Eccentric exercise markedly increases c-Jun NH$_2$-terminal kinase activity in human skeletal muscle

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STUDIES CONDUCTED IN MAMMALIAN cells have established the existence of four homologous yet distinct mitogen-activated protein kinase (MAPK) intracellular signaling pathways, including the c-Jun NH$_2$-terminal kinase (JNK) intracellular signaling pathway has been characterized as a stress-activated pathway based on its ability to respond to environmental stressors, including proinflammatory cytokines (35), osmotic shock (16), shear stress (25), and stretch (21, 27). Activation of this cascade involves the sequential phosphorylation of a series of proteins, including the MAPK kinase kinase 1 (26), MAPK kinase 4 (MKK4) (26) and/or MAPK kinase 7 (MKK7) (29), and JNK. JNK and other members of the MAPK family become fully active when they are phosphorylated on conserved threonine and tyrosine residues, and these kinases translocate to the nucleus where they can phosphorylate transcription factors, such as c-Jun (33) and ATF-2 (20). This leads to a complex and poorly defined series of alterations in gene transcription and expression, presumably contributing to the molecular adaptations that occur in response to stress.

Physical activity results in mechanical and metabolic disturbances similar to those observed with other forms of cellular stress. A single bout of moderate-intensity exercise can activate multiple intracellular signaling pathways in skeletal muscle, including the JNK cascade (1, 17, 40). JNK activity is increased three- to fourfold throughout 60 min of moderate-intensity treadmill running in rat skeletal muscle (17), sixfold after submaximal cycling exercise in the human vastus lateralis muscle (1), and sevenfold in response to contraction of rat hindlimb skeletal muscle in situ (2). Increases in MKK4 activity have been observed after electrical stimulation and contraction of the rat hindlimb (2) and after 60 min of cycling exercise in human skeletal muscle (40). These data suggest that the JNK signaling cascade is involved in the molecular adaptations that occur in response to exercise and contraction in skeletal muscle.

Skeletal muscle force production during dynamic exercise is dependent on both concentric (shortening) and eccentric (lengthening) contractions of individual sarcomeres. The eccentric component of a muscle contraction results in increased muscle soreness 24–48 h postexercise (4), ultrastructural myofibrillar damage (15, 31), increased myocellular enzyme release (9), prolonged muscle proteolysis (14), and inflammation (13). The immediate and prolonged presence of proinflammatory cytokines has also been observed with eccentric exercise (8), which appears to be involved in

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mediating skeletal muscle protein catabolism in vivo (13). The intracellular mechanisms responsible for these acute and prolonged alterations in skeletal muscle after eccentric exercise are not known. Because the JNK signaling cascade has been shown to be activated by numerous stressors, including skeletal muscle injury (3), proinflammatory cytokines (35), and stretch (21, 27), we postulated that eccentric exercise would result in a greater activation of JNK signaling compared with concentric exercise. Therefore, we examined the effects of maximal concentric and eccentric exercise on JNK activity in skeletal muscle of young, healthy male and female subjects.

METHODS
Subjects. This study was approved by the Sargent College Institutional Review Board at Boston University. Informed consent was obtained from each subject after the potential risks and procedures of the study were fully described to them. Twelve healthy subjects, seven men and five women, age 19–27 yr, were screened by a medical and fitness history questionnaire and physical examination. Exclusion criteria included any clinical evidence of cardiac, pulmonary, or hematologic abnormalities. Subjects were sedentary and were not participating in routine resistance or endurance aerobic training before participation in the study. All subjects selected were then assigned to either the concentric or eccentric exercise group (concentric n = 7: 4 men, 3 women; eccentric n = 5: 3 men, 2 women). Subjects were instructed to refrain from performing exercise (48 h) and from taking analgesics (10 days) before testing. Anthropometric measurements were collected, including height, weight, and percent body fat (Table 1). Percent body fat was determined by using skinfold calipers.

Experimental protocols. On day 1, after an overnight fast, a basal percutaneous needle biopsy was obtained from the vastus lateralis muscle from all subjects by using a 5-mm-diameter side-cutting Bergstrom needle with applied suction. Basal biopsies were obtained from either the nondominant or dominant leg in a random manner. On day 2, after an overnight fast, all subjects performed a total of 20 sets of 10 repetitions (100 repetitions/leg) of maximal concentric or eccentric knee extensions on a KinCom isokinetic dynamometer. Before the subjects began the exercise session, maximum torque was measured by using KinCom software. Subjects were encouraged to generate and maintain maximum torque throughout each set. Exercise was performed on both legs separately, beginning with the leg used to obtain the basal needle biopsy. Subjects were given a 1-min rest period between sets and a 5-min rest period before beginning exercise in the opposite leg. All subjects were able to complete the exercise immediately on completion of exercise, the subjects moved to a separate treatment room, and within 10 min a second needle biopsy was obtained from the leg that was not used to acquire the basal biopsy. A third needle biopsy was obtained 6 h postexercise from a separate site in the leg that was used to acquire the basal biopsy.

Blood samples were collected from all subjects in the basal state, immediately postexercise, and 3, 6, and 24 h postexercise for analysis of circulating creatine kinase (CK) concentrations. Serum CK activities were determined by using an enzymatic assay (Sigma Chemical, St. Louis, MO). Serum samples were also analyzed for interleukin (IL)-6 concentrations, which were measured in duplicate by using a high-sensitivity sandwich enzyme immunoassay technique on 96-well microtiter plates (R & D Systems, Minneapolis, MN).

Muscle processing. Approximately 100 mg of the vastus lateralis muscle obtained from muscle biopsies were homogenized (Polytron; Brinkman Instruments, Westbury, NY) in ice-cold buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na2VO4, 1% Triton X-100, 10% glycerol, 10 mM leupeptin, 3 mM benzamidine, 5 mM pepstatin A, 10 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Homogenates were rotated for 1 h at 4°C and centrifuged at 13,000 g for 68 min at 4°C. Samples were quickly frozen in liquid nitrogen and stored at −80°C. Protein concentrations of the muscle lysates were measured by using a kit purchased from Bio-Rad (6).

Kinase activity assays. For the JNK activity assays, muscle lysates (250 μg protein) were immunoprecipitated with 1.0 μg of anti-J NK1 and 50 μl of prewashed Protein A beads. After immunoprecipitation, the JNK immune complexes were washed and resuspended in 30 μl kinase assay buffer, and kinase reactions were carried out in a reaction mixture containing 3 μg inactive glutathione S-transferase (GST)-c-Jun as substrate, 3.75 mM MgCl2, 50 μM ATP, and 10 μCi [γ-32P]ATP. The GST-c-Jun fusion protein was prepared as previously described (24). For the MKK4 activity assay, muscle lysates (500 μg protein) from a subset of samples were immunoprecipitated with 3 μg of anti-MKK4 and 50 μl of prewashed Protein A beads. The immune complexes were washed extensively and resuspended in a reaction mixture containing kinase assay buffer (25 mM HEPES, 10 mM MgCl2, 2 mM DTT), 50 μM ATP, 10 μCi [γ-32P]ATP, and 1 μg inactive GST-J NK1 fusion protein as substrate. For both assays, reactions were terminated with Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) containing 400 mM DTT, samples were heated to 60°C, and labeled reaction products were resolved on 10% SDS-polyacrylamide gels. To visualize the proteins, gels were stained in Fast Green FCF Concentrate (F-6141, Sigma Chemical) diluted 1:1, destained in 30% ethanol and 10% glacial acetic acid, dried, and exposed to a PhosphorImager screen for 3 days. Bands were quantitated by using a PhosphorImager analysis system (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting. To determine JNK protein phosphorylation and expression, muscle lysates (100 μg protein) were solubilized in Laemmli sample buffer containing 400 mM DTT and boiled for 5 min. Samples were then resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with either 5% nonfat milk or 5% BSA, and immunoblotted with either an anti-J NK1 antibody (1:2,000) or a phosphospecific J NK antibody (1:5,000), which only recognizes J NK when dually phosphorylated at threonine residue 183 and tyrosine residue 185. After incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000), immunoreactive proteins were detected by using enhanced chemiluminescence.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Concentric Group</th>
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<tr>
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<td>21±1</td>
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<tr>
<td>Body fat, %</td>
<td>23.2±1.2</td>
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Values are means ± SE; n = 7 in concentric group and n = 5 in eccentric group. M, male; F, female; BMI, body mass index.
Concentric vs. eccentric-induced increases in JNK activity. Figure 1A shows the c-Jun reaction products from JNK activity assays from two representative subjects undergoing either concentric or eccentric exercise. The magnitude of change in JNK activity immediately postexercise ranged from no change to eightfold above basal in the concentric group and six- to 28-fold above basal in the eccentric group. Overall, the mean increase in JNK activity was fourfold above basal in the eccentric group and twofold above basal in the concentric group. Therefore, JNK activity increased 15-fold in the eccentric group immediately postexercise (Fig. 1B). Concentric vs. eccentric exercise resulted in either no change or a threefold increase above basal in the eccentric group. Therefore, JNK activity was greater in the eccentric group compared with the concentric group immediately postexercise (P < 0.05). At 6 h postexercise, JNK activity was back to near baseline levels, with activity levels decreasing to 1.4-fold above basal in the concentric group and twofold above basal in the eccentric group. JNK activity was not detected if anti-JNK or c-Jun substrate was omitted from the assay (negative controls). The greater activation of JNK with eccentric exercise was also demonstrated in experiments showing a greater phosphorylation of JNK by immunoblotting with a phosphospecific JNK antibody (Fig. 2A). The increase in JNK activity after exercise was not due to an increase in the amount of JNK protein in the muscle lysates (Fig. 2B).

MKK4 activity after concentric and eccentric exercise. As a result of the dramatic and significant increase in JNK activity observed immediately after eccentric exercise, we next determined if MKK4, the upstream regulator of JNK, was correspondingly activated in a subset of representative samples. MKK4 activity immediately postexercise resulted in either no change or a threefold increase above basal for the two concentric subjects examined and ranged from a 40% to 2.6-fold increase above basal for all subjects in the eccentric group. Interestingly, MKK4 activity levels were minimally altered for each subject at 6 h postexercise. Therefore, these data demonstrate that, although there was a trend for a similar increase (twofold) in both groups, MKK4 activation did not differ between concentric and eccentric exercise groups (Fig. 3).

Circulating CK and IL-6. CK is a myocellular enzyme released from damaged muscle and is considered a marker for skeletal muscle injury after exercise (12, 30). IL-6 is a cytokine released into the circulation during endotoxemia, trauma, and acute infection (23). In the present study, plasma CK and IL-6 concentrations did not change in the subjects who performed concentric exercise. However, we noted a dramatic increase in CK and IL-6 concentrations in two of the five subjects who performed eccentric exercise. Interestingly, these two subjects also had the highest JNK activities recorded immediately postexercise (Fig. 4).
This study demonstrates that eccentric exercise dramatically increases JNK activity in human skeletal muscle. Eccentric exercise increased JNK activity 15.4-fold immediately postexercise compared with the basal state, and this increase was significantly higher than the 3.5-fold increase observed immediately after concentric exercise. The transient increase in JNK activity immediately after exercise occurred in the absence of changes in the expression of JNK in the muscle.

Widegren et al. (40) recently demonstrated that MKK4 activity was elevated twofold immediately, 15 min, and 60 min after moderate-intensity cycling exercise. In the present study, we did not observe a statistically significant increase in MKK4 activity, which was likely due to the low number of subjects used to examine MKK4 activity. The increase in MKK4 activity observed with exercise is small and appears to be similar in both studies, regardless of the type of exercise or level of exercise intensity. These data suggest that MKK4 activation is not dramatically increased in response to either submaximal aerobic or maximal injury-producing resistance exercise. It is possible that MKK4 is activated to a comparable extent as JNK (15-fold) during eccentric exercise but then becomes dephosphorylated and deactivated by a phosphatase within seconds after exercise is stopped. Alternatively, a 1:1 stoichiometry between MKK4 and JNK may not exist, and only a small increase in MKK4 may be necessary to evoke a large increase in JNK activity.

Studies conducted in vitro have reported that MKK7, a recently identified and cloned isoform of MKK4, may be the primary upstream regulator of JNK after exposure to specific stressors, such as proinflammatory cytokines (29). Moriguchi et al. (29) demonstrated that tumor necrosis factor-α (TNF-α) stimulation in vitro rapidly and markedly activated MKK7, whereas tumor necrosis factor-α stimulation activated MKK4 slowly and to a limited extent. Therefore, it is possible that the stressor (cytokine accumulation, stretch) associated with eccentric exercise in our study activated not only MKK4, but also a distinct upstream regulatory protein that then led to enhanced JNK activation. Future studies will need to address the contribution of MKK7 and other upstream molecules in the activation of JNK after exercise.

Ostrowski et al. (32) recently reported that marathon running dramatically increased plasma CK and IL-6 concentrations and provided evidence that injury to the skeletal muscle fibers triggered the release of IL-6, a cytokine that is hypothesized to have both proinflammatory and anti-inflammatory effects (43). Intravenous injection of rat IL-6 in vivo results in a dramatic increase in JNK phosphorylation in the rat liver (P.-R. Ling and R. J. Smith, personal communication). Other data also suggest that eccentric exercise, but not concentric exercise, significantly increases CK and IL-6 concentrations (7). In the present study, we measured CK and IL-6 concentrations and found that there was a marked increase in both CK and IL-6 in two of the five subjects who performed eccentric exercise. Interestingly, these two subjects also had the highest JNK activities, suggesting that muscle injury above a specific threshold results in exaggerated alterations in JNK activity, CK release, and IL-6 production. The relationship between JNK activity, CK, and IL-6 after injury-producing exercise remains to be elucidated.

Although the primary stimulus for the increase in JNK activity with exercise is not known, stretch and proinflammatory cytokines are two possible candidates responsible for this activation. Static stretch applied to isolated rat soleus muscles in vitro increases JNK activity 20-fold, compared with a twofold increase with in vitro contraction (M. D. Boppart, R. A. Fielding, and...
The biological consequences of increased JNK activity after exercise are not known. Most studies using cultured cell models provide evidence that JNK is primarily involved in transcriptional activation. When complexed as a heterodimer c-Fos, the c-Jun/c-Fos heterodimer binds to activator protein-1 sites located in the promoter regions of genes implicated in the processes of inflammation and extracellular matrix turnover, including metalloproteinases (22). Guan et al. (18, 19) have also shown that IL-1β-induced activation of JNK leads to a concomitant increase in prostaglandin E2 production and cyclooxygenase-2 expression, two markers for inflammation. Recent studies have suggested that JNK is a direct inhibitor of the glucocorticoid receptor, a receptor known for its anti-inflammatory effects (37). In addition, the JNK cascade has been shown to be activated by a variety of proteins associated with inflammation, including ceramide (36) and spingosine (34). In addition to its potential role in the inflammatory response, JNK has also been implicated in mediating hypertrophy (10, 39) and apoptosis (5).

In summary, our data show that JNK activity is highly activated in response to injury-producing exercise. It is possible that signaling through the JNK cascade contributes to the acute and/or chronic adaptations that occur in response to exercise-induced skeletal muscle injury. Determining a role for JNK activation in skeletal muscle will be an important and exciting area of future investigation.

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