## MATERIALS AND METHODS

### Experimental animals

Eight 19-week-old male ICR mice were assigned randomly to either a sedentary control (control, n=4) or voluntary running exercise (exercise, n=4) group. All mice were individually housed in similar cages, except that there was no running wheel in the cage for the control group. The mice in the exercise group exercised voluntarily in the running wheels for 30 days. Food and water were provided *ad libitum*. The mice were kept in a controlled environment with fixed 12: 12 h light: dark cycles (lights off from 19 : 00 to 07 : 00) and room temperature maintained at 22± 2 ℃. All procedures were approved by the University Committee for the Care and Use of Animals for Research Purposes and followed the Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals.

### Running wheel apparatus

A running wheel apparatus was developed in which the load and running distance could respectively be controlled and monitored electronically[12]. This apparatus includes a standard plastic cage and a running wheel (width 5.0 cm, diameter 25.5 cm) attached vertically to a freely rotating shaft inserted into a metal controller box that is supported on a metal base. The running wheel rotates on the shaft whenever the mouse walks or runs in either direction in the running wheel, and the number of revolutions of the running wheel is continuously recorded. A transducer in the controller box connected to the running wheel produces an electric signal for each revolution of the running wheel. This signal is then sent to, and subsequently stored by, a computer which is equipped to continuously monitor the number of signals from up to 20 running wheels simultaneously. The time interval for data collection is set by a time-mark generator (from 3 sec to 24 h).

### Tissue preparation

At the end of the 30-day exercise period, the mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). The tibialis anterior muscles were removed, cleaned of excess fat and connective tissue, and wet weighed. Each muscle was placed on cork, stretched to its *in vivo* length, and immediately frozen in isopentane cooled in liquid nitrogen. Serial 10 $\mu$m thick transverse sections from the midbelly region of the muscle were cut in a cryostat set at −20 ℃. The sections were brought to room temperature, air-dried, and then stained for SDH activity, an indicator of mitochondrial oxidative potential[10,11,16,18]. The SDH activity was determined in an incubation medium containing 100 mM phosphate buffer (pH 7.6), 0.9 mM sodium azide, 0.9 mM 1-methoxyphenazine methylsulfate, 1.5 mM nitroblue tetrazolium, 5.6 mM EDTA disodium salt, and 48 mM succinate disodium salt. The reaction was stopped by multiple washings in distilled water and the sections were dehydrated in a graded series of ethanols, passed through xylene, and then coverslipped. Histochemical control sections, in which either succinate disodium salt or nitroblue tetrazolium was excluded from the incubation medium, showed no positive SDH staining.

### Tissue analysis

The cross-sectional areas and SDH activities of muscle fibers from the deep (close to the bone), middle (between the deep and superficial regions of the muscle), and superficial (away from the bone) regions of the muscle were examined using a computer-assisted image processing system (Neuroimaging System)[5,9,13]. These muscle regions were selected for analysis, because the tibialis anterior muscle shows an increasing gradient of muscle fibers having a high oxidative enzyme activity proceeding from the superficial to the deep region of the muscle[6,19]. Tissue sections were digitized as gray level images on a computer-assisted image processing system. Each pixel on the computer was quantified as one of 256 gray levels. A gray level value of zero was equivalent to 100% transmission of light, and that of 255 was equivalent to 0% transmission. The optical density (OD) value was determined by gray levels. The cross-sectional areas of muscle fibers were determined automatically, i.e. via edge detection defined by the gradients of OD values, from the boundary of the muscle fiber outlined in digitized images of the SDH-stained sections. The mean OD value of all pixels within a muscle fiber was determined using a calibration tablet which had 21 steps of gradient density ranges and corresponding diffused density values.

### Statistics

Means, standard deviations, and correlations were calculated from individual values using standard procedures. Values were expressed as mean ± standard deviation (SD). An analysis of variance was used to determine significant differences for increases in

the running distance with time and among animals. The Student's $t$-test was used to determine significant differences in the cross-sectional area and SDH activity between the control and exercise groups. All statistical analyses were performed using Statview.

## RESULTS

### Running distance

The daily running distance in the exercise group increased progressively (Fig. 1). The mean running distances of four animals during a 30-day exercise period were 4184 (no. 1), 5801 (no. 2), 4838 (no. 3), and 4119 (no. 4) m/day. There were no differences in the daily increase in the running distance among these four animals.

### Body and muscle weights

There were no differences in the mean body weight between the control ($31.6\pm3.7$ g, $n=4$) and exercise ($29.6\pm1.3$ g, $n=4$) groups after a 30-day exercise period.

There were no differences in the mean tibialis anterior muscle weight between the control ($55.8\pm3.1$ mg, $n=4$) and exercise ($59.0\pm5.4$ mg, $n=4$) groups after a 30-day exercise period.

### Relationship between muscle fiber cross-sectional area and SDH activity

There was an inverse relationship between cross-sectional area and SDH activity of muscle fibers in the middle region of the control group ($r=-0.970$, $p<0.05$) (Fig. 2).

### Muscle fiber cross-sectional area and SDH activity

There were no differences in the mean cross-sectional area of muscle fibers between the control and exercise groups, irrespective of the muscle region (Fig. 3).

The mean SDH activities of muscle fibers in the middle and superficial regions, but not in the deep region, were greater in the exercise group than in the control group (Fig. 3).

## DISCUSSION

In the present study, voluntary running exercise was used as an increased muscle activity model for experimental animals. Running distances in all exercised animals increased during the 30-day exercise period. In addition, similar increase patterns in the running distance during the exercise period were observed in these animals (Fig. 1). In the present study, we compared mean cross-sectional area and mean SDH activity of fibers in the different regions of the muscle in the exercise group with those in the control group (Fig. 3), because the exercised animals showed similar running patterns during the exercise period.

The tibialis anterior muscle was examined in the present study, because it has a mixture of muscle fibers having different oxidative enzyme activities proceeding from the superficial to the deep region of the muscle[6,19]. Therefore, it would be expected that muscle regional specific adaptations should be observed following the increased muscle activity. The primary finding in the present study was that the muscle fibers did not hypertrophy, irrespective of the muscle region, in response to the running exercise. This result is consistent with previous studies using rat muscles[19-21]. Previous studies[4,14,15,23-25,28,30] reported that fiber hypertrophy in skeletal muscles was observed only after an anaerobic (high-intensity and short-duration) type of exercise and functional overload by muscle removal of synergists.

The second finding was that an increased oxidative enzyme activity of muscle fibers in the middle and superficial regions, but not in the deep region of the muscle, was observed after the voluntary running exercise. One previous study[8] observed that voluntary running exercise for 45 days increased percentages of high-oxidative fibers in the fast-twitch plantaris muscle in rats, while no change in the slow-twitch soleus muscle was observed after voluntary running exercise. Similarly, Werning et al.[29] observed that the proportion of high- and low-oxidative fibers in the slow-twitch soleus muscle of mice did not change after voluntary running exercise. These results indicate that slow-twitch muscles, i.e. the soleus muscle, are less responsive to voluntary running exercise, because such muscles presumably consist of high-oxidative fibers and have sufficient aerobic capacity to meet the increased energy demand needed for running exercise. In contrast, the activation of different types of muscle fibers, such as the recruitment of low-oxidative muscle fibers, is needed during running exercise, because the increased muscle activity was sufficient to incrementally recruit more muscle fibers having low-oxidative enzyme activities in the middle and superficial regions of the muscle to accomplish a given condition of activity.

In conclusion, increased muscle activity with voluntary running exercise results in aerobic adaptations in the skeletal muscle, as confirmed by observation of

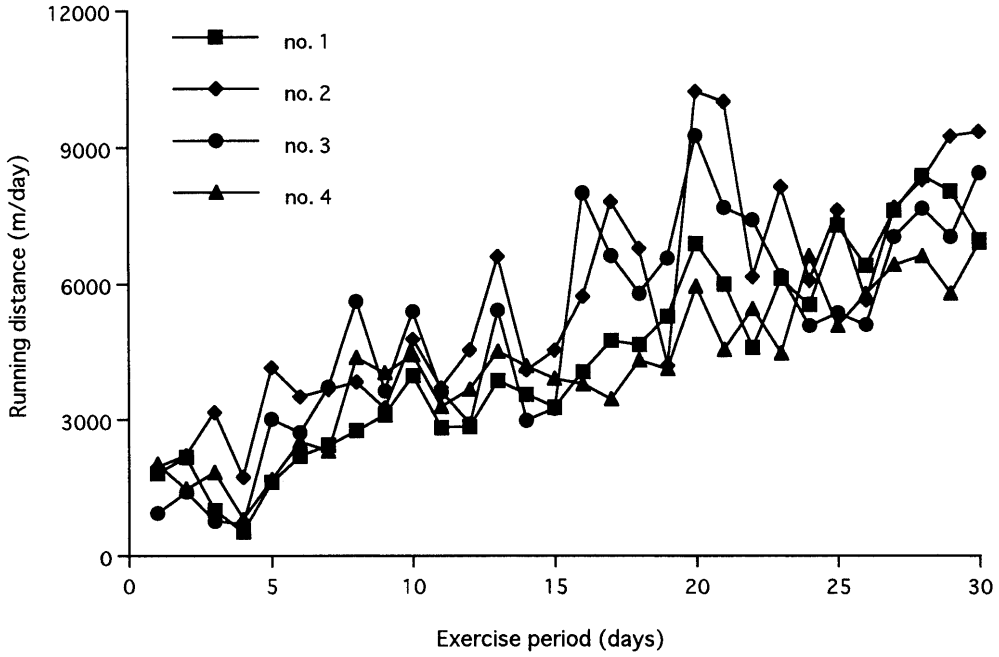


**Fig. 1.** Changes in the daily running distance of four mice in the exercise group. The running distances in all mice increased progressively during the exercise period and similar increased patterns in the running distance were observed in these mice.

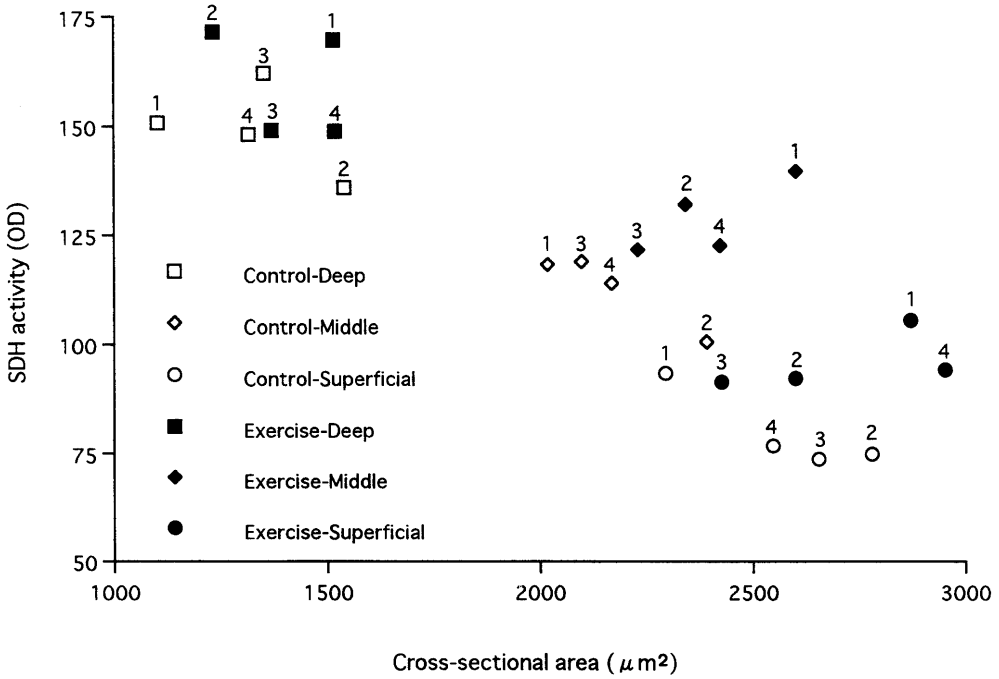


**Fig. 2.** The relationship between cross-sectional area and succinate dehydrogenase activity of fibers in the different regions of the tibialis anterior muscle in the control and exercise groups. Numbers on the symbols show the serial number of the animal. SDH, succinate dehydrogenase; OD, optical density. The correlation coefficients of the deep, middle, and superficial regions of the muscle in the control group were $-0.495$ ($n=4$), $-0.970$ ($n=4$, $p<0.05$), and $-0.913$ ($n=4$), respectively, while those in the exercise group were $-0.354$ ($n=4$), 0.768 ($n=4$), and 0.596 ($n=4$), respectively.

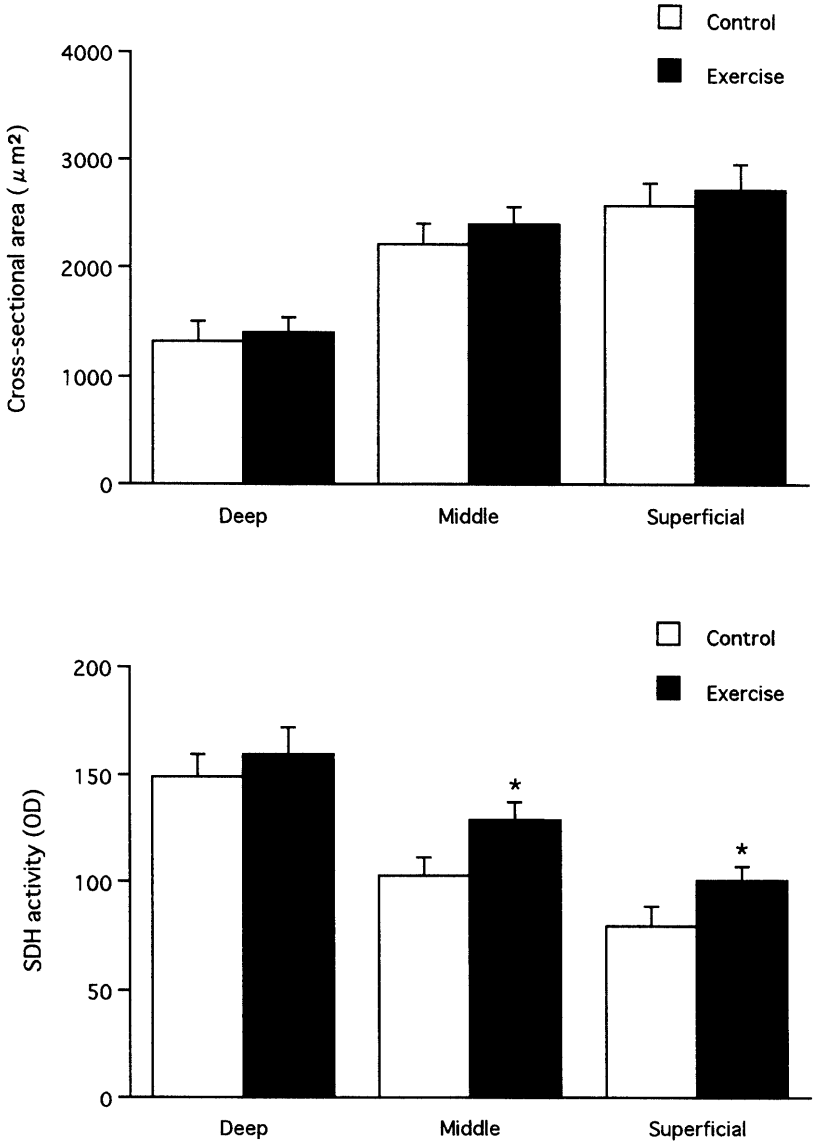


**Fig. 3.** Mean cross-sectional areas and mean succinate dehydrogenase activities of fibers in the different regions of the tibialis anterior muscle in the control and exercise groups. Values are means±SD from four animals. SDH, succinate dehydrogenase; OD, optical density. *$p<0.05$ compared with the value of the control group.

an increased oxidative enzyme activity of low-oxidative muscle fibers in the middle and superficial regions in the tibialis anterior muscle.

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