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Effectiveness of Caloric Restriction in Preventing Age-Related Changes in Rat Skeletal Muscle

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The dihydropyridine receptor (DHPR) and ryanodine receptor (RYR1) are needed for excitation–contraction coupling in skeletal muscle. Previous studies from this laboratory have shown DHPR–RYR1 uncoupling in 33-month-old Fischer 344 × Brown Norway F1 (F344BNF1) rats fed *ad libitum*. The purpose of the present study is to determine whether caloric restriction prevents age-related impairments in skeletal muscle function and expression of DHPR and RyR1. Bundles of *soleus* and *extensor digitorum longus* (EDL) were studied from rats fed *ad libitum* and on 60 percent caloric restriction. Significant differences were found in peak twitch or tetanic tension between the *ad libitum* and calorie-restricted groups in *soleus* and EDL muscles. A significant increase in the expression of DHPR and RyR1 was observed in caloric restricted rats. These results show that calorie restriction preserves the mechanical properties of aging hind-limb skeletal muscle and maintains the level of DHPR and RyR1 in aged F344BNF1 rats fed *ad libitum*.

As humans age, decreases in skeletal muscle mass and strength may impact an individual's quality of life by contributing to disability and dependence. The mechanisms underlying these changes in skeletal muscle are poorly understood. We have proposed that excitation-contraction (EC) uncoupling is a process that accounts for a significant fraction of the decay in skeletal muscle force with aging [1] [2]. Two proteins are involved in the transduction of sarcolemmal excitation to sarcoplasmic reticulum (SR) calcium release during muscle contraction. The dihydropyridine receptor (DHPR), a voltage-gated calcium channel, is located at the transverse tubule and plays the role of voltage-sensor [3] [4]. The ryanodine receptor (RyR1), a calcium–activated calcium channel, is located at the SR and is coupled to the DHPR [5]. The interaction of these receptors increases myoplasmic calcium, thus causing muscle contraction. The DHPR-RyR1 coupling a key step in muscle force development and the regulation of the level of these receptors and their interaction has profound impact on muscle functional performance [1]. It has been postulated that age-related loss of functional capacity is associated with accumulation of oxidative damage induced by such molecules as $^{\bullet}O_2$, H_2O_2, and $^{\bullet}OH$ [6] [7]. In the present study we test the hypothesis that the prevention of age-related DHPR-RyR1 uncoupling results in improvement of hind-limb skeletal muscles mechanical output.

MATERIALS AND METHODS

Muscle force determination. Two populations of rats were used in the experiments: (1) Fischer 344 X BNF1 ad libitum-fed (AL) rats (weight: 369g ± 25g), and (2) Fischer 344 X BNF1 caloric-restricted (CR) rats (mass: 219g ± 12g), which received 60 percent of the AL diet. Animals were obtained from SPF Aging colony at the National Center for Toxicological Research (The Bionetics Corporation, Jefferson, AR). Rats were housed in a pathogen-free area at Wake Forest University School of Medicine (WFUSM). Animal handling and procedures followed an approved protocol by the Animal Care and Use Committee of WFUSM. The extensor digitorum longus (EDL) and the *soleus* were dissected and preserved in an isotonic solution of 121mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.5mM $MgCl_2$, 0.4mM NaH_2PO4, 24mM $NaHCO_3$, 5.5mM glucose, 0.1mM EDTA at room temperature, and bubbled with a mixture of 95% O_2 and 5% CO_2. Muscles from both hind-limbs were used for functional recordings. In a separated group of experiments muscles from one leg were used for contraction test whereas contralateral muscles were used for high-affinity ligand binding assay. For contraction studies bundles of fibers from *soleus* and EDL muscles were dissected. We used bundles of fibers to facilitate oxygenation of the core fibers and ameliorate pH and ionic gradients from the outer to the inner layers of the muscle [8, 9]. The weight of the *soleus* and EDL bundles of fibers was 33.4 ± 1.7 mg and 39.5 ± 1.9 mg, respectively. The contraction experiments were conducted in the isotonic solution described above. Each bundle was attached to a force transducer and platinum electrodes were aligned parallel to the bundle to deliver current pulses. The optimum resting fiber length (L_0) was determined by delivering tetanic supramaximal pulses. Contraction tension in response to 0.2ms pulses (P_t) was studied. For the analysis of

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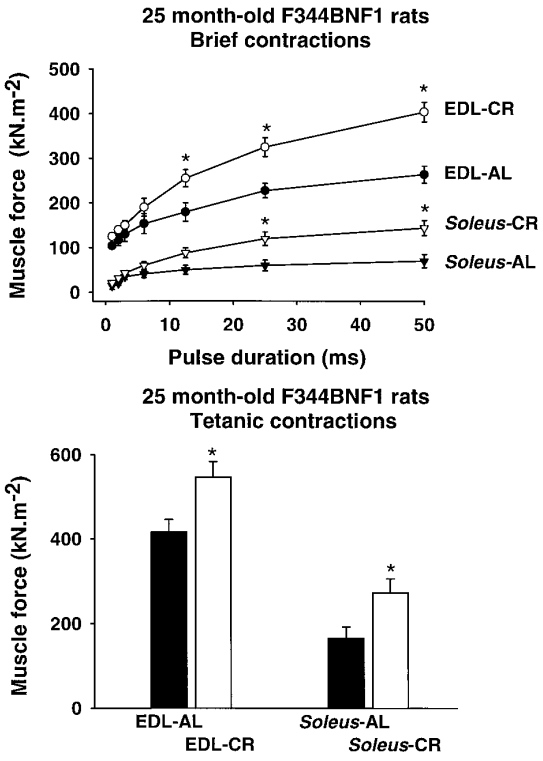

FIG. 1. Muscle force – pulse duration relationship (upper panel) and tetanic tension (lower panel) for EDL and *soleus* muscle bundles in caloric restricted (CR) and *ad libitum* fed 25 month-old F344BNF1 rats. Asterisks indicate statistically significant differences ($P < 0.05$).

tetanic tension (Po), electrical stimulation for 1.5s and 10s at different frequencies were applied to EDL and *soleus* bundles, respectively. Fixed intervals of 1s or 2s between brief pulses or tetanus, respectively were used. EDL and *soleus* muscle bundles were tested alternatively. In this way the time after dissection influenced similarly both EDL and *soleus* muscles. Also, recordings from different animals were comparable. For data acquisition and analysis pClamp 6.3 software was used (Axon Instruments, Foster City, CA). Data were sampled at 2 kHz. Muscle bundle cross sectional area (cm^2) was calculated by dividing muscle wet mass by the product of fiber length and muscle density (1.06 g/cm^3) [9, 10]. Muscle force is expressed in kN/m^2.

Muscle homogenate and radioligand binding to DHPR and RyR1. EDL, *soleus* and whole leg muscles were studied. Muscles were homogenized as described [11]. Protein concentration was determined by Coomassie protein assay with bovine serum albumin as the protein calibration standard. DHPR and RyR1 concentrations were determined using the radioligands [^{3}H]PN200-110 and [^{3}H]ryanodine, respectively. Homogenate (1-2 mg/ml protein) were incubated either with 0.05-5 nM [^{3}H]PN200-110 for 1 hr at 23°C in 50 mM Tris-HCl, pH 7.5, 10 μM Ca^{2+}, 1 mM diisopropyl fluorophosphate (DIFP) and 5 μM leupeptin or 0.5-50 nM [^{3}H]Ryanodine for 24-48 h at 10°C in 20 mM pipes-NaOH, pH 7.0, 1.0 M NaCl, 100 μM Ca^{2+}, 5 mM AMP, 1 mM DIFP, and 5 μM leupeptin. Membrane bound [^{3}H]PN200-110 and [^{3}H]Ryanodine were determined by filtration through Whatman GF/B filters using a Millipore unit (XX2702550, Millipore corporation, Bedford, MA). Filters were rinsed three times with 5 ml of ice cold 200 mM choline chloride, 20 mM Tris-HCl, pH 7.5. Non-specific [^{3}H]PN200-110 and [^{3}H]Ryanodine binding were assessed in the presence of 10 μM unlabelled nifidepine (Sigma Chemical Company, St. Louis, MO) or PN200-110 (Sandoz Pharmaceutical, East Hanover, NJ) and 10 μM unlabelled ryanodine (Calbiochem, San Diego, CA) respectively. Radioligand concentrations used resulted in occupancy of >95% of the high affinity binding sites [12]. Linear regression and non-linear least square analysis were used to calculate non-specific and total binding of the radioligands to the receptors. Specific binding of [^{3}H]PN200-110 and [^{3}H]Ryanodine at each concentration was calculated by subtracting the non-specific binding from the total binding obtained from the above analysis. The following equation: $y = (x\ a)/((x + b) + (x\ c))$ where, a = receptor number, (B_{max}); b = K_d, dissociation constant; c = the non-specific binding or the low affinity site, was used to fit the binding isotherm. Data were also given in a graphical representation of the Scatchard plot. There was no significant difference in B_{max} values obtained from the curve fit or Scatchard plot [11]. All values are mean ± SEM. Data were analyzed for statistical significance using unpaired Student t-test.

RESULTS

Muscle Force Determination

We examined the mechanical performance of hindlimb fast-twitch (EDL) and slow-twitch (*soleus*) muscles of adult and old rats AL or CR fed. Twenty EDL and 18 *soleus* muscles for 32 month-old rats and 15 EDL and 14 *soleus* muscles from 25 month-old rats were studied. A range of 26 to 32 bundles was examined for each group. Fig. 1 shows muscle bundle contractions in response to brief or prolonged stimulation

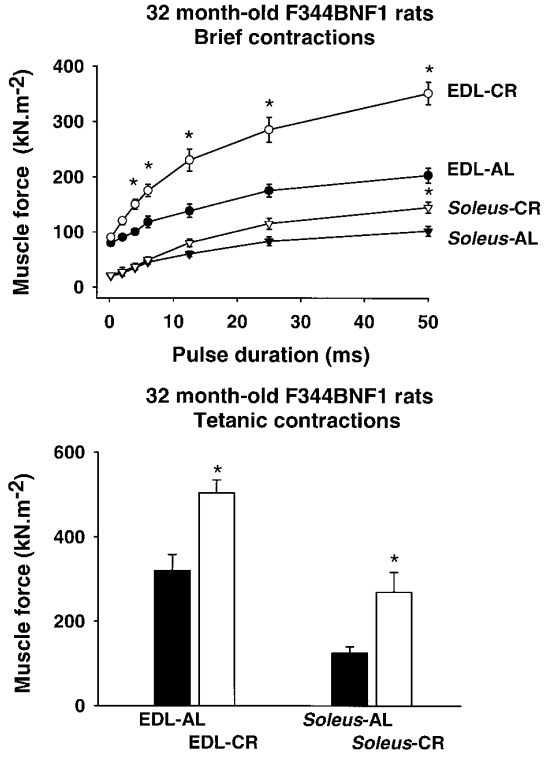

FIG. 2. Muscle force – pulse duration relationship (upper panel) and tetanic tension (lower panel) for EDL and *soleus* muscle bundles in caloric restricted (CR) and *ad libitum* fed 32 month-old F344BNF1 rats. Asterisks indicate statistically significant differences ($P < 0.05$).

TABLE 1

High-Affinity [^{3}H]PN200-110 and [^{3}H]Ryanodine Binding to Skeletal Muscles of 32 Month Old F344BNF1 Rats Fed either *ad libitum* or on 60% Calorie Restriction

	DHPR		RyR1		Ratio
Calorie intake	B_{max}	K_D	B_{max}	K_D	DHPR/RYR1
Ad-libitum	1.57 ± 0.16	1.67 ± 0.53	2.70 ± 0.12	38 ± 5	0.58 ± 0.03
60% calorie restriction	4.10 ± 0.18 ($P < 0.03$)	1.77 ± 0.22	4.26 ± 0.13 ($P < 0.001$)	30 ± 5	0.97 ± 0.29 ($P < 0.05$)

Note. Values of B_{max}, K_D are mean ± S.E.M. and expressed as pmole/mg protein and nM, respectively.

in 25 month-old rats. Contraction tension in both muscles was significantly improved in CR compared with AL fed rats in response to pulses longer than 10ms. Prolonged stimulation resulted in significant potentiation of muscle tension in EDL and *soleus* bundles. The frequency needed to attain a maximum tetanic contraction in AL and CR *soleus* muscle bundles from 25 month-old rats was 56 ± 5.5 Hz and 53 ± 6.5 Hz, respectively. The frequency needed to maximally activate AL and CR EDL muscle bundles from 25 month-old rats was 83 ± 6.9 and 88 ± 7.8 Hz, respectively (non-statistically significant difference). The Pt/Po ratio for AL and CR *soleus* muscle bundles was 0.16 ± 0.01 and 0.09 ± 0.01 ($P < 0.05$) and for AL and CR EDL bundles was 0.26 ± 0.01 and 0.14 ± 0.02 ($P < 0.05$). Fig. 2 shows brief and tetanic contractions in both muscle groups. It is obvious from the comparison of the 25 and 32 month-old AL fed rats that a decrease in about 30% in specific tension occurred in the older group. The decrease in specific tension was recorded in response to brief and prolonged stimulations. It is also apparent that the mechanical force developed by EDL and *soleus* muscle bundles in response to brief pulses or trains of pulses was potentiated in CR rats. The frequency needed to attain a maximum tetanic contraction in AL and CR *soleus* muscle bundles from 32 month-old rats was 52 ± 6.1 Hz and 57 ± 5.6 Hz, respectively. The frequency needed to maximally activate AL and CR EDL muscle bundles from 32 month-old rats was 86 ± 7.5 and 83 ± 6.9 Hz, respectively. Differences between groups were not statistically significant. The Pt/Po ratio for AL and CR *soleus* muscle bundles was 0.15 ± 0.01 and 0.08 ± 0.01 ($P < 0.05$) and for AL and CR EDL bundles was 0.24 ± 0.02 and 0.17 ± 0.01 ($P < 0.05$). Differences in the Pt/Po ratio are due to a significant potentiation of the tetanic contraction in AL fed rats.

DHPR/RYR1 Ratio in Mixed Fiber-Type Skeletal Muscles

The DHPR was quantified by radioligand analysis using the high-affinity probe [^{3}H]PN200-110. The RYR1 was assessed using the high affinity probe [^{3}H]ryanodine [11]. The binding studies were done in predominantly *soleus*, EDL and in a pool of both fast and slow-twitch muscles. [^{3}H]PN200-110 and [^{3}H]ryanodine binding to mixed fiber skeletal muscle homogenate in 32-month-old AL and CR F344BNF1 rats are shown in Table 1. The B_{max} for [^{3}H]PN200-110 and [^{3}H]ryanodine binding in AL and CR rats in individual muscles (*soleus* and EDL) are shown in Table 2. There are 62 percent fewer PN200-110 receptors and 37 percent fewer ryanodine receptors in AL fed rats than in calorie restricted rats. The B_{max} values correspond to a PN200-110/ryanodine binding ratio of 0.58±0.03 in the

TABLE 2

High-Affinity [^{3}H]PN200-110 and [^{3}H]Ryanodine Binding to Skeletal Muscles of 32 Month Old F344BNF1 Rats Fed either *ad libitum* or on 60% Calorie Restriction

	Soleus muscle			EDL muscle		
Calorie intake	DHPR	RYR1	DHPR/RYR1	DHPR	RYR1	DHPR/RYR1
Ad-libitum	1.15 ± 0.30	3.24 ± 0.72	0.34 ± 0.05	2.95 ± 0.25 (ns)	3.57 ± 0.84 ($P < 0.02$)	0.84 ± 0.05
60% calorie restriction	1.96 ± 0.21 ($P < 0.05$)	4.10 ± 0.56 ($P > 0.05$)	0.50 ± 0.05 ($P < 0.03$)	5.19 ± 0.59 ($P < 0.03$)	5.35 ± 0.52 ($P < 0.01$)	0.98 ± 0.09 ($P > 0.05$)

Note. Values of B_{max} are mean ± S.E.M. and expressed as pmole/mg protein.

AL group and 0.97±0.29 in the caloric-restricted group. These results are significantly different ($P<0.05$), indicating receptor uncoupling in the AL fed animals.

Binding studies were also performed in homogenates of only *soleus* or EDL muscles to determine whether the increase in DHPR-unlinked RyR1 in the muscle pools of AL-fed animals was due to uncoupling in slow-twitch (*e.g.* soleus) and/or fast-twitch (*e.g.* EDL) muscles. Table 2 includes [^{3}H]PN200-110 and [^{3}H]ryanodine binding to *soleus* and EDL from AL and CR animals. In the *soleus,* the AL fed rats showed 41 percent fewer DHPR and 21 percent fewer RYR1 than the CR rats. Thus, the DHPR/RYR1 ratio in the *soleus* is significantly lower in the AL group compared to the CR group. In the EDL, the AL fed rats showed 43 percent fewer DHPR and 33 percent fewer RYR1 than CR rats. Despite these reductions, the DHPR/RYR1 ratio in the EDL is not significantly different between the AL and the caloric-restricted groups.

DISCUSSION

The aim of this work was to study the magnitude of age-dependent decay in hind-limb muscle force and the effectiveness of chronic CR in preventing these changes. Pure fast-twitch (EDL) and slow-twitch (*soleus*) muscles from adult and old F344BNF1 rats were used to determine primary effects of aging and/or CR on a specific muscle fiber subtype. The results of this work can be summarized as follows. (1) *Soleus* and EDL muscles force declines in 30% in old compared to adult rats. (2) CR EDL and *soleus* muscle bundles exhibited a significant increased in tension compared to AL fed animals. (3) High-affinity radioligand binding assays done in contralateral *soleus* or EDL muscles used for contraction studies and in pool of fast- and slow-twitch muscles demonstrated a significant increase in DHPR and RyR1 in CR animals of both age groups. The decrease in specific muscle tension with age is consistent with previous studies in aging humans and rodents [10, 13]. The significant increase in EDL and *soleus* muscle tension in CR animals reported here implies that fast- and slow-twitch muscles from hind-limbs are altered by oxidative stress and both muscles are similarly responsive to CR. These results also imply that the age-related impairment in muscle function is more manifest in fast-twitch muscles from the limbs than in non-limb fast-twitch muscles such as the lateral omohyoideus [14]. The finding that CR improves the mechanical performance of the *soleus* muscle does not confirm previous observations [14]. Although the explanation for this discrepancy is not obvious we found that the order in which the bundles of *soleus* and EDL are tested influences importantly the statistics. Because muscle function deteriorates with time, in the present work, *soleus* and EDL bundles were tested alternatively following the same time schedule.

EC uncoupling has been identified as a basic mechanism contributing to the decline in muscle force with aging [1, 2, 15, 16]. The increase in the number of DHPR and RyR1 determined by radioligand binding assay supports that CR regulates the expression of these molecules. The preservation of the level of these molecules may play a role in the stronger contractions recorded in CR EDL and *soleus* muscle bundles. This phenomenon may be associated with modulation of other molecules involved in the cell signaling leading to muscle contraction evoked by DHPR and RyR1 activation.

CR has been identified as a powerful intervention with effects on hind limbs muscle mass and structure [17–21]. An indication of the beneficial effects of CR is that muscle injury can be alleviated by using free radicals scavengers [22]. Although CR attenuates dityrosine cross-linking of proteins in mice contributing to the deterioration of skeletal muscle function with advancing age [23], the specific molecular mechanisms underlying the preservation of DHPR and RyR1 in older muscles need further investigation.

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