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Research Article

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Effect of Fasting, Hypocaloric Feeding, and Refeeding on the Energetics of Stimulated Rat Muscle as Assessed by Nuclear Magnetic Resonance Spectroscopy

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Abstract

A previous study suggested that muscles from hypocalorically fed rats were limited in their ability to rephosphorylate ADP. During muscle contraction hydrolysis of ATP results in an increase in phosphorus, free ADP, ΔG_{ATP} , and a reduction in phosphocreatine levels that is reversed during rest by rephosphorylation of ADP to ATP and the resynthesis of phosphocreatine by ATP. We therefore hypothesized that these changes would be restored more slowly during postcontraction rest in hypocalorically fed rats as compared with controls. We compared controls fed ad lib to 2-d fasted and hypocalorically fed rats, losing 20% of their weight. We also compared hypocalorically fed rats that had been refed ad lib for 7 d with age-matched controls fed ad lib. The results showed that ATP, muscle pH, and total muscle creatine levels were not different in all groups. The raised phosphorus and ΔG_{ATP} levels and lower phosphocreatine/phosphorus ratio at the end of contraction changed more slowly during rest in the hypocaloric rats. These abnormalities were partially corrected by refeeding. The data taken as a whole support the concept of impaired rephosphorylation of ADP in malnourished muscle that is not completely restored by refeeding in stimulated muscle. (*J. Clin. Invest.* 1993; 92:114–121.) Key words: malnutrition • function • adenosine triphosphate • phosphocreatine • energy

Introduction

In a previous study (1) using ^{31}P nuclear magnetic resonance (NMR)¹ we showed that in resting rat gastrocnemius muscle, 2-d fasting (2DF) and hypocaloric feeding (HYPO) associated

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1. *Abbreviations used in this paper:* CONT, controls; Cr, free creatine; CREF, control group refed animals; 2DF, 2-d fasting; FADP, free ADP; HYPO, hypocaloric feeding; K_{ck} , equilibrium constant of creatine kinase reaction; NMR, nuclear magnetic resonance; PCr, phosphocreatine; Pi, free phosphorus; Q, unloaded quality factor; REF, refed animals; TCr, total creatine.

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with a 25% loss of body weight resulted in a reduction of phosphocreatine (PCr) but not ATP levels as compared with ad lib-fed controls (CONT). In that study, we showed that in HYPO rats there was a significant rise in free ADP (FADP) levels, a fall in the free energy change of ATP hydrolysis (ΔG_{ATP}), minimal change in muscle pH and normal arterial PO_2 , pH, and PCO_2 . Refeeding the HYPO rats for a week corrected these abnormalities. The data were unlikely to be caused by changes in fiber type and/or anoxia, but they suggested a limitation of the rephosphorylation of ADP to ATP, indicating that malnutrition may inhibit mitochondrial function and/or limit glycolysis (1). The limitation of ATP synthesis (1) could result from low levels of phosphofructokinase and succinic dehydrogenase (2, 3) observed in muscles from malnourished animals. During muscle contraction, ATP is hydrolyzed to ADP and free phosphorus (Pi). Hence at the end of active contraction there is an increase in ADP and Pi levels in muscle. During rest these levels fall as a result of rephosphorylation of ADP to ATP. We therefore hypothesized on the basis of our previous observation that in the malnourished muscle the high levels of Pi at the end of a maximal tetanic contraction would fall more slowly during rest in the malnourished muscle as compared with those from controls or refed animals. It has been shown that the oxygen consumption by mitochondria is stimulated during muscle contraction by a rise in FADP, and until oxygen delivery is limiting, there is a linear relationship between FADP and oxygen consumption (4, 5). These facts together with our previous observations lead us to also hypothesize that the FADP levels after muscle activity will be higher and fall more slowly during rest and that the ΔG_{ATP} would be less negative and change more slowly in malnourished muscle as compared with muscles from controls and refed rats. Since it has been shown that the Pi/PCr ratio determined by NMR reflects the changes in FADP levels (5), this ratio would also be expected to be higher after muscle contraction and fall more slowly during relaxation. The raised FADP is even more significant when related to the lower absolute force of the malnourished muscle that would be expected to consume less oxygen during a tetanic contraction.

Methods

Animals and study protocol. Male Wistar rats (Charles River Laboratories, Montreal, Canada) weighing between 245 and 255 g were obtained for the study. On arrival from the supplier the rats were individually housed in an environmentally controlled atmosphere at an ambient temperature of 22°C with a 12-h light-dark cycle. The animals were fed purina rat chow (Ralston Purina Co., St. Louis, MO) containing 23% protein, 4.5% fat, 51% carbohydrate, 7.2% ash, 5.8% fiber, 2.5% added minerals, and 6% water. The rats were randomly assigned to one of five groups referred to as CONT, 2DF, HYPO, control group for refed animals (CREF), and refed animals (REF).

All groups had access to water ad lib. The CONT group (nine ani-

mals) were the controls and received food ad lib for 7 d before the study, during which time they had gained about 25% of their initial weight. The 2DF rats (seven animals) were given food ad lib for 5 d and then fasted for 48–56 h before study. They gained 19% of their initial body weight during the 5 d and lost 20% of the day 5 weight during the fast. The HYPO rats (eight animals) were fed 5 g/d of rat chow (~25% of CONT) for 7 d. Animals in this group lost 25% of their initial weight. The CREF group (five animals) were the controls for the REF group. They were given chow ad lib for 14 d. They gained 43% of their initial weight. The REF group (seven animals) were given 5 g/d of chow daily for 7 d and then allowed to eat ad lib for another 7 d. They lost 25% during the first 7 d and then regained 20% during the next 7 d.

NMR protocol. The rats were anesthetized with pentobarbital at an initial dose of 0.1 ml/100 g body wt administered intraperitoneally. A needle was inserted into the peritoneum and connected to a catheter. Through this catheter, additional doses of 0.05 ml pentobarbital were given every 30 min while the animals were studied. With this technique, we were able to keep the rats stable and well oxygenated, as shown previously (1), for the duration of the experiment. The rat was enclosed in an insulated jacket to preserve body heat while in the bore of the magnet. Before being placed in the magnet the right hind limb was shaved and the skin was reflected from the heel to expose the common tendon of the gastrocnemius, soleus, and plantaris. The tendon was dissected from the heel and was securely tied with a silk suture (no. 00; Ethicon Ltd., Peterborough, Ontario, Canada) and a length left free to be later attached to a transducer. It was not necessary to remove the skin over the muscles because we had previously shown (1) that the skin did not contribute to the ^{31}P -NMR signals received from the leg.

The sciatic nerve was exposed and was surrounded by two flat electrodes (Dantec 13L20; Dantec Electromedical and Scientific Equipment Ltd., Scarborough, Ontario, Canada). The edges of the electrodes were sewn together to ensure good contact at all times. The electrodes were connected by a cable (Dantec 13L02/12; Dantec Electromedical and Scientific Equipment Ltd.) to a stimulus isolation unit that was connected to a stimulator (model no. S-48; Grass Instrument Co., Quincy, MA). We found that the cables attached to the stimulator acted as antennae and introduced noise into the NMR spectrum. This noise was eliminated by shunting a 500-pF capacitor across the leads and by enclosing the stimulator unit in a grounded aluminum box and enclosing the stimulator cable leading to the rat in a copper pipe. This created a closed environment for the stimulation electronics.

The rat was laid on its side on a Perspex board. The prepared hind limb was secured to the board by a plastic screw that had been driven through the femur near the knee joint. The leg was then inserted into the solenoid coil, and the coil was centered over the bulk of the gastrocnemius muscle. The suture tied to the common tendon referred to above was attached to a force displacement transducer (model no. FT03; Grass instrument Co.). The transducer was attached by a cable to a Gould single-channel chart recorder (model no. 2200; Gould-Statham Inc., Cleveland, OH). The loose skin over the abdomen and the other leg was kept away from the coil by taping to the board. The board was placed in the magnet, and warm air was also blown into the bore to keep the ambient temperature at ~25°C, which, together with the jacket, kept the rat leg at normal body temperature.

Muscle stimulation protocol. The stimulator delivered square wave pulses 75 μs in duration. In separate experiments it was determined that 15–16 V resulted in maximal twitch tension. Using a supramaximal voltage of 20 V the L_0 was determined by adjusting the muscle tension until the maximal twitch tension was achieved. At the optimal L_0 it was determined that maximal tetanic force was achieved when the sciatic nerve was stimulated at a rate of 100 Hz. It was, therefore, decided to stimulate the muscle for 500 μs at 100 Hz, repeated at intervals of a minute, during data acquisition period of 32 min. The period of data acquisition was kept constant for the groups to ensure that they received the same number of stimuli.

Solenoid coil. The coil was made of five turns of a polyvinyl chloride, insulated, 50% flattened 12-gauge copper wire wound around a

former, 25 mm in diameter and 30 mm in length. The coil was tuned to 34.64 MHz and matched to 50 ω . Copper strips formed a Faraday shield between the animal leg and the coil. The presence of the shield degrades the unloaded quality factor (Q) of the coil to 125, but greatly reduces the loading effects of the animal. The loaded Q of the coil was 115.

Magnetic field shimming. Shimming of the static magnetic field was done before each day's run using the ^{31}P signal from a phantom which filled the coil and containing 85% phosphoric acid. A Lorentzian line 6–8 Hz (0.2–0.3 ppm) wide was achieved by the shim. The ^{31}P signal from a rat leg was insufficient for shimming and the Q of the coil was too great to permit the use of a ^1H signal.

Spectral acquisition. The 100-W driver of the spectrometer (CSI Omega 2.0 Tesla; General Electric NMR Instruments, Fremont, CA) was used to deliver a 90° pulse in 40 μs . The digitizing band width was chosen as 5 kHz composed of 1,024 points for the phosphorus spectrum of 1 kHz width.

NMR data acquisition was regulated by the rate of muscle stimulation. The muscle was stimulated as indicated above every 60 s. Each acquisition cycle begins with the stimulus lasting 500 μs that also triggers the spectrometer. A 200- μs delay was introduced between the triggering of the spectrometer and the first RF pulse to allow for recovery from leg movement. The eight spectra were acquired at 6-s intervals during the poststimulation recovery period referred to as time 0, 6, 12, 18, 24, 30, 36, and 42 s. This period of 0–42 s will be referred to as the "stimulation-recovery period." The data were acquired in a multiblock file. An extra RF pulse was inserted exactly 6 s before the first RF pulse of the next cycle to ensure that all eight spectra have the same magnetization recovery. The delay between the last data acquisition and this recovery compensating pulse was of variable duration to correct for inaccuracies in the dial setting of the stimulator cycle. The cycle is timed to the nearest 1/10th s and used to calculate the pulse sequence timings. The cycle was repeated 32 times. There were eight spectra, each composed of 32 acquisitions.

Spectral analysis. A broad background resulting from the bone was observed in the spectrum as demonstrated previously (1). We removed this background by applying an exponential filter of 500 Hz to the raw data and subtracting the filtered spectrum from the original data. The resulting spectrum was filtered through a 5-Hz filter. The result was a spectrum which had a consistent baseline. The areas under the peaks were determined by a computer program.

Correction for saturation. Correction for saturation was done by taking the ratio of the fully relaxed spectra from unstimulated muscle and similar spectra taken at the experimental rate of 6 s. The saturation factors were found to be 1.03 for beta-ATP, 1.21 for PCr, and 1.16 for Pi.

pH calculation. The difference in the ppm between the PCr and the Pi peaks was measured ($PCr - Pi$) and used in the following formula:

$$pH = 6.75 + \text{Log}_{10} \{ [(PCr - Pi) - 3.27] / [5.67 - (PCr - Pi)] \}$$

This formula was validated by using test solutions as described previously (1).

Biochemical methods. The muscle tissue was obtained by freeze clamping the contracting gastrocnemius muscle after the NMR spectrum had been obtained. Another biopsy was similarly obtained from the unstimulated leg. Each biopsy was immediately frozen in liquid nitrogen. It was lyophilized and biochemical analysis was undertaken within 24 h for the following metabolites: ATP, PCr, free creatine (Cr), and lactate by the fluorimetric methods of Lowry and Passoneau (6).

Absolute values of ATP, PCr, Cr, Pi. The absolute value of ATP was determined fluorimetrically as indicated above. Total creatine was determined by adding biochemically determined PCr and Cr. The values of PCr and Pi were calculated from the ratios of PCr/ATP and PCr/Pi, using the ATP levels determined fluorimetrically. Thus $PCr = PCr/ATP \times ATP$, and using the PCr levels so determined, $Pi = 1/(PCr/Pi) \times PCr$. Free creatine was then calculated by subtracting the NMR calculated PCr from total creatine.

Free magnesium. Since the relative separation between the alpha and the beta of ATP are proportional to the amount of ATP bound to Mg^{2+} , these separations were used as an index of the amount of ATP complexed to Mg^{2+} as indicated previously (1). They were also used to calculate the ratio of free Mg/bound Mg using the relationship according to Gupta (7). While there is uncertainty about the exact dissociation constant of Mg-ATP (ATP complexed to magnesium) used to convert this ratio to an absolute value (8) of free Mg^{2+} , the results can nevertheless be used to detect a relative change between the four groups studied. Since the main effect of the free Mg^{2+} levels is to alter the equilibrium constants of the creatine kinase reaction (K_{ck}), the calculated ΔG_{ATP}^0 and ΔG_{ATP} (free energy change of ATP hydrolysis), and also because the published free Mg^{2+} levels have been reported to vary between 0.2–0.4 mM (9, 10) and 1 mM (11), and 2.5 mM (12), we used a range of these values. The actual values used were 0.5 mM derived from our calculations, based on the observed separation of the three ATP peaks using the equation of Gupta et al. (7), and a K_d^{MgATP} of 50 μM (10), to higher values reported in the literature of 1.0 and 2.5 mM. These ranges were used to calculate K_{ck} , ΔG_{ATP}^0 , ΔG_{ATP} and, free ADP to determine whether our conclusions about the relative changes between control, malnourished, and refed groups were going to be affected by the possible variation in the whole possible range of free Mg^{2+} levels.

Free ADP. The free ADP levels can be calculated from the assumed equilibrium constant K_{ck} of the creatine kinase reaction using the following relationship:



$$ADP = (ATP)(Cr)/(PCr)(H^+)K_{ck} \quad (2)$$

The constant K_{ck} is dependent upon the pH and free Mg^{2+} . Therefore a range of values was calculated from the equations of Lawson and Veech (11). Using a computer spread sheet, the values of different pHs observed in our study and the assumed free Mg^{2+} values were entered into the above equations to obtain a range of K_{ck} .

ΔG_{ATP} . The free energy change of ATP hydrolysis ΔG_{ATP} can be calculated as follows:

$$\Delta G = \Delta G^0 + 2.58 \ln (ADP)(Pi)/ATP$$

However, the absolute ΔG_{ATP} depends on an estimation of the ΔG^0 , which is dependent on pH, free Mg^{2+} , and temperature. The equations of Alberthy (13) were used to calculate a range of ΔG_{ATP}^0 at a temperature of 37°C and pH measured together with a range of free Mg^{2+} levels as given above. The last term of the equation requires the measurement of the free ADP/ATP ratio, which cannot be done directly from experimental data. However, since the creatine kinase reaction is in equilibrium (14) in rat muscle, the FADP can be derived from equation (2).

Free energy change for ATP hydrolysis =

$$\Delta G^0 + 2.58 \ln (ADP)(Pi)/ATP \quad (3)$$

substituting the right side of (2) for ADP in (3),

$$= \Delta G^0 + 2.58 \ln (Cr)(Pi)/(PCr)(H^+)K_{ck}$$

Statistical analysis. The data were reported as mean \pm SE. The differences between the CONT, 2DF, and HYPO rats were tested by ANOVA for statistical significance. If the ANOVA was significant, the differences between CONT and the 2DF, CONT, and HYPO, as well as 2DF and HYPO was tested by the Tukey-Kramer test for unplanned comparisons. The differences between CREF and REF were tested by the unpaired *t* test. The effect of stimulation and recovery over the 42 s of observation after contraction was compared between CONT, 2DF, and HYPO by an analysis of covariance of the regressions between a parameter (pH, ATP, PCr, PCr/ATP, FADP, and ΔG_{ATP}) or its logarithm transform (for Pi and PCr/Pi, which showed an exponential fall) and time. If the slopes were not significantly different then significant differences between the intercepts showed that there was an overall

significant difference in the levels of these parameters during the stimulation-recovery period. If the slopes were significantly different, then the rate of recovery was considered to be different.

Results

Rat weight

The mean initial weights at the time of entry to the study were not significantly different between the groups by ANOVA. The CONT rats gained weight over 7 d of study from 247.0 \pm 2.2 to 313.8 \pm 2.5 g ($P < 0.01$). The weights of the 2DF were not changed significantly at the end of 7 d and those of the HYPO rats fell significantly from 255.8 \pm 4.5 to 192 \pm 3.5 g over the 7 d period. The weights of the REF rats (305 \pm 9.0 g) were significantly lower than the CREF (366.4 \pm 3.7 g; $P < 0.05$) after 14 d of study.

Qualitative changes in NMR spectra between CONT and HYPO rats

The data are illustrated in Fig 1. Note that the PCr peak is lower and the area smaller in HYPO rats. Note that the separation between the Pi and PCr peaks and between the alpha- and beta-ATP peaks are the same between the CONT and HYPO spectra showing that the pH and free Mg^{2+} levels were the same in the two nutritional groups.

Muscle pH

The pH of muscle did not change significantly during the stimulation-recovery period in any of the groups studied. The mean pH were not different between CONT (7.15 \pm 0.001), 2DF (7.19 \pm 0.002), and HYPO (7.19 \pm 0.001). Also by the same criteria the mean pH of the CREF (7.18 \pm 0.001) rats was not different from that in REF (7.14 \pm 0.002) rats.

Ratio of PCr to ATP

The PCr/ATP ratio at time 0 during the stimulation-recovery period as determined by the intercept of the regression was

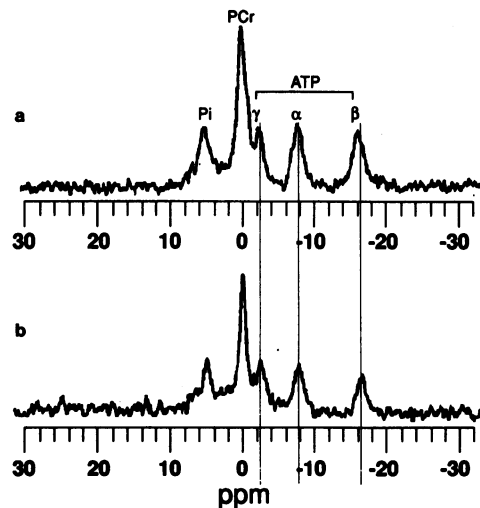


Figure 1. (a) NMR spectrum of CONT rats 200 μs after the end of stimulation (time 0). Pi, PCr, and ATP peaks. The separation between the ATP peaks are similar to those in HYPO rats (b). b shows NMR spectrum of HYPO rats 200 μs after the end of stimulation (time 0). Pi, PCr, and ATP peaks. The separation between the ATP peaks are similar to those in CONT rats (a).

significantly lower in 2DF (2.55 ± 0.005) and HYPO (2.47 ± 0.006) as compared with CONT (3.45 ± 0.001 ; $P < 0.03$). At time 0, the PCr/ATP ratio of the CREF (3.28 ± 0.003) rats was significantly higher than that in REF (2.96 ± 0.001) rats ($P < 0.01$).

Ratio of PCr to Pi

Analysis of covariance showed that the PCr/Pi ratio of muscle increased significantly ($P < 0.01$) during the stimulation-recovery period in all the groups studied. The slope of this regression was significantly lower for HYPO ($P < 0.05$) as compared with both CONT and 2DF (Fig. 2 a). The ratio in relation to time also increased significantly ($P < 0.01$) during the stimulation-recovery period in the CREF and REF rats. The slope of this regression was significantly lower for REF as compared with CREF rats ($P < 0.05$; Fig. 2 b).

Ratio of ATP/total phosphorus, PCr/total phosphorus and Pi/total phosphorus

In CONT rats, the ATP/total phosphorus was 0.162 ± 0.016 and 0.178 ± 0.015 at time 0 and 42 s, respectively ($P = \text{NS}$). The PCr/total phosphorus was 0.672 ± 0.013 and 0.768 ± 0.013 at time 0 and 42 s, respectively ($P < 0.01$). The Pi/total phosphorus was 0.164 ± 0.02 and 0.053 ± 0.009 at time 0 and 42 s, respectively ($P < 0.01$).

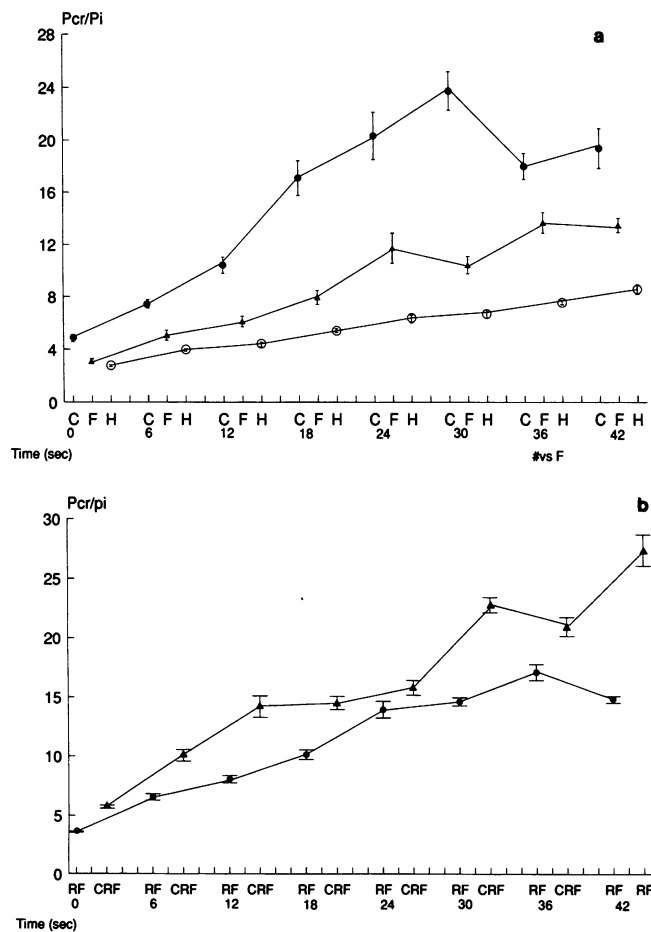


Figure 2. (a) PCr/Pi ratio of muscle of CONT (C), 2DF (F), and HYPO (H) rats measured 200 μs after the end of stimulation (time 0) and at 6-s intervals to 42 s. (b) PCr/Pi ratio of muscle of CREF (CRF) and REF (RF) rats measured 200 μs after the end of stimulation (time 0) and at 6-s intervals to 42 s.

phorus was 0.164 ± 0.02 and 0.053 ± 0.009 at time 0 and 42 s, respectively ($P < 0.01$).

Muscle free Mg^{2+}

This was estimated indirectly from the separation of the alpha and beta peaks of the ATP spectrum (7), this distance is independent of pH changes (7). The separations were 282.1 ± 4.7 , 275.1 ± 3.6 , and 286.5 ± 3.9 Hz for CONT, 2DF, and HYPO rats, respectively, which were not significantly different by ANOVA. In addition a more exact estimate of free Mg^{2+} was made using the relationship given by Gupta et al. (7) and the apparent dissociation constant K_d^{MgATP} given by them of $50 \mu\text{M}$ (10). The mean calculated value of $536 \mu\text{M}$ was similar to that we had published earlier (1). Also the calculated values were not significantly different between the groups.

Effect of pH and Mg^{2+} differences on K_{ck} and the ΔG^0 in ATP hydrolysis

There were no statistically significant differences in the pH and the Mg^{2+} between the different groups. Table I shows the the calculated K_{ck} and ΔG_{ATP}^0 values for the pH range in our study using the mean Mg^{2+} calculated from our spectra of 0.5 mM , those used by Lawson and Veech (11) of 1 mM and the higher one of 2.5 mM used by Wu et al. (12). Although the level of free Mg^{2+} alters the K_{ck} and ΔG_{ATP}^0 , the relative differences between the groups remain the same. Furthermore, when these various values for free Mg^{2+} are used to calculate the free ADP levels and the ΔG_{ATP} of the experimental groups, they did not change the significance of the differences observed between them, because they are constants in the equation given on pages 12 and 13. Therefore, in the figures and tables, we have illustrated the results for free ADP and ΔG_{ATP} by displaying data calculated using a Mg^{2+} of 0.5 mM (our calculated value).

Muscle ATP, PCr, Cr, total creatine, Pi, and lactate levels

Muscle ATP. There are no significant differences in ATP levels between the different groups by ANOVA (Fig. 3). We also did not find any significant differences between the ATP levels in the muscles from stimulated and unstimulated legs.

Total creatine. There was no significant difference by ANOVA among CONT, 2DF, and HYPO. The total creatine was 32.2 ± 1.9 , 33.5 ± 1.0 , and $35.7 \pm 1.8 \mu\text{mol/g}$ wet wt. There was no significant difference in the total creatine content of muscle between CREF (33.9 ± 1.1) and REF ($36.4 \pm 2.9 \mu\text{mol/g}$ wet wt).

Phosphocreatine. At the end of the stimulation (time 0) given by the intercept of the regression of PCr, the PCr levels of

Table I. Effect of pH and Free Mg^{2+} Differences on the Equilibrium Constant of the Creatine Kinase Reaction and ΔG^0 of ATP Hydrolysis

Free Mg^{2+}	pH	$K_{ck} (\times 10^9)$	ΔG_{ATP}
mM			kJ/mol
0.53	7.14	1.239	36.51
0.53	7.19	1.236	36.69
1.00	7.14	1.681	36.08
1.00	7.19	1.677	36.27
2.50	7.14	2.328	35.87
2.50	7.19	2.322	36.08

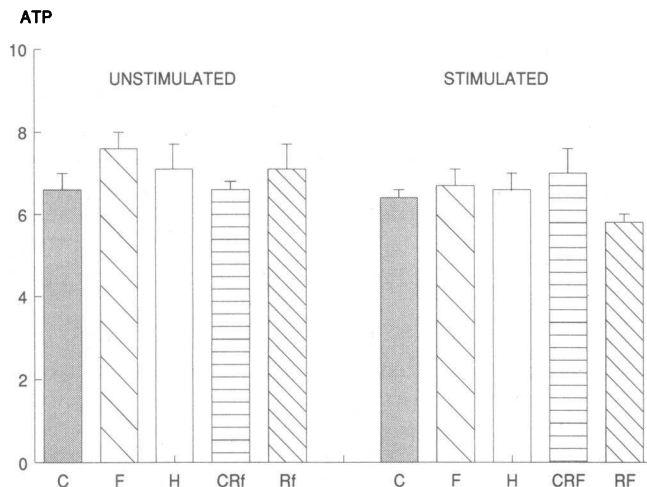


Figure 3. ATP levels ($\mu\text{mol/g}$ wet wt) of unstimulated and stimulated muscle. The unstimulated muscle was freeze clamped from the unstimulated leg at the end of the study. The stimulated muscle was freeze clamped during the muscle contraction at the end of the study. C, CONT; F, 2DF; H, HYPO; CRF, CREF; and RF, REF.

CONT ($26.6 \pm 1.4 \mu\text{mol/g}$) was significantly higher ($P < 0.01$) than 2DF ($20.6 \pm 2.5 \mu\text{mol/g}$) and HYPO ($18.3 \pm 1.7 \mu\text{mol/g}$), the latter two were not significantly different from each other. The PCr at the end of the stimulation (time 0) given by the intercept of the regression was significantly higher ($P < 0.01$) in CREF rats ($27.4 \pm 2.6 \mu\text{mol/g}$) as compared with REF ($20.2 \pm 0.89 \mu\text{mol/g}$).

Pi levels. The Pi levels at the end of the stimulation (time 0) given by the intercept of the regression of Pi (Fig. 4 a) was significantly higher in the HYPO rats than in CONT or 2DF ($P < 0.03$). The Pi levels fell significantly during the stimulation-recovery period in all the above three nutritional groups ($P < 0.01$), and the slope of the HYPO rats were significantly lower than CONT and 2DF rats (Fig. 4 a). The Pi levels at the end of stimulation were not significantly different between the CREF and REF groups. The Pi levels fell significantly during the stimulation-recovery period, but the rate of decline was not different between the CREF and REF groups (Fig. 4 b).

Lactate levels. The lactate levels in the stimulated leg were 1.06 ± 0.29 , 0.92 ± 0.26 , and $1.85 \pm 0.33 \mu\text{mol/g}$ wet wt in CONT, 2DF, and HYPO muscles, and they were significantly different by ANOVA ($P < 0.05$). The HYPO rats were significantly higher than CONT and 2DF ($P < 0.05$). The lactate levels in the unstimulated leg were 0.87 ± 0.26 , 0.39 ± 0.11 , and $1.64 \pm 0.21 \mu\text{mol/g}$ wet wt in CONT, 2DF, and HYPO rats. The higher levels in the HYPO rats were not significantly different between the stimulated and unstimulated legs. The lactate levels were not significantly different between the CREF and REF rats.

FADP levels. The mean FADP levels calculated based on a Mg^{2+} level of 0.5 mmol/liter , were the lowest for CONT ($2.08 \pm 0.22 \text{ mol} \times 10^{-5}$), the highest for HYPO ($5.95 \pm 0.50 \text{ mol} \times 10^{-5}$), and the 2DF levels ($3.57 \pm 0.25 \text{ mol} \times 10^{-5}$) were between the other two. The differences were significant between each of the three groups ($P < 0.03$) with the other two. These differences were found to be significant irrespective of the assumed free Mg^{2+} level. The FADP at the end of stimulation was significantly lower in CREF ($2.77 \pm 0.25 \text{ mol} \times 10^{-5}$), as compared with that in REF ($4.64 \pm 0.32 \text{ mol} \times 10^{-5}$).

ΔG_{ATP} . The calculated ΔG_{ATP} levels at the end of stimulation (time 0) are given by the intercept of the regression (Fig. 5 a). The CONT levels were the highest, the HYPO were the lowest, and the 2DF levels stood between the other two. At time 0, the HYPO and 2DF were significantly lower than controls. At 42 s during the stimulation-recovery period, the differences were significant between each of the three groups ($P < 0.03$) with the other two (Fig. 5 a). These differences were found to be significant irrespective of the assumed free Mg^{2+} level. The ΔG_{ATP} at time 0 was significantly lower in REF as compared with the CREF rats (Fig. 5 b), and the levels of ΔG_{ATP} recovered more slowly in REF rats during the stimulation-recovery period.

Discussion

In this study, we had three different models of nutrition. Controls of two different durations (CONT and CREF), an acute fast (2DF), a chronic weight-losing state (HYPO), and recovery from this state (REF). In addition, the study was designed to examine the question of the effect of the added energy expenditure of muscle contraction on the energetics in relation to the nutritional status. In this study, we observed two different

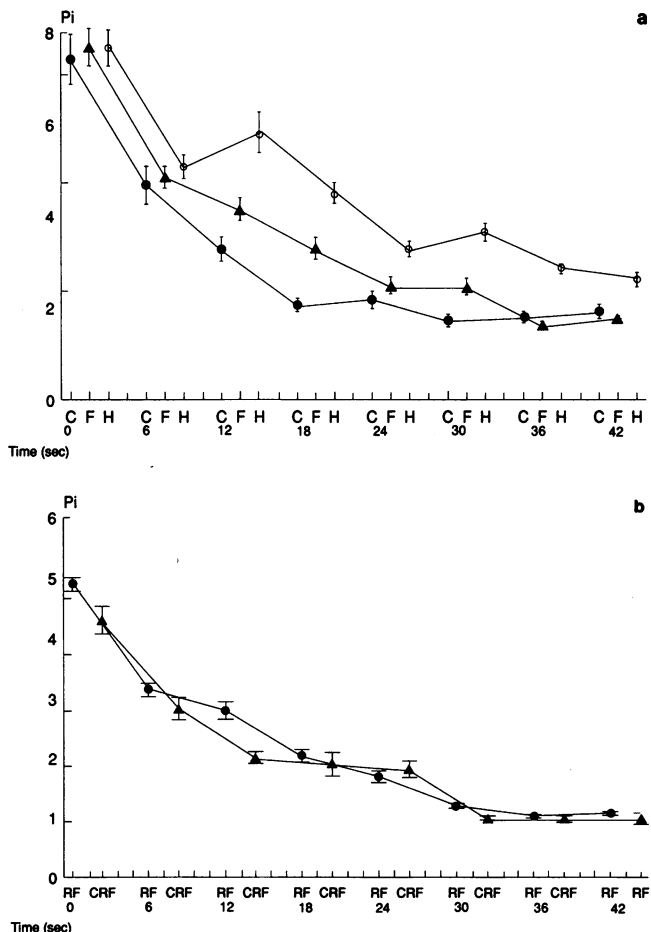


Figure 4. (a) Pi levels ($\mu\text{mol/g}$ wet wt) of muscle of CONT (C), 2DF (F), and HYPO (H) rats measured $200 \mu\text{s}$ after the end of stimulation (time 0) and at 6-s intervals to 42 s. (b) Pi levels ($\mu\text{mol/g}$ wet wt) of muscle of CREF (CRF) and REF (RF) rats measured $200 \mu\text{s}$ after the end of stimulation (time 0) and at 6-s intervals to 42 s.

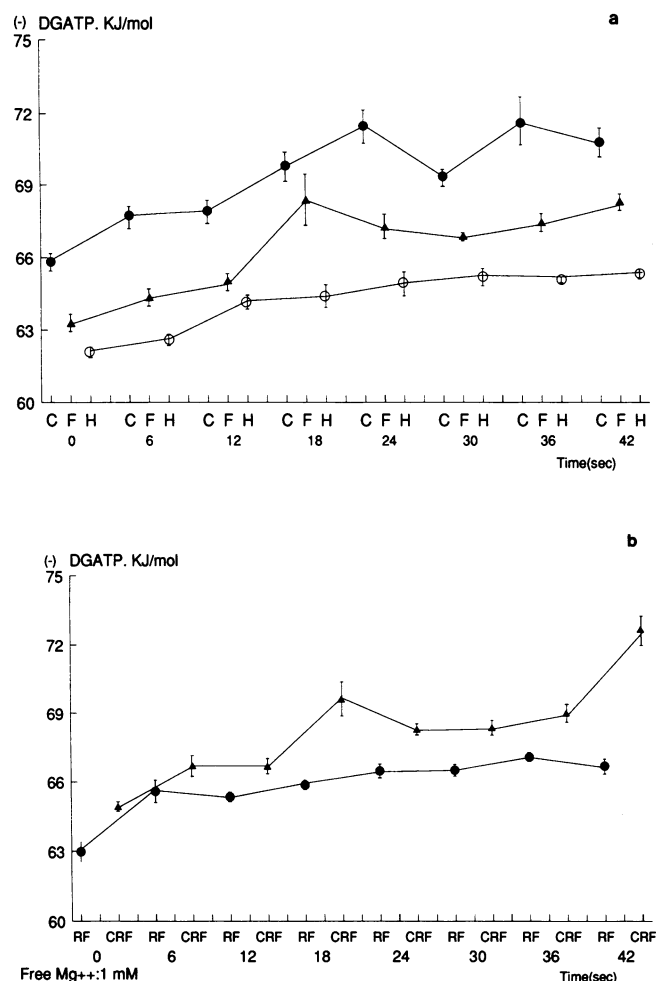


Figure 5. (a) ΔG_{ATP} levels (kJ/mol of ATP) of muscle of CONT (C), 2DF (F), and HYPO (H) rats measured 200 μ s after the end of stimulation (time 0) and at 6-s intervals to 42 s. (b) ΔG_{ATP} levels (kJ/mol of ATP) of muscle of CREF (CRF) and REF (RF) rats measured 200 μ s after the end of stimulation (time 0) and at 6-s intervals to 42 s.

stimulated states. First, the effect of chronic stimulation with only a 1-min recovery observed over the entire study period, and second, the acute recovery after a stimulus during the period of 1 min. We have already published the energetic status in these nutritional states in the completely unstimulated state (1).

CONT and CREF

Previously, we had studied muscle energetics in the unstimulated muscle in an identical model of nutritional manipulation (1). The only difference is the inclusion of CREF REF animals. The biochemical techniques used in this study are identical to those used previously (1) and the NMR techniques substantially the same, but modified for the fact that these muscles required stimulation. In our previous study, we showed that in control animals, the ATP, PCr, Pi, pH, ratios of ATP, PCr, and Pi/total phosphorus (ATP + PCr + Pi) were comparable to those published earlier (15) for the cat biceps a comparable fast twitch muscle. In addition, the calculated FADP and ΔG_{ATP} were also comparable to those published by others (15, 16) if

the free Mg^{2+} is assumed to be the same as in those studies, namely 1 mM.

In this study, the CONT ATP, PCr, and Pi levels 42 s after the stimulus, the time of maximal rest before the next stimulus, were similar to those published earlier for unstimulated muscles (1). Correspondingly, the mean PCr/total phosphorus of 0.768, Pi/total phosphorus 0.053 and ATP/total phosphorus were similar to those published by us earlier for unstimulated muscle in controls (1) and by Meyer et al. (15). At time 0, just after the muscle was stimulated, while the mean ATP/total phosphorus ratio (mean = 0.162) was not significantly different from that at 42 s after the stimulus, ratios of the Pi/total phosphorus (mean = 0.164), and PCr/total phosphorus (mean = 0.672) were significantly different. These findings show that the CONT muscles returned to the unstimulated state before the next stimulus. The results were similar in the CREF muscles. The FADP and the ΔG_{ATP} levels in controls at 42 s after stimulation were comparable to those published by Veech et al. (16) and Meyer et al. (15).

These findings suggest that in our model, we were able to reproduce the metabolite concentrations in controls at 42 s after stimuli comparable to those observed in unstimulated muscle.

Effect of stimulation on rats on a 2-d fast and on those receiving hypocaloric feeding

ATP, PCr and Pi levels. Just after the stimulation (time 0) the Pi levels were elevated and significantly higher in HYPO as compared with 2DF and CONT. The Pi levels subsequently fell exponentially during the stimulation-recovery period (Fig. 4 a). However, the rate of fall of Pi in the HYPO group was significantly slower than in CONT or 2DF showing that oxidative phosphorylation was significantly affected by hypocaloric feeding. These conclusions are supported by the fact that PCr levels were significantly lower in the 2DF and HYPO rats. Since the total creatine (TCr) levels were not different between the groups, therefore, the lower PCr is clearly caused by a change in the ratio of phosphorylated to free creatine. Meyer (17) noted that the fall and recovery of PCr induced by contractions at rates of between 0.25 to 0.75 Hz was monoexponential and consistent with a model in which the PCr is proportional to ΔG_{ATP} . Our observed lower levels of PCr in HYPO rats would suggest the presence of a lower cytosolic ΔG_{ATP} . The defect in rephosphorylation is supported by the higher FADP levels seen in 2DF and HYPO animals as compared with CONT. In addition, the FADP levels progressively rose from CONT to HYPO rats with the 2DF animals having intermediate levels. Hence, there is an abnormality of rephosphorylating ADP with 2-d fasting and this abnormality is intensified by hypocaloric feeding over a longer term with weight loss. The data based on calculated FADP is supported by similar changes in the absolute PCr/Pi ratio and its restitution during the stimulation-recovery period. This ratio is the lowest in the HYPO muscle and in addition the slope of the recovery of this ratio is the slowest in the HYPO muscle. This ratio has been shown to be a function that relates work to the concentration of control chemicals, called the transfer function (5). Since the ATP levels were comparable to those seen in controls and there were no significant differences in ATP levels as a result of stimulation, the creatine kinase reaction maintained normal ATP levels despite an abnormality of rephosphorylation of ADP. Finally, there

was also a fall in the ΔG_{ATP} , which was significantly higher in CONT as compared to 2DF and lowest in the HYPO rats (Fig. 5 a).

Effect of refeeding hypocalorically fed rats

ATP, PCr, and Pi levels. Refeeding did not completely restore the PCr levels but did restore the rate of fall of Pi during the stimulation-recovery period (Fig. 4 b). Hence, rephosphorylation of ADP appeared to have partly recovered. The higher FADP and lower ΔG_{ATP} in REF as compared with CREF suggest that rephosphorylation of ADP had not completely recovered with refeeding (Fig. 5 b). Furthermore, the free energy change of ATP hydrolysis fell at the end of stimulation (ΔG_{ATP} levels increased or less negative) in both REF and CREF rats. However, during the stimulation-recovery period, the REF rats did not restore the ΔG_{ATP} levels at the same rate as the CREF animals, indicating a residual abnormality (Fig. 5 b).

TCr, pH, and intracellular Mg^{2+} . The TCr content of muscle in relation to wet weight were not different in the groups. This finding suggests that the muscle composition in relation to the contractile elements were comparable in all groups. The pH, likewise, was not different in the groups, nor was the relative Mg^{2+} content. Since the K_{ck} and ΔG^0 are altered by the pH and the Mg^{2+} of muscle, therefore, the kinetics of the creatine kinase reaction, the calculated ΔG^0 are comparable among the groups, making the relative comparison of FADP and ΔG_{ATP} between the groups possible as we had also shown previously (1).

Significance of the observed findings. The findings confirm our previous observation in unstimulated muscle that hypocaloric feeding reduces PCr levels, increases FADP levels, and reduces ΔG_{ATP} levels. In addition, by observing the effect of stimulation, it was noted that hypocaloric feeding reduces the rate of rephosphorylation during recovery. Also, unlike the unstimulated muscle, refeeding did not restore the lower PCr, higher FADP, and lower ΔG_{ATP} levels to normal. Furthermore, the recovery of ΔG_{ATP} after stimulation was slower. The data are consistent with a reduction of oxidative phosphorylation caused by mitochondrial dysfunction for the following reasons: Similar changes have been observed in dystrophic muscle by Barbiroli et al. (18), who noted low PCr/ATP and high Pi/ATP ratios in the muscles of patients with this condition. In addition, they noted that the initial postexercise recovery of Pi was slower in patients than in controls. In addition, similar changes have been observed in other mitochondrial myopathies (19). Although the reduced PCr could be caused by a rise in the proportion of oxidative type I fibers noted to occur in malnutrition, the slow recovery of the PCr/Pi ratio noted herein is not consistent with this possibility. Type I fibers with a high oxidative activity show a very rapid postexercise recovery of the PCr/Pi ratio (20). It is not caused by anoxia, since the muscle pH did not fall. While muscle lactate measured at the end of the experiment was significantly higher in HYPO muscles, the difference could not be caused by anoxia, since it was similarly raised in the unstimulated limb and was not increased by stimulation. Ruderman and his colleagues have shown that during starvation, raised ketones may inhibit pyruvate dehydrogenase (21, 22) and alter the redox of muscle (23). The cause of the mitochondrial abnormality remains speculative, however Barbiroli et al. postulated that increased Ca^{2+} in dystrophic muscle may have altered mitochondrial function (18) in

their patients by influencing the activity of mitochondrial dehydrogenase enzymes. It is of interest that we had previously observed increased muscle Ca^{2+} and altered stimulated muscle function in obese individuals subjected to a 400-kcal diet for 2 wk and in rats on a hypocaloric diet (24, 25). In addition, in humans, we also showed that hypocaloric feeding with 400 kcal/d resulted in vacuolation of mitochondria observed by electron microscopy in muscle biopsies (24). Another possible cause for the observed changes may be the altered thyroid function seen in malnutrition. Malnutrition results in a decrease in T_3 and an increase in the less active reverse T_3 (26), and it has been shown that the ratio is lower and is restored more slowly postexercise in hypothyroid patients (27).

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