INTRODUCTION

Skeletal muscular contractions of the limbs and abdomens from strenuous physical activity may result in muscle damage (1). The damage may involve injured sarcomers focally or more extensively. However, it is unclear if running can induce injury to diaphragm muscle. The diaphragm is a unique skeletal muscle that is considered to be two muscles in one. This fact, based on anatomical and functional differences between the costal and crural regions (2), includes different composition in muscle fiber types. The crural diaphragm is dominated by type IIb, fast twitch muscle fibers, whereas the sternal/costal diaphragm contains equally type I (slow twitch) and type IIb fibers (3).

Given that the diaphragm is basically two separate muscles, we used the diaphragm as the muscle of interest to determine the impact of moderate (slow speed) or intensive (fast speed) exercise training on injury susceptibility of the extracellular matrix (ECM). This was done by measuring the level of metalloproteinase-2 (MMP-2). The extracellular matrix supports and maintains the structural integrity and composition of the cell (4-6). Previous reports have indicated that the zinc-dependent MMPs play the pivotal role in ECM remodeling during muscle regeneration (7), by digesting the ECM components (8, 9), and contribute to the ECM remodeling in muscle and other tissues (10-12). Activation of the isoform MMP-2 has been implicated in various myopathic and inflammatory conditions (13). MMP-2 (72-kDa, gelatinase A) belongs to a group of calcium and zinc endoproteinases that play a pivotal role in maintaining the ECM during morphogenesis, proliferation, and cell apoptosis (14-18).

Skeletal muscle responds and adapts to mechanical strain by deformation and differentiation (19). Changes in stimulation, such as immobility (20) and overloading (21, 22) activate the MMP-2. Strenuous exercise, especially fast speed running, is known to cause intra- and extra-myofiber damage (23-25). High-intensity running increased both mRNA and protein levels of MMP-2 as previously described by Carmeli et al. (26). The effect of running on the expression level of MMP-2 in diaphragm muscles has not yet been investigated. Therefore, in the present study we set out to investigate the expression of MMP-2 in type I fibers (slow twitch, high oxidative) and type IIb fibers (fast twitch, high glycolytic) in diaphragm muscles following fast and slow speed running in order to study the effects of training on injury susceptibility.
Superoxide dismutase-1 (Cu,Zn-SOD-1) is predominantly localized in the cytosol and it is one of the most important enzymes in the antioxidant defense system. SOD-1 converts the toxic superoxide free radical into oxygen and hydrogen peroxide. The enzyme, whose turnover rate is in milliseconds, is abundant in type I muscle fibers. Increased production of superoxide anions and their derivatives can cause muscle injury. Expression of Cu/Zn-SOD-1 confers significant protection against inflammation, apoptosis, and oxidative stress. Acute exercise may cause oxidative damage in the rat diaphragm through the activation of inflammatory pathways; thereby could lead to upregulation of Cu,Zn-SOD-1 activity, indicating the potential for improvement of resistance toward intracellular reactive oxygen species.

The aim of the present study was to investigate the effects of training on injury susceptibility by examining the expression of MMP-2 and Cu,Zn SOD-1 in type I fibers (slow twitch, high oxidative) and type IIb fibers (fast twitch, high glycolytic) in the diaphragm muscle following fast and slow speed running.

MATERIAL AND METHODS

Animals

A local Ethics Committee approved the study protocol. Pathogen-free Sprague-Dawley female rats were used for the study (4 month-old, body weight 240±21 g at the beginning of the experiment). The animals were kept at room temperature (22°C) and humidity (40%), at a natural night-day cycle (12/12 hour light cycle), and were fed with standard rat chow and water ad libitum. The rats were randomly assigned to three different experimental groups: 5 animals in each of the running groups and 3 animals in the control group. All animals were kept according the principles of laboratory animal care formulated by Florida University (USA) and Tel Aviv University (Israel).

Experimental procedure

Treadmill running: Animals were adapted to treadmill running during the first 5 days for 10 min at 15 m/min speed. Habituation was followed by 3 weeks, 5 consecutive days per week, of continuous running at different speeds as follows: running group I - slow speed (SSR), 18 m/min (~50% VO2) 0% incline (n=5); running group II - very fast speed (FSR) 32 m/min (~75% VO2), 0% incline (n=5). In both groups, treadmill exercise was preceded by 20 min training, with daily increases by 10 min until a total of 50 min was achieved. Immediately after the last running session, an intraperitoneal injection of pentobarbitol sodium (150 mg/kg) was administrated to the animals, followed by a surgical procedure of careful removal of the diaphragm. The cranial and sternal/costal regions were separated and the material was immersed in liquid nitrogen (-165°C) for 10 min and then stored at -85°C until assay.

RT-PCR

Total RNA was isolated from 100 mg muscle tissue using EZ-RNA isolation kit (Biological Industries; Beit Haemek, Israel; 20-400-100). The RNA was used as a template for RT-PCR reaction (Access Quick™ RT-PCR system, Promega A1702, USA) using MMP-2 primers: sense CCACATTCTGGCCTGAGCTCCC and anti-sense GATTTGTATGGCTTTCAAACCTCAC, and alpha tubulin primers (as a reference): sense ATCACAGGCAAGGA AGATGC and anti-sense ATTGACATCTTGTGGGGACCA (Sigma, St. Louis, Maryland, USA). The reaction products were run on 1.2% agarose gel.

SDS-PAGE and Western blot analysis

100 mg muscle tissue was homogenized (20 sec homogenization and 10 sec pause x 3 times) in cold buffer containing 42 mM trizma base, 0.3 M KCl, 2.5 mM MgCl2, 0.1% Triton X-100 and protease inhibitor cocktail (Sigma, St. Louis, Maryland, USA; cat. #P-8340), and centrifuged (14000 x g for 10 min at 4°C). The supernatants were collected, and total protein concentration was measured using Bradford reagent (Bio-Rad, California, USA, cat. #500-0006). Equal amounts of the supernatants were suspended in protein sample buffer containing 5% beta-mercapto-ethanol, vortexed, boiled, and centrifuged. The supernatant were subjected to 10% SDS-PAGE. Proteins from polyacrylamide gels were transferred onto nitrocellulose membranes.

Blots were blocked with 2.5% skim milk (Bio-Rad, CA; cat. #170-6404) in PBST (PBS containing 0.05% Tween 20) for 1 hr, reacted with MMP-2 specific goat polyclonal antibody (Santa Cruz, California, USA; sc-6383) and alpha tubulin specific mouse monoclonal IgG2a antibody (Santa Cruz, California, USA; sc-5286) for 1 hr; washed three times with 2.5% skim milk in PBST for 30 min (3 x 10 min), reacted with bovine anti-goat IgG-HRP (Santa Cruz, California, USA; sc-2350) or donkey anti-mouse IgG-HRP (Santa Cruz, California, USA; sc-2314) respectively, for 30 min, and washed once with 2.5% skim milk in PBST for 10 min and three times with PBST for 9 min (3 x 3 min). The membranes were developed using Super Signal (West Pico Chemiluminescent substract) (Pierce 34080; BioRad, Tel Aviv, Israel) followed by exposure to X-ray films (Fuji, Tokyo, Japan).

The resulting labeled bands were quantified by using a PC. The scans were created using an image scanner (600 dpi transparency module, Mirror Technologies; Madison, Wisconsin, USA) connected to the computer. The scans were subsequently digitized and imported into an image analysis software program (Scion Image Version 4.0.2 Beta, Scion Cooperation; Frederick, Maryland, USA), and the quantification of MMP-2 was performed by calculating the density of each individual band sample.

Protein isolation

Frozen muscles were powdered and total cellular protein was isolated by homogenization of the muscles samples in lysis buffer (120 mM Tris, HCl and 5% glycerol, no protease inhibitor). After homogenization, Triton X100 was added to a final concentration of 0.01%.

Cu,Zn-SOD specific activity level

Cu,Zn-SOD specific activity level was assessed spectrophotometrically at 320 nm by measuring the inhibition of 10 μM cytochrome c reduction by the 3.5 microunits/ml xanthine oxidase and 50 μM xanthine (xanthine/xanthine oxidase system). Both cytochrome oxidase and Cu,Zn-SOD were inhibited by 1 mM KCN. Superoxide dismutase units in each fraction were calculated by extrapolation from the calibration curve with titrated commercial Cu,Zn-SOD. According to the standard curve using homogenous human Cu,Zn-SOD (Biotechnology General, Rehovot, Israel), normal values are 30-40 μmol/g tissue.

Statistical analysis

All values are reported as means ± SE. For MMP-2 levels among experimental and control groups, analysis was conducted using one-way ANOVA and Tukey’s post hoc test (PRISM-Graph pad). P<0.05 was accepted as indicative of significant changes.
RESULTS

Body weight

The effect of running on body weight is demonstrated in Table 1. After 3 weeks of slow or fast speed running, body weight of the rats did not appreciably change.

To address the question of whether altered MMP-2 expression at the mRNA level is associated with protein levels, Western immunoblotting and RT-PCR were performed. In accord with the PCR results, a signal at the molecular size of 72 kDa (latent MMP-2 proform) was detected particularly in the crural muscle fibers type IIb, following fast and slow speed running (Figs. 1 and 2).

Concerning the Cu,Zn-SOD-1 activity, we observed a significant reduction of its level during fast speed running only in the crural diaphragm muscle (Table 2).

Table 1. Body and muscle weights following three weeks of slow and fast treadmill running.

<table>
<thead>
<tr>
<th></th>
<th>Control Group (n=3)</th>
<th>Before/after 3 wk SSR (n=5)</th>
<th>Before/after 3 wk FSR (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>252 ±20</td>
<td>241 ±23/244 ±21</td>
<td>255 ±20/258 ±19</td>
</tr>
</tbody>
</table>

SSR, slow speed running; FSR, fast speed running. Values represent means ±SD.

Table 2. Cu,Zn-SOD activity (µmol/g tissue) in the crural and sternal parts of the diaphragm muscle.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SSR</th>
<th>FSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crural</td>
<td>33.2 ±3.0</td>
<td>39.5± 4.0</td>
<td>41.2 ±4.0</td>
</tr>
<tr>
<td>Sternal</td>
<td>34.4 ±3.0</td>
<td>41.2 ±4.0</td>
<td>50.7 ±5.0</td>
</tr>
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Fig. 1. Qualitative MMP-2 expression by Western immunoblotting in diaphragm muscle fibers in the slow speed running (SSR) (Panel A) and fast speed running (FSR) (Panel B) groups of rats. In both groups, MMP-2 visibly increased in type IIb fibers compared with type I fibers, following exercise. The quantitative results of MMP-2 levels and their significance are shown in Fig. 2 (Panels A and B).

Fig. 2. Quantification of activated MMP-2 expression by Western immunoblotting in diaphragm muscle fibers in the slow speed running (SSR) (Panel A) and fast speed running (FSR) groups of rats. In both groups, MMP-2 activity in type IIb fibers significantly increased compared with type I fibers following exercise. Also, in both fiber types, expression of MMP-2 increased vs. control. Values represent group means ±SE. Significant differences vs. control are indicated by asterisks *P<0.05, **P<0.001, ***P<0.001.
DISCUSSION

It is well known that MMP-2 is a key regulatory molecule in the formation, remodeling, and degradation of extracellular matrix components in both physiological and pathological processes (20, 12, 28). MMP-2 is a collagenase with a special ability to degrade collagen type IV, the most prevalent protein in skeletal muscle basal lamina. It is present in low levels of normal ECM of muscle tissue and its expression is regulated by cytokines and growth factors. It has also been implicated in successful regeneration of experimentally damaged muscle fibers in the mouse (29).

Although production of proteolytic enzymes is known to be associated with myopathies and inflammatory conditions, their involvement in different fiber types under different loading stimulation had been little investigated. Physical exercise could affect the expression of MMP-2 in skeletal muscle by producing local ischemia, and increases in muscle stretching and shear stress (30, 31). In addition MMP-activity was demonstrated to be crucial for exercise-induced skeletal muscle angiogenesis in the rat (32).

The results of this study show that fast speed running leads to a significant expression of the MMP-2, 72 kDa in the crural region of the diaphragm and, therefore, the running increases capacity for ECM degradation. This is consistent with previous reports on exercise in both rat (33) and human (34).

The costal and crural diaphragm regions differ in their responses to exercise training (35). In contrast to the costal region, where the maximum training-induced changes are observed during low intensity training, in the crural region moderate and high intensity training of long duration results in the elevation of oxidative capacity (34). Type Iib fibers are not recruited during normal ventilatory behaviors in the costal diaphragm and serve as a protective strategy against fatigue in the diaphragm muscle. However, high intensity exercise requires an additional recruitment of motor units within the crural region (36). Supported by these facts, we assumed that the observed differences between the two parts of the diaphragm are due to differences in the muscles fiber type composition.

Studies measuring changes in muscle oxidative potential following endurance training show an increase in oxidative enzyme capacity of approximately 9-25% (37). In the present study, following 3 weeks of slow or fast running, the findings suggest that a physical load intensity increase from sedentary to intense exercise status resulted in the expression of MMP-2 in diaphragm. On the other hand, a gradual transfer from sedentary life style to low work intensity did not end up with a major expression of MMP-2, which suggests that changes in ECM might happen mainly under highly intense efforts. We assume that the 32 m/min speed we used constitutes an ‘aggressive’ protocol that may have created such a high level of fatigue and injury which ended up with increasing MMP-2 expression causing some degradation of extracellular matrix.

It has been reported that slow twitch muscles contain more collagen IV (38) than fast twitch muscles and that the concentration of ECM is higher around slow than fast skeletal muscle fibers in rats (39). This was settled by Koskinen et al (40) who demonstrated higher levels of MMP-2 in the soleus muscle which includes mostly slow fibers. In contrast, in the present study, a relative increase in gelatinolytic activity of MMP-2 was higher in the fast twitch muscle fibers than in the slow twitch fibers particularly in the FSR group. Previous works have shown that MMP-2 can be inhibited by tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1,2), which are secreted by the same myofiber that secretes MMP-2 (41). We confirmed that TIMP-1,2 activity changes in parallel with MMP-2 activity, as also previously reported (40).

In the current study, every muscle fiber type showed a unique response pattern to running speed measured by the MMP-2 expression level. We could observe that type Iib fibers were more susceptible to ECM degradation than type I muscle fibers. Moreover, a degree of muscle tissue change is fiber type specific, and appears more pronounced in type Iib fibers. A greater change in MMP-2 value, observed in type Iib fibers following 3 weeks of fast speed running, suggests a higher protein degradation in fast twitch (type Iib) than in slow twitch muscle fibers (type I).

There are potentially three explanations for the differences in MMP-2 expression between the muscle fibers. First, in rat skeletal muscles, type Iib fibers are at least twice as big as type I fibers, which probably explains a higher volume of collagen; therefore, ‘white’ fibers (fast twitch) require more MMP-2 to maintain its integrity than ‘red’ fibers (slow twitch). Secondly, ‘white’ fibers demonstrate a better muscle plasticity than ‘red’ fibers and show faster adaptation to exercise training. Under intensified training, fast fibers may undergo transition to intermediate muscle fibers (type Ia) with corresponding changes in ECM composition (42). Following the fast (32 m/min) and prolong running (50 min for 15 days), the anaerobic type Iib muscle fibers are thought to be more susceptible to oxidative stress in order to produce a greater aerobic energy. Thirdly, as mention before by Powers et al (43), the diaphragm differs from locomotor skeletal muscle in adaptation to exercise. The recruitment of coastal fibers increases from rest-to-low intensity, but is not maintained from low-to-high intensity of exercise. The costal region reaches a plateau in motor unit recruitment before meeting the ventilatory demand during intense exercise. This suggests that type Iib fibers are not recruited during normal ventilatory behavior. Therefore, in contrast to type I, high intensity performance of type Iia provides a trigger for type Iib intra- and extracellular adaptation.

In summary, treadmill exercise can serve as a model to demonstrate different damage pattern to the two separated parts of the diaphragm muscle due to running. The integrity and composition of the ECM were affected through expression of degrading enzymes, such as MMP-2, and changes in the collagen synthesis particularly in type Iib muscle fibers.

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Conflict of interests: None declared.

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