Fasting modulates creatine entry into skeletal muscle in the mouse

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Summary. [^{14}C]-Creatine entry into skeletal muscle of mice is inhibited by feeding creatine or by fasting. Plasma creatine specific activity after an i.p. injection of [^{14}C]-creatine is decreased by feeding creatine and increased by fasting. These results indicate that creatine enters muscle via a saturable process that is modulated by fasting.

Creatine is synthesized in kidney, pancreas, and liver from arginine, glycine, and methionine[2,3] and carried by the blood to muscle where it is extracted and concentrated[2]. In resting muscle, more than half of the creatine is present as phosphocreatine[4]. Two specialized processes, a saturable entry process and intracellular trapping, are known to be involved in maintaining high concentrations of creatine and phosphocreatine in muscle of certain species[5,6]. Only the entry process has been studied in detail. In the rat it is specific for amidino compounds including creatine, β-guanidinopropionic acid, and β-guanidinobutyric acid[7,8], and it is inhibited by hypoxia and metabolic poisons[6].

Initial studies suggesting that the entry process has functional importance in vivo[8] were verified by feeding rats β-guanidinopropionic acid[9] and β-guanidinobutyric acid[7]. Both compounds deplete muscle of creatine and phosphocreatine[7,9]. β-Guanidinopropionic acid competitively inhibits creatine entry[8] and serves to a small extent as a substrate for creatine kinase[10]. β-Guanidinobutyric acid inhibits creatine entry without serving as a substrate for creatine kinase[7]. When β-guanidinopropionic acid is fed to rats, white (fast-twitch, glycolytic) fibers atrophy[11,12] and red (slow-twitch, oxidative) fibers exhibit abnormal contractile characteristics[13]. Feeding β-guanidinobutyric acid to chickens causes a necrotizing myopathy[14].

In contrast to the results of studies of rats, attempts to find a saturable entry process for creatine using isolated extensor digitorum longus muscles from mice have been unsuccessful[15]. Nevertheless, creatine and phosphocreatine concentrations are reduced in mice by feeding β-guanidinopropionic acid[12] or β-guanidinobutyric acid (unpublished observations) just as in rats, raising the possibility that a saturable entry process operates in vivo despite the failure to detect it in vitro. The present experiments were designed to demonstrate more definitively the existence of a saturable entry process in vivo.

Materials and methods. Young, male, albino, outbred mice, either Hla: (SW) or Sas:CFI, were routinely provided with Purina Laboratory Chow and water ad libitum. To evaluate the response to increasing plasma creatine concentrations, mice received 2% creatine in their diet for 4 days. This special diet was prepared by dissolving creatine in water and mixing it with powdered Chow to form a thick paste. The paste was spread on cookie sheets, dried in an oven at 55 °C, and cut into small pieces for feeding. Control mice were fed Chow similarly treated except for omission of creatine. At the end of the feeding period, each mouse was injected i.p. with 1 μCi of 1-[^{14}C]-creatine (4.7 μCi/μmole, New England Nuclear Corp.) dissolved in 0.1 ml of 0.154 M NaCl. Subsequently, anesthesia was induced with ether at selected time intervals, blood was collected, the mice were killed by cervical dislocation, and tibialis anterior muscles were obtained for measurement of creatine concentration and radioactivity. One μCi of [^{14}C]-creatine did not cause a detectable increase in plasma creatine concentrations in control or creatine-fed mice.

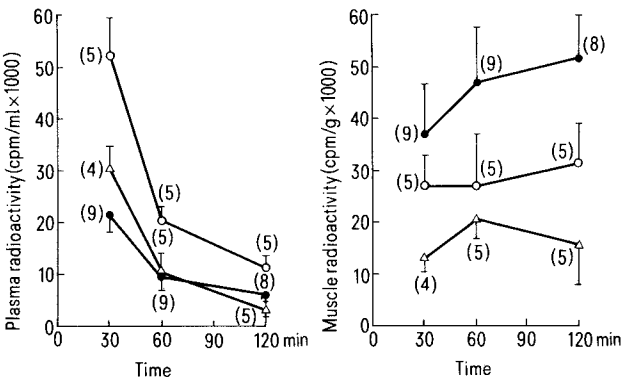

Figure 2. Time-course of [^{14}C]-creatine distribution between plasma and tibialis anterior muscles. Means ± SD and numbers of mice are shown. Control mice, ●; fasted mice, ○; creatine-fed mice, △.

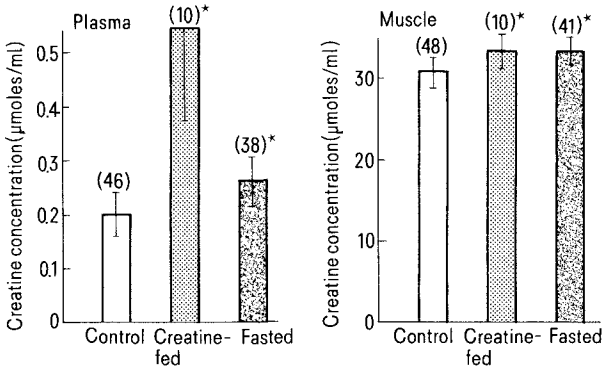

Figure 1. Creatine concentrations in plasma and tibialis anterior muscles. Means ± SD and numbers of mice are shown. The asterisk indicates $p < 0.01$ by Student's t-test.

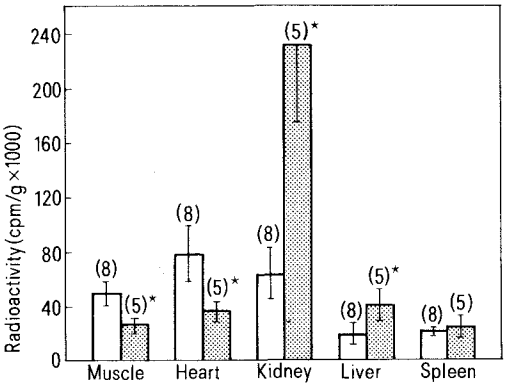

Figure 3. Effect of a 24-h fast on the distribution of [^{14}C]-creatine to tibialis anterior muscles and other tissues. The tissue samples were obtained 60 min after i.p. injection of 1 μCi of 1-[^{14}C]-creatine. Means ± SD and numbers of mice are shown. The asterisk indicates $p < 0.01$ by Student's t-test. Control mice, open bars; fasted mice, cross-hatched bars.

To evaluate the response of creatine entry to fasting, groups of mice were fasted for 24 h but permitted free access to water. Corresponding control groups were treated identically except they were provided Chow ad libitum. At the end of the fasting period, individual mice from each of the groups were injected i.p. with 1 μCi of 1-[^{14}C]-creatine, and blood and tissue specimens were obtained for analysis at selected time intervals. The effect of fasting on loss of creatine from muscle was evaluated using mice that had been injected i.p. with [^{14}C]-creatine prior to dividing them into fasting and control groups.

When both creatine content and radioactivity were measured, protein was precipitated from 0.2 ml of plasma or an appropriate volume of tissue homogenate by adding 3 ml each of aqueous solutions of barium hydroxide (50 g of $Ba(OH)_2 \cdot 8\ H_2O$ per l of freshly boiled water) and of zinc sulfate (50 g of $ZnSO_4 \cdot 7\ H_2O$ per l). These 2 solutions were balanced against each other to ensure that neither was added in excess. This method of extracting creatine was compared to a previously tested alcoholic, perchloric acid method[16] and was found to yield quantitative recovery of radioactivity and total creatine (free creatine plus phosphocreatine). In addition, some of the tissue extracts were subjected to paper chromatography to verify that all of the radioactivity comigrated with authentic creatine[17]. No degradation of [^{14}C]-creatine was detected. When only radioactivity was to be measured, trichloroacetic acid was added to tissue homogenates to achieve a final concentration of 6% (weight/volume), protein was removed by centrifugation, and the clear supernatant fluid was used for counting. This method also gave quantitative recovery of radioactivity.

Creatine concentrations were measured using the diacetyl reaction[18]. Radioactivity was measured using a xylene:-dioxane:2-ethoxyethanol based counting solution[19] and a liquid scintillation spectrometer equipped with an automatic external standard for quench correction. The counting error was 3% or less. Creatine concentrations and radioactivity are given per ml of plasma or per g wet wt of tissue.

Results. Creatine feeding caused only a slight increase in muscle creatine concentration (fig. 1), as has been reported previously[20], but the plasma creatine concentration more than doubled. This rise in plasma creatine was associated with little or no change in plasma radioactivity from an injection of [^{14}C]-creatine (fig. 2). Consequently, the specific activity (cpm/μmole) of plasma creatine was always significantly less in creatine-fed than in control mice. The entry of [^{14}C]-creatine into muscle of creatine-fed mice (fig. 2) was inhibited to approximately the same extent as the decrease in plasma creatine specific activity.

As was true for creatine feeding and in agreement with previous studies of the rat[21], fasting caused only a slight increase in muscle creatine concentrations (fig. 1). In contrast to creatine feeding, fasting caused a large increase in plasma radioactivity after an injection of [^{14}C]-creatine (fig. 2) and only a small increase in plasma creatine concentration (fig. 1). Consequently, the plasma creatine specific activity was always much greater in fasted than in control mice. Nevertheless, the entry of [^{14}C]-creatine into muscle of fasted mice was inhibited by 40% or more (fig. 2). [^{14}C]-Creatine entry into the heart also was inhibited by fasting (fig. 3) and there were reciprocal increases in radioactivity in liver and kidney (fig. 3). Fasting had no effect on the amount of radioactivity in the spleen (fig. 3).

In mice that were prelabeled with [^{14}C]-creatine (table), fasting caused proportionate increases in the concentrations of nonradioactive creatine and of [^{14}C]-creatine in muscle (table). Indeed, calculation of specific activities yielded identical values for fasted and control mice, indicating that [^{14}C]-creatine in muscle was not being diluted significantly by the entry of nonradioactive creatine from plasma. This result agrees with the inhibitory effect of fasting on creatine entry. In addition, as expected, the amount of radioactivity in plasma increased in fasted mice due to the release of [^{14}C]-creatine from tissue stores, undoubtedly including skeletal muscle. The fasted mice were active and appeared to be healthy, but they invariably lost weight (table). Muscle weight apparently also decreased, as the ratio of tibialis anterior muscle weight to body weight was approximately the same in fasted and control mice (table).

Discussion. Although the process of creatine entry into skeletal muscle of mice is nonsaturable in vitro[16], the results of the present experiments demonstrate that it is saturable in vivo. Thus, in agreement with data obtained from rats[7], [^{14}C]-creatine entry into muscle was inhibited by exogenous nonradioactive creatine. Moreover, the extent of inhibition corresponded closely to the extent of dilution of [^{14}C]-creatine by nonradioactive creatine in plasma. These observations are consistent with the existence of a saturable transport process for creatine which is operating near its maximal capacity at physiological concentrations of creatine in plasma.

Additional support for saturable transport of creatine into muscle in vivo is provided by the results obtained from fasting mice. These mice exhibited only a modest increase in plasma creatine concentrations in comparison to creatine-fed mice. Yet, the incorporation of radioactivity in muscle after administration of [^{14}C]-creatine was reduced. In this case, the radioactivity in plasma was much greater than it was in control mice (fig. 2). Therefore, the reduced amount of radioactivity in muscle cannot be attributed to dilution of [^{14}C]-creatine in plasma with nonradioactive creatine. Clearly, the transport of creatine into muscle is reduced during fasting, despite higher than normal plasma creatine concentrations. We conclude that creatine not only enters the muscle of mice by a saturable process in vivo, but also that the maximal capacity of this process is modulated by the physiological state of the animal. This conclusion is consistent with the lack of a transport maximum for creatine in mouse extensor digitorum longus muscles in vitro because these muscles would be out of their normal environment, which must include one or more regulatory factors. Further work is needed to identify these regulatory factors. The fasting mouse may be a useful model for this purpose.

In addition, the fasting mouse may provide a model for studies of the process of intracellular trapping of creatine. Our data suggest that creatine is lost from muscle during fasting (table); but, if so, the rate of loss must be relatively less than that of other muscle constituents to account for the observed increase in muscle creatine concentration. In the absence of creatine synthesis in muscle, the only other possible way for the concentration to increase would be for the transport of creatine into muscle to increase, which does

Effect of fasting on creatine loss from tibialis anterior muscles

Measurement	Control	Fasted
Initial body weight (g)	30.3 ± 1.3[a]	32.6 ± 1.0
Final body weight (g)	30.5 ± 1.3	28.0 ± 1.6[b]
Muscle weight (mg)	48.2 ± 2.0	46.7 ± 2.1
Muscle creatine (μmoles/g)	32.0 ± 0.6	34.0 ± 1.8[c]
Muscle radioactivity (cpm/g × 1000)	204 ± 15.8	217 ± 12.5
Plasma creatine (μmoles/ml)	0.24 ± 0.03	0.30 ± 0.04[c]
Plasma radioactivity (cpm/ml)	1273 ± 190	1845 ± 320[c]

Mice were each injected with 2 μCi of 1-[^{14}C]-creatine 4 days prior to dividing them into control and fasting groups. At the end of a 24-h fast, blood and tibialis anterior muscles were obtained for analysis. [a] Means ± SD are shown. There were 5 mice in each group. [b] $p < 0.01$ by the paired t-test for the loss of body weight during fasting. [c] $p < 0.01$ by Student's t-test.

not occur with fasting. These considerations emphasize the importance of the trapping mechanism in the maintenance of muscle creatine concentrations. Further work is needed to determine whether creatine is trapped primarily because it is phosphorylated, bound to a muscle constituent, or compartmentalized or because the process that moves creatine across muscle membranes serves only for entry.

1 Acknowledgment. This work was supported in part by grant AM 16623 from the National Institutes of Health. To whom reprint requests should be addressed.

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Inhibition of erythrocyte ATPase activity by aclacinomycin and reverse effects of ascorbate on ATPase activity

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Summary. We studied the effect of aclacinomycin on human erythrocyte membrane enzymes. Aclacinomycin inhibited ATPase, including Na-K-dependent ATPase, ouabain insensitive ATPase and Ca-ATPase. However acetylcholinesterase was not inhibited by aclacinomycin. The ATPase activities were not inhibited by aclacinomycin if ascorbate was added to the incubation mixture. However other reducing agents, α-tocopherol, superoxide dismutase and catalase had no effect on ATPase activity. Ascorbate may protect membrane proteins and lipids from peroxidate damage.

In 1975, a new anthracycline antibiotic, aclacinomycin, was isolated from a culture filtrate of *Streptomyces galilaeus*. The compound has shown wide antitumor activity against ascitic and solid forms of various malignancies in animal tumor systems. Objective responses to aclacinomycin were observed in patients with lymphoma, acute leukemia, chronic leukemia, breast cancer, ovarian cancer, lung cancer, gastric cancer and bladder carcinoma[1]. The cardiotoxicity of aclacinomycin was estimated as approximately $^1/_{15}$ that of doxorubicin.

Henderson demonstrated that anthracycline antibiotics generated oxygen radicals in erythrocytes[2]. Doxorubicin has the potential of inhibiting erythrocyte ATPase activity, including both Na^+-K^+-ATPase and ouabain insensitive ATPase, at concentrations not inhibitory to other enzymes. The net Na^+ content increased and K^+ content decreased after incubation of erythrocytes with doxorubicin. Such effects of doxorubicin on membrane ATPase and cation permeability may explain, in part, the cardiotoxicity observed in its clinical use and also the potential hemolyzing effect on erythrocytes[3].

The purpose of this study was to obtain further knowledge of the effects of aclacinomycin on erythrocyte membrane enzymes and the effects of radical scavengers on the enzyme activities.

Materials and methods. Commercially available aclacinomycin from Yamanouchi Pharma Co. was dissolved in isotonic NaCl and added to erythocyte suspensions in varying concentrations.

Adenosine triphosphate (ATP-Na), ouabain, acetylcholine iodide, 5,5′-dithio(bis)nitrobenzoic acid, etyleneglycolbis(2-aminoethylether)tetracetate (EGTA), ascorbate, α-tocopherol, coenzyme Q, riboflavin, catalase and superoxide dismutase were of the highest purity commercially available.

Heparinized blood was washed 3 times with isotonic NaCl and the buffy coat was removed after each concentration.

Erythrocyte ghosts were prepared by the method of Dodge et al.[4]; the protein concentration was measured by biuret method; ATPase activity was measured by the method of Nakao et al.[5]. All tubes contained Tri-HCl 40 mM pH 7.7, NaCl 140 mM, KCl 14 mM, $MgCl_2$ 5 mM, ATP-Na 3 mM and erythrocyte ghost 200 µg at a final volume of 1 ml. The reaction was started at 37 °C by the addition of ATP-Na. At the end of the incubation period (30 min) 2.0 ml of trichloracetic acid was added to each tube and the mixture was shaken vigorously. All the tubes were centrifuged at 2000×g for 15 min and the concentration of inorganic phosphorus in an aliquot of the supernatant solutions was measured by the method of Fiske and SubbaRow[6]. The activity of Na^+-K^+-dependent ATPase was determined by the difference between total activity and that in the presence of ouabain. Ca^{++}-ATPase activity was estimated by following procedures. All tubes contained Tris-maleate buffer 50 mM pH 7.0, $MgCl_2$ 5 mM, $CaCl_2$ 0.47 mM, EGTA 0.5 mM, KCl 100 mM, ATP 40 mM and erythrocyte ghost 200 µg at a final volume of 1 ml. The reaction was started by the addition of ATP. At the end of the incubation period (4 min) 1.0 ml of trichloroacetic acid (10%) was added and centrifuged. The concentration of inorganic phosphorus in the supernatant solutions was measured by the method of Martin and Doty[7].