Jensen *et al*., 1988). Such a reduction in body size generally results in a lowering of whole-body energy expenditure because of reduced maintenance requirements (Drew *et al*., 2003).

Amino acids and insulin are the most potent anabolic factors, are known to increase synergistically muscle protein synthesis in human adults (Wolfe 2002; Volpi *et al.,*2000). The protein synthetic response, which occurs in disparate tissues but is most profound in skeletal muscle (Hillier *et al.,* 1998), is independently induced by the postprandial rise in insulin and amino acids (Bennet 1990). In the present study, the skeletal muscle protein synthesis was reduced in 10 to 30 days calorie restricted groups and it was maintained in CR 5 group. Here we observed decreased EAA and BCAA levels in CR 10, CR 15 and CR 30 groups and no change was observed in EAA and BCAA levels in CR 5 group. However, the change in the levels of EAA and BCAA levels and reduced IGF-1 levels may lead to decreased skeletal muscle protein synthesis rate. Insulin levels were unaltered in CR rats.

Another important finding of this study is mitochondrial ATP production rate was unaltered during 5 to 15 days calorie restricted rats but it was decreased in 30 days CR rats. Mitochondria in skeletal muscle convert energy from nutrients into ATP by oxidative phosphorylation. Normal ATP production in skeletal muscle is of key importance not only in sustaining contractile activities, but also for the high energetic demand of particular metabolic pathways such as protein turnover. Our result correlates with some of the published reports (Drew *et al*.,2003; Sreekumar *et al.,* 2002; Bevilacqua *et al.,* 2004), dietary restriction of both protein and calorie intakes had no effect on skeletal muscle ATP production rate. The capacity of skeletal muscle mitochondria to generate ATP was preserved despite the CR, probably because ATP requirements were in balance with ATP synthesis. Drew *et al*. (2003) studied the effects of aging and lifelong CR on ATP content and the rate of ATP production in rat skeletal muscle and heart. They examined 12-mo-old ad libitum-fed rats, 26-mo-old ad libitumfed rats, and 26-mo-old CR (40% restriction) rats and demonstrated decreased ATP content and ATP production with age in skeletal muscle (50% decrease in gastrocnemius) but not in heart.

Assessment of mitochondrial complex activities showed a significant decrease in enzymes of complexes I, II and III, with no significant change in complex IV In agreement with the data on ATP production with total CR. The reduced energy intake may have slowed electron flow through the ETC. This hypothesis is supported by the reduced CS activity following total CR. CS is the starting point of the Krebs cycle, and it defines the supply of electrons from reduced substrates to the mitochondrial respiratory chain. Therefore, the decrease in CS activity may be the rate-limiting step in energy production.

As the primary function of skeletal muscle is contraction, it seems logical that an improvement in energetic production and protein turnover rate would affect muscle strength. We demonstrated that energy restriction with maintenance of protein intakes leads to a significant increase in the grip force of CR rats. It is remarkable that despite the absence of nutritional treatment-related changes in muscle mass, both grip force and grip force-to-body size ratio unaltered in the CR groups. However, because the method of measuring grip force is not the best representation of muscle strength, more studies are needed to estimate the effect of CR on muscle strength in young rats.

CONCLUSION

In conclusion, the data of the present study suggests that the moderate calorie restriction (40%) for shorter term (5 days) was able to maintain the skeletal muscle protein synthesis rate along with muscular strength. CR of medium term (10-30 days) decreased the skeletal muscle protein synthesis rate but without affecting the muscular strength and these groups are under negative nitrogen balance.

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