Activation pattern of MAPK signaling in the hearts of trained and untrained rats following a single bout of exercise

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Iemitsu, Motoyuki, Seiji Maeda, Subrina Jesmin, Takeshi Otsuki, Yoshitoshi Kasuya, and Takashi Miyauchi. Activation pattern of MAPK signaling in the hearts of trained and untrained rats following a single bout of exercise. J Appl Physiol 101: 151–163, 2006. First published February 16, 2006; doi:10.1152/japplphysiol.00392.2005.—Since exercise training causes cardiac hypertrophy and a single bout induces mechanical stress to the heart, the present study aimed to characterize the activation patterns of multiple MAPK signaling pathways in the heart after a single bout of exercise or chronic exercises. The hearts of untrained rats received 5, 15, and 30 min of treadmill running exercise (Ex5 to Ex30) and rested for 0.5, 1, 3, 6, 12, and 24 h (PostEx0.5 to PostEx24) before subjecting them to the following different experiments. Activation of MAPKs (ERK, JNK, and p38) and MAPKKs (MEK1/2, SEK, and MKK3/6) increased immediately after acute exercise in a time-dependent manner, with ERK, JNK, and p38 peaking at Ex15, Ex15, and Ex30, respectively. Expression of immediate early genes (c-fos, c-jun, and c-myc) was augmented and activator protein-1 DNA binding activity was enhanced in untrained rats immediately after a single bout of exercise. The elevated levels of MAPKs declined to the resting levels within 24 h after exercise. In another set of experiments, following 4, 8, and 12 wk of exercise training, the rats exhibited significant cardiac hypertrophy by week 12. Activation of MAPKs in the 4-wk-trained rats increased after a 30-min single bout of exercise but decreased in the 8-wk group. Finally, the activity of MAPKs signaling in the 12-wk-trained rats exposed to an acute bout of exercise was unaltered. We conclude that exercise induces the activation of multiple MAPK (ERK, JNK, and p38) pathways in the heart, an effect that gradually declines with the development of exercise-induced cardiac hypertrophy.

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) are ubiquitous signaling proteins involved in the control of cell growth, function, and adaptation. The MAPK family is divided into three major cascades, namely, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK (p38) (35). MAPKs are activated through phosphorylation by MEK1/2, SEK, and MKK3/6 (35). Mechanical stretch causes activation of MAPK signaling cascades, resulting in the induction of immediate early gene (c-fos, c-jun, and c-myc) expression, and subsequently these induced immediate early genes augment the expression of their target molecules related to hypertrophy of cardiomyocytes in vitro (18, 29–31, 43). MAPKs are well known to play an important role in pathological cardiac hypertrophy due to pressure overload caused either by hypertension or aortic banding in vivo (4, 5, 8, 19). Pressure overload-induced activation of MAPK in the heart of guinea pig was sustained for 8 wk after aortic banding, and this sustained activation of MAPK led to cardiac hypertrophy (38). Moreover, an elevated wall stress in the heart induced an increase in mRNA and protein expression of c-fos and c-jun in the rat heart (5).

Exercise training causes cardiac hypertrophy, which is considered as a favorable adaptation to the cardiovascular system (i.e., increased cardiac function during exercise, decreased resting heart rate, decreased submaximal heart rate during exercise, and increased filling time and venous return) (24, 26, 28). In addition, a single bout of exercise increases the myocardial mechanical load, accompanied by a positive alteration in heart rate, stroke volume, and sympathetic nervous system activity, which means that even a single bout of exercise can induce mechanical stretch to cardiac muscle in vivo (10, 22). Despite these cardiac benefits of exercise training, the molecular mechanisms underlying the exercise training-mediated cardiac adaptation are unclear. Because MAPK signaling cascades play a crucial role in cardiac growth, we hypothesized that activation of multiple MAPK signaling pathways by a bout of exercise contributes to the development of exercise training-induced cardiac hypertrophy. Indeed, a single bout of exercise enhanced the activation of JNK signaling pathway, a component of MAPKs, in the heart of untrained rats, whereas a 6-wk exercise training failed to activate JNK signaling (3). Thus cardiac MAPKs are differentially activated based on duration and intensity of exercise. To date, no study has characterized in depth the activation pattern of the different components of cardiac MAPKs. This includes upstream as well as the immediate early genes induced either by a single bout of exercise or by an exercise training of different durations and that induces cardiac hypertrophy. The present study is the first to characterize alterations in the activation pattern of multiple MAPK signalings, including ERK, JNK, and p38, and their upstream pathways MEK1/2, SEK, and MKK3/6, and expression of immediate early gene mRNA (c-fos, c-jun, and c-myc) after a single bout of exercise of different time points. Because c-Fos and c-Jun form activator protein-1 (AP-1), we also investigated the alteration of the transcriptional activity of AP-1 in the heart by a single acute bout of exercise. To further confirm the
alteration in AP-1 in the present experimental setting, we also
determined the changes in the myocardial mRNA expression of
endothelin-1 (ET-1) (15, 44) and brain natriuretic peptide
(BNP) (27), two important target genes of AP-1, after exercise
during different time durations. Last, to gain further mechanistic
insight of exercise training-induced cardiac adaptation, we
investigated the changes in the activation pattern of different
components of MAPKs, namely ERK, JNK, and p38 in the
heart of chronically exercise trained rats (4, 8, and 12 wk) after
an acute bout of exercise.

METHODS

Animals. The experimental protocols were approved by the Com-
mittee on Animal Research at the University of Tsukuba. Male
10-wk-old Sprague-Dawley rats were obtained from Charles River
Japan (Yokohama, Japan) and cared for according to the Guiding
Principles for the Care and Use of Animals based on the Helsinki
Declaration. All rats were maintained on a 12:12-h light-dark cycle
and received food and water ad libitum.

Experimental protocol of initial studies. Sixty rats were familiar-
ized with running for 2 days on a motor-driven treadmill for 10 min
at a speed of 15 m/min with no incline (0% grade). The other six rats
remained at rest for the training period, i.e., no training on a treadmill
for 2 days (NTC), to confirm whether treadmill exercise for 2 days
affects basal activation of signaling pathways in the rat heart. Resting
systolic arterial pressure and heart rate of the animals were measured
using a tail-cuff sphygmomanometer (model MK-1030, Muromachi
Kikai, Tokyo, Japan) 48 h before the experiment. The body weight of
the animals was also measured 48 h before the experiment. On the day of
the experiment, the rats were randomly divided into 10 groups:
resting control (RC); 5, 15, and 30 min of exercise (Ex5, Ex15, and
Ex30, respectively); and 0.5, 1, 3, 6, 12, and 24 h after 30 min of exercise
(PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and Post-
Ex24, respectively). Rats were run on a treadmill for 5 (Ex5), 15
(Ex15), and 30 min (Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6,
PostEx12, and PostEx24) at a speed of 30 m/min. NTC and RC rats
remained at rest for 30 min on the treadmill. Immediately after each
exercise time point, animals were anesthetized with diethyl ether, and the
heart was rapidly excised and washed thoroughly with cold saline to
remove contaminating blood, then the left ventricle was separated from
the right ventricle and atria. The left ventricle was weighed, frozen in
liquid nitrogen, and stored at −80°C for determination of the activation
of ERK1/2, JNK1/2, and p38 proteins by Western blotting analysis.
The soleus muscles in the sedentary rats and RC rats for 4, 8, or 12 wk
were also excised, frozen in liquid nitrogen, and stored for measurement
of citrate synthase activity. Sedentary rats, rested for 4, 8, and 12 wk, were
killed at the same time point as each age-matched trained rats (14, 18,
and 22 wk old).

Myocyte surface area. For determining myocyte surface area, the
frozen heart tissues were cut into 8-μm-thick sections. Then hema-
toxylin and eosin-stained slides were prepared by using standard
methods (16). At least 16 sections were taken from each sample, and
at least 64 microscopic fields were examined at ×400 magnification.
Myocyte scan area was calculated using ImageJ 1.33 software (NIH)
as previously described (16).

Muscle oxidative enzyme activity. Citrate synthase activity, a
marker of mitochondrial content and training adaptation, was mea-
sured in the whole soleus muscle homogenate using the spectropho-
tometric method (11).

Immunoblot analysis. Heart tissues were homogenized with 10
volumes of 20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 3 mM EDTA,
3 mM EGTA, 1 mM Na3VO4, 2 mM DTT, 20 mM β-glycerophos-
hate, 0.6% Nonidet P-40, 0.5 mM PMSF, 60 μg/ml aprotinin, and 1
μg/ml leupeptin on ice using a teflon homogenizer. The homogenate
was gently rotated for 30 min at 4°C and then centrifuged at 13,000
for 10 min at 4°C, and the protein concentration of the resulting
supernatant was determined. The samples (20 μg of protein) were
then subjected to heat denaturation at 96°C for 7 min with Laemmli
buffer. Western blot analysis for detection of phosphorylated and
upregulated forms of MAPK and MAPKK was performed according
to the method described in our laboratory’s previous paper
with minor modification (14). Briefly, each sample was separated on
10% SDS-polyacrylamide gel and transferred to a polyvinylidene
difluoride (Millipore, Billerica, MA) membrane. The membrane was
incubated in blocking buffer 5% skim milk in PBS containing 0.1%
Tween 20 for 12 h at 4°C, followed by incubation with primary
antibody, polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204), anti-
ERK1/2, anti-JNK, anti-phospho-p38 (Thr180/Tyr182), anti-p38,
anti-phospho-MEK1/2 (Ser277/272), anti-MEK1/2, anti-phospho-
SEK (Thr261), anti-SEK, anti-phospho-MKK3/6 (Ser189/207), anti-
MKK3/6, or G9 monoclonal anti-phospho-JNK (Thr183/Tyr185) an-
tibody (1:1,000 dilution with blocking buffer, Cell Signaling, Beverly,
MA) for 1 h at room temperature. The membrane was washed with
PBS containing 0.1% Tween 20 three times, and incubated with
horseradish peroxidase-conjugated secondary antibody, which was an
anti-rabbit or anti-mouse IgG (1:2,000 dilution with blocking buffer,
Cell Signaling), for 1 h at room temperature. After being washed, as
described above, the expression level of each molecule was deter-
mined using ECL detection reagents (Amersham Pharmacia Biotech)
followed by exposure to Hyper film (Amersham Biosciences).

Quantitative RT-PCR to determine levels of mRNA expression
in heart. Total tissue RNA was isolated with Isogen reagent
(Nippon Gene, Toyama, Japan), according to the method described in
our laboratory’s previous papers (11, 12). Briefly, the tissue was homog-
ienized in Isogen (50 mg tissue/1 ml Isogen) with a Polytron tissue
homogenizer (model PT1025K/35, Kinematica, Lucerne, Switzerland).
Total RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. Total RNA was treated with an RNase-free DNase kit (QIAGEN, Tokyo, Japan) and further purified with an RNaseasy mini kit (QIAGEN). Single-strand cDNA from prepared RNA (2 μg) was synthesized with oligo(dT) primer at 37°C for 60 min.

The mRNA expression levels of c-fos, c-jun, c-myc, ET-1, BNP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the left ventricle were analyzed by real-time quantitative PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA). Real-time quantitative PCR was performed according to the method described in our laboratory’s previous paper with minor modification (11).

Gene-specific primers and TaqMan probes were synthesized using our laboratory’s previous paper with minor modification (11). Primers and probe sequences used in the current study included the following: c-fos forward: 5′-CTGGTGTCAGCCACCTGCT-3′; reverse: 5′-CTCCCGCTCTGCGTAAAGC-3′; fos probe: 5′-CCCCATGGACGACCC-3′; c-jun forward: 5′-GAGCCAGAATCCGGGACCTT-3′; c-jun reverse: 5′-CCATGGTGAGTGCAG-3′; c-myc forward: 5′-CCCCGTAGCAACCATCA-3′; reverse: 5′-CCATACG-3′; ET-1 forward: 5′-ACTCCACGATGCAGAAGCT-3′; reverse: 5′-ACAATCCACGATGCAGAAGCT-3′; ET-1 probe: 5′-CCCGCTGACCCAACATCA-3′; GAPDH forward: 5′-CCCGCTGACCCAACATCA-3′; GAPDH reverse: 5′-CCACGGTTGACAACATCG-3′; GAPDH probe: 5′-GATCTGGGTCAACACT-3′, 5′-CTGGTGCAGC-3′.

The expression of GAPDH mRNA was determined as an internal control. The PCR mixture (25-μl total volume) consisted of 450 nM of both forward and reverse primers for c-fos, c-jun, c-myc, ET-1, BNP, and GAPDH, 200 nM of FAM-labeled primer probes (Perkin-Elmer Applied Biosystems), and TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems). Each PCR amplification was performed in triplicate, following the profile: 1 cycle at 95°C for 10 min and 40 cycles at 94°C for 15 s and 60°C for 1 min.

In the analysis of the present study, the slope of the standard curve was between −3.0 and −4.0, and the correlation coefficient was ≥0.95. The amount of target cDNA product in the myocardial tissue sample was calculated automatically by plotting each sample on the standard curve. The quantitative values of c-fos, c-jun, c-myc, ET-1, and BNP mRNA were normalized by that of GAPDH mRNA expression.

**Electrophoretic mobility shift assay.** Heart tissues were homogenized with 10 volumes of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, 1 mM DTT, 20 mM β-glycerophosphate, 0.5 mM PMSF, 60 μg/ml aprotinin, and 2 μg/ml leupeptin on ice using a teslon homogenizer. After the addition of Nonidet P-40 to 0.6%, the homogenate was rotated for 30 min at 4°C and centrifuged at 3,000 g for 10 min at 4°C. The precipitated nuclear fraction was resuspended in 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, 0.2 mM DTT, 20 mM β-glycerophosphate, 0.5 mM PMSF, 60 μg/ml aprotinin, 2 μg/ml leupeptin, and 20% glycerol and centrifuged at 18,000 g for 10 min at 4°C, and the protein concentration of the resulting supernatant was determined. Electrophoretic mobility shift assay using the myocardial nuclear extracts was performed, according to the method described in our laboratory’s previous paper with minor modification (13, 14). Briefly, samples of left ventricular nuclear extracts (15 μg of protein) were incubated with 50,000 counts/million of a32P-labeled double-stranded oligonucleotide probe containing the consensus AP-1 binding sequence (5′-CGCGTGTAGGATCGCCGAAA-3′) for 20 min at room temperature in 10 μl of binding buffer, consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl2, 0.5 mM DTT, 4% glycerol, and 0.05 μg/ml poly[d]I·[d]C. We designed a mutant AP-1 oligonucleotide (5′-CCGTTGATGCGCCGAAA-3′) for a competitive experiment. For supershift experiments, 1 μg of anti-c-fos and anti-c-jun polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The DNA-protein complexes were separated from free DNA probe by electrophoresis in nondenaturing 4% polyacrylamide gel in 0.5× Tris-borate-EDTA at 4°C. After drying, the gel was subjected to autoradiography and analyzed by a bioimaging analyzer ( BAS-5000, Fuji Film, Tokyo, Japan).

**Statistical analysis.** Values are expressed as means ± SE. Statistical analysis was carried out by analysis of variance followed by Scheffe’s F-test for multiple comparisons. Values of P < 0.05 were accepted as significant.

**RESULTS**

**First series: a single bout of acute exercise in untrained rats.** There was no significant difference in body weight, left ventricular weight, resting heart rate, and resting systolic and diastolic blood pressure among the NTC, RC, Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24 rats (Table 1).

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<th>Table 1. Body weight, left ventricular weight, resting heart rate, and resting blood pressure in untrained rats</th>
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Values are means ± SE; n, no. of rats. Postexercise: recovery point from 30-min treadmill running exercise; LVW, left ventricular weight.
There were no significant differences in the activation of MAPK and MAPKK and the mRNA expression of c-fos, c-jun, c-myc, preproET-1, and BNP in the heart between NTC and RC rats, suggesting that treadmill running for 2 days did not affect the basal activation of cardiac hypertrophic signaling (data not shown).

Figure 1A shows representative immunoblots of the time course alteration of MAPK in the heart immediately after a single bout of acute exercise and the 24-h postexercise period. Activation of ERK1/2 (p44/p42) in the untrained heart increased time dependently after acute exercise. Activation of ERK-p44 at the time points of Ex15, Ex30, PostEx0.5, and PostEx1 and activation of ERK-p42 at the time points of Ex15 and Ex30 were significantly higher than in the RC group, and peak activation of p44 and p42 was observed in Ex15 (Fig. 1B). Activation of JNK1/2 (p54/p46) in the untrained heart increased time dependently after an acute bout of exercise. Activation of JNK-p54 at the time points of Ex15 and Ex30 and activation of JNK-p46 at the time point of Ex15 were significantly higher compared with the RC group, and a peak activation of p54 and p46 was evident in Ex15 (Fig. 1B). Activation of p38 in the untrained heart was augmented time dependently after a single bout of acute exercise and was significantly higher at the time points of Ex15 and Ex30 than in the RC group (Fig. 1B). Peak activation of p38 was seen in Ex30 (Fig. 1B). These activations were restored to normal levels time dependently after the respective peak points and returned to the level in RC rats by 12 or 24 h after acute exercise in untrained rats (Fig. 1).
Figure 2A shows representative immunoblots of the time-course alteration of MAPKK, the upstream of MAPK, in the untrained heart immediately after acute exercise and the 24-h postexercise period. An augmented activation of MEK1/2 in the untrained heart was seen time dependently after acute exercise (Fig. 2B). Activation levels of MEK1/2 were significantly higher at the time points of Ex15, Ex30, PostEx0.5, PostEx1, PostEx6, and PostEx12 than in the RC group, and a peak activation was observed in Ex30 (Fig. 2B). Activation of SEK in the untrained heart was enhanced time dependently after an acute bout of exercise, and significantly higher activation was detected at the time point of Ex15 than in the RC group (Fig. 2B). A time-dependent increased activation of MKK3/6 was seen in the untrained heart immediately after acute exercise, and a significantly higher level of activation was found at the time points of Ex15 and Ex30 compared with the RC group with a peak activation in Ex30 (Fig. 2B). These activations returned to the level in RC rats by 24 h postexercise (Fig. 2).

A time-dependent elevation of c-fos mRNA expression was observed in the untrained heart and increased immediately after acute exercise (Fig. 3). As can be seen in Fig. 3, the expression of c-fos mRNA was significantly higher at the time points of Ex15 and Ex30 than in the RC group with a peak expression in Ex30 (Fig. 3). Immediately after an acute exercise bout, expression of c-jun mRNA in the untrained heart was increased time dependently (Fig. 3). Expression of c-jun mRNA was significantly higher at the time points of Ex15, Ex30, PostEx0.5, and PostEx1 than in the RC group, with a peak expression level in Ex30 (Fig. 3). Expression of c-myc mRNA in the

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**Figure 2.** Changes in activity of mitogen-activated protein kinase kinase (MEK-1/2, SEK, and MKK3/6) proteins in untrained heart (left ventricle tissue) from RC, Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24. **A:** representative immunoblots of phospho-MEK1/2, MEK1/2, phospho-SEK, SEK, phospho-MKK3/6, and MKK3/6 proteins. **B:** results of densitometric analysis of activation levels of MEK1/2, SEK, and MKK3/6 proteins. Values are means ± SE. Statistical analysis was done by post hoc Scheffé’s F-test for multiple comparisons (RC vs. Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24; n = 6 for each group). *P < 0.05 vs. RC rats.
untrained heart was also increased time dependently just after an acute exercise bout (Fig. 3). Expression of c-myc mRNA was significantly higher at the time points of Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24. Expression of GAPDH mRNA was used as an internal control. Values are means ± SE. Statistical analysis was done by post hoc Scheffe’s F-test for multiple comparisons (RC vs. Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24; n = 6 for each group). *P < 0.05 vs. RC rats.

untrained heart was also increased time dependently just after an acute exercise bout (Fig. 3). Expression of c-myc mRNA was significantly higher at the time points of Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, and PostEx6 than in the RC group, with peak expression in PostEx0.5 (Fig. 3). These expressions of immediate early genes were decreased time dependently after respective peak and returned to the level in RC rats by 12–24 h postexercise (Fig. 3).

Figure 4 shows representative films of gel mobility shift assay of myocardial AP-1 DNA binding in RC and Ex30 rats. The activity of AP-1 DNA binding was decreased time dependently after respective peak and returned to the level in RC rats by 12–24 h postexercise (Fig. 3).

of unlabeled (competitive) AP-1 consensus oligonucleotide in a dose-dependent manner and was almost undetected with the addition of mutant AP-1 oligonucleotide (Fig. 4). Furthermore, the AP-1 complex band was supershifted by the addition of anti-c-fos or anti-c-jun antibody (Fig. 4). Thus these data indicate that this band represented specific AP-1 DNA binding and that specific AP-1 DNA binding complex contained c-fos and c-jun protein in the heart. The activity of myocardial AP-1 DNA binding was significantly higher in the Ex30 group than in the RC group (Fig. 4).

The mRNA expression level of preproET-1 and BNP, two important target genes of AP-1, was significantly higher in the untrained rats at the time point of PostEx1 than in the RC group

Fig. 3. Changes in mRNA expression of c-fos (top), c-jun (middle), and c-myc (bottom) in untrained heart (left ventricle tissue) from RC, Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24. Expression of GAPDH mRNA was used as an internal control. Values are means ± SE. Statistical analysis was done by post hoc Scheffe’s F-test for multiple comparisons (RC vs. Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24; n = 6 for each group). *P < 0.05 vs. RC rats.

Fig. 4. Typical examples of gel mobility shift assay to demonstrate the level of activator protein-1 (AP-1) DNA binding activity in untrained heart after 30 min of exercise and in resting control heart (nuclear extract of left ventricle tissue was used). Top: each competitive assay for AP-1 DNA binding (lanes 1–5) was carried out in the presence of a 2-, 10-, or 100-fold molar excess of each unlabeled AP-1 oligonucleotide (competitor). Arrow of AP-1 indicates AP-1 complex. The specificity of AP-1 DNA binding was determined by addition of mutant AP-1 oligonucleotide (lane 6), and the super shift of AP-1 DNA binding complex was determined by addition of c-Jun and c-Fos antibodies (lanes 7 and 8, respectively). Bottom, left: representative photographs of gel mobility shift assay to show the AP-1 DNA binding activity level in untrained heart (left ventricular nuclear extracts) from resting control (C) and after 30 min exercise (E) rats using AP-1 oligonucleotide probe. Arrow of AP-1 indicates AP-1 complex. Bottom, right: results of densitometric analysis of AP-1 DNA binding activity level in the untrained heart (resting and after 30 min exercise). Data are means ± SE. NS, nonspecific binding; F, free probe. Statistical analysis was performed using Student’s t-test to compare between the groups (each group, n = 6). Values of P < 0.05 were accepted as significant.
Second series: a single bout of acute exercise in chronically exercise-trained rats. Left ventricular weight and myocyte surface area were significantly higher in the 12-wk-trained rats compared with the age-matched sedentary rats but unchanged in 4- or 8-wk exercise-trained rats (Table 2). Left ventricular weight-to-body weight ratio (LVW/BW) was significantly higher either in the 8- or 12-wk-trained rats than in the respective age-matched sedentary rats, whereas 4-wk exercise training had no effect on LVW/BW (Table 2). Resting heart rate was significantly lower either in the 8- or 12-wk-trained rats than in the respective age-matched sedentary rats but was not modified by 4 wk of exercise training (Table 2). There was no significant difference in body weight and resting systolic and diastolic blood pressure between the trained rats of 4, 8, or 12 wk and respective age-matched sedentary rats (Table 2). Citrate synthase activity in the soleus muscle was significantly higher in the 8- or 12-wk-trained rats compared with the age-matched sedentary rats but was unchanged by 4 wk of exercise training (Table 2).

There was no significant difference in the activation of ERK1/2, JNK1/2, and p38 in the heart of different age groups of sedentary rat (n = 6 for each group) (the time duration of exercise training), suggesting that the basal MAPK phosphorylation states in the sedentary rat heart did not depend on the age (see supplementary figure at http://jap.physiology.org/cgi/data/00392.2005/DC1/1).

There was no significant difference in the activation of MAPK (ERK1/2, JNK1/2, and p38) in the heart between sedentary and trained rats of 4, 8, and 12 wk at resting state, except JNK-p46 protein, which was higher in trained-rest rats of 4 or 8 wk compared with the respective age-matched sedentary rats (Fig. 6–8).

Activation of ERK1/2 (p44/p42) in the heart of 4-wk exercise-trained rats was increased after a 30-min bout of exercise but not changed either in 8- or 12-wk-trained rats (Fig. 6). Activation of JNK-p54 in the heart of 4-wk exercise-trained rats was increased after a 30-min bout of exercise but unchanged both in the 8- and 12-wk-trained rats (Fig. 7). On the other hand, a 30-min bout of exercise could not induce any significant activation of JNK-p46 in the heart of 4-, 8-, or 12-wk exercise-trained rats.

Fig. 5. Changes in mRNA expression of endothelin-1 (ET-1; top) and brain natriuretic peptide (BNP; bottom) in untrained heart (left ventricle tissue) from RC, Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24. Expression of GAPDH mRNA was used as an internal control. Values are means ± SE. Statistical analysis was done by post hoc Scheffe’s F-test for multiple comparisons (RC vs. Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24; n = 6 for each group). *P < 0.05 vs. RC rats.

Table 2. Body weight, left ventricular weight, hemodynamic parameters, and citrate synthase activity in trained and sedentary rats

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<th>4 wk Training</th>
<th>8 wk Training</th>
<th>12 wk Training</th>
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<td></td>
<td>Sedentary</td>
<td>Trained</td>
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<tr>
<td>n</td>
<td>6</td>
<td>12</td>
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<tr>
<td>Body weight, g</td>
<td>463.3 ± 2.8</td>
<td>458.6 ± 3.7</td>
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<tr>
<td>LVW, g</td>
<td>0.93 ± 0.04</td>
<td>0.93 ± 0.02</td>
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<td>LVW/BW, mg/g</td>
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<td>Myocyte surface area, μm²</td>
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<td>Heart rate, beats/min</td>
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<td>387.0 ± 5.3</td>
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<td>Blood pressure, mmHg</td>
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<td>Diastolic</td>
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<td>Soleus citrate synthase activity, nmol/min/mg tissue (n = 6)</td>
<td>41.6 ± 3.1</td>
<td>48.6 ± 2.5</td>
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</tbody>
</table>

Values are means ± SE. BW, body weight. *P < 0.05 and †P < 0.01 vs. each age-matched sedentary control.
12-wk exercise-trained rats compared with the respective age-matched sedentary rats (Fig. 7). Activation level of p38 in the heart of 4-wk exercise-trained rats was significantly increased after a 30-min bout of exercise but unchanged in 8- or 12-wk exercise-trained rats (Fig. 8). Thus the activation of MAPKs was time dependently decreased by chronic exercise training in a period of 0–12 wk and returned to the level in trained-rest rats (Fig. 6–8).

Figure 9A shows representative immunoblots of the time-course alteration of MAPK in the heart immediately after acute exercise and the 24-h postexercise period in 12-wk exercise-trained rats. There was no difference in the activation of ERK1/2, JNK1/2, and p38 in the heart between the trained-rest rats and trained (Ex5, Ex15, Ex30, PostEx0.5, PostEx1, PostEx3, PostEx6, PostEx12, and PostEx24) rats (Fig. 9B).

DISCUSSION

We here show that a single bout of acute exercise causes a time-dependent transient activation of multiple cardiac MAPK (ERK1/2, JNK1/2, and p38) and MAPKK (MEK1/2, SEK, and MKK3/6) signaling in untrained rats, which are associated with upregulation of immediate early gene mRNA (c-fos, c-jun, and c-myc). Moreover, there was also an increase in AP-1 DNA binding activity, and mRNA expression of ET-1 and BNP, two important target genes of AP-1. This study is the first to...
provide a detailed characterization of cardiac MAPK signaling induced by a single bout of exercise in untrained rats.

Activation of multiple cardiac MAPK signaling cascades by a single bout of exercise in untrained rats could be one of the factors that induce upregulation of molecules associated with cardiac adaptation. Moreover, the activation pattern seen in the different components of cardiac MAPK by a single bout of exercise was not observed in trained rats, even though rats were subjected to a 30-min bout of exercise. Interestingly, chronic exercise training for 4–12 wk attenuates activation of cardiac MAPK signaling pathways by a single bout of exercise. After 12 wk of exercise training, the activation response of MAPK to a single acute bout of exercise disappears, although significant cardiac hypertrophy is evident. Thus we also suggest that acute exercise-induced activation of multiple cardiac MAPK pathways gradually declines with development of exercise training-induced cardiac hypertrophy.

It has been shown that activation of MAPKK and MAPK and expression of immediate early genes in cardiac myocytes is augmented by mechanical stretch, adrenergic agonist, and ET-1, leading to cardiac myocyte hypertrophy that is mediated by cell hypertrophy-related molecules (14, 18, 30, 43). A bout of exercise increases the myocardial mechanical load, accompanied by a parallel alteration in heart rate, stroke volume, and sympathetic nervous system activity in vivo (10, 22). Acute exercise does not only cause induction of catecholamine but also secretion of neurohumoral factors, i.e., ET-1 (23). Thus a number of factors could contribute to the positive response of cardiac MAPK signaling to a single bout of exercise in the untrained rats, as observed in the present study.

Fig. 7. Changes in activity of JNK1/2 (p54 and p46) protein in 4- (A), 8- (B), and 12-wk (C) exercise-trained heart (left ventricle tissue) from Trained-Rest, Trained-Ex30, and Sedentary groups. Left: representative immunoblots of phospho-JNK1/2 and JNK1/2 proteins. Right: results of densitometric analysis of activation levels of JNK1/2 (p54 and p46) protein. Values are means ± SE. Statistical analysis was done by post hoc Scheffé’s F-test for multiple comparisons among 3 experimental groups as mentioned above (n = 6). Values of P < 0.05 were accepted as significant.
Although a single bout of exercise activated cardiac MAPKs in untrained and 4-wk-trained rats, as revealed by the present data, the LVW/BW, and the cardiomyocyte surface area were unchanged compared with sedentary rats. The activation response of cardiac MAPK to an acute bout of exercise in 8-wk-trained heart was attenuated, but at that time there was a modest increase in LVW/BW (9% increase compared with sedentary rats) as well as cardiomyocyte surface area (4% increase compared with sedentary rats). This fact suggests development of an early stage of cardiac hypertrophy. Subsequently, cardiac hypertrophy (left ventricular weight was a 16% increase and the cardiomyocyte surface area was an 8% increase compared with sedentary rats) was evident when the rats received 12 wk of exercise training. However, at this stage, the response of cardiac MAPKs to a single bout of acute exercise was lost. Taken together, these findings suggest that myocardial MAPK signaling pathways are activated before the development of exercise training-induced cardiac hypertrophy. Instead, myocardial MAPK activation fades away in a time-dependent fashion during cardiac hypertrophy development. Indeed, Boluyt et al. (3) showed that the activation of JNK in the 6-wk-trained rat heart was not increased by an acute bout of exercise. Thus the present findings, together with the findings of Boluyt et al. (3), led us to speculate that activation of cardiac MAPK signaling pathways precedes cardiac hypertrophy and requires augmentation of MAPK target molecules related to cardiac hypertrophy. Furthermore, by the time exercise training-induced cardiac growth is established, further cardiac adaptation mediated by MAPK-dependent molecular signaling may not be necessary. However, the effect of longer exposure of exercise training should be investigated before drawing any definite conclusion. The time-course phenomenon of myocardial MAPK signaling pathways to a single bout of exercise may be helpful to understand the regulation of molecular signaling of exercise training-induced cardiac adaptation.

Several studies have reported that the pattern of gene and protein expressions of various cardiovascular regulatory molecules in the heart differs between exercise training-induced cardiac hypertrophy and that induced by hypertension (7, 9, 12). Here, we reveal that acute exercise-induced activation of MAPK signaling cascades and its downstream signaling is transient, resetting within 24 h after exercise. Moreover, the activation of MAPKs by a single bout of exercise was lost with the development of chronic exercise training-induced cardiac hypertrophy. On the other hand, pathological cardiac hypertrophy, caused by pressure overload, continuously enhanced activation of cardiac MAPK (38). Thus there appear to be etiological-based patterns of cardiac hypertrophic signaling mediated by MAPK pathways.

The intensity of treadmill-running exercise (30 m/min) applied to the rats in the present study causes ~80% of the maximal oxygen consumption (36), and this intensity of exercise training could induce cardiac hypertrophy (34, 39). Moreover, an interval training protocol of high exercise intensity (~80% of maximal oxygen consumption) markedly enhanced the development of cardiac hypertrophy (1, 42). In the present investigation, cardiac hypertrophy was clearly evident in 12-wk exercise-trained rats. In addition, these trained rats with cardiac hypertrophy and bradycardia exhibited no change in blood pressure. Moreover, the level of citrate synthase activity in soleus muscle, which is a potential marker of the treadmill training adaptation, corresponded to the extent of cardiac adaptation. Finally, we measured brain weight of different trained rats and sedentary rats, because there was a slight but statistically insignificant decrease in body weight in the 12-wk exercise-trained rats. In addition, there was no significant difference in the brain weight between the trained rats of 4, 8, or 12 wk and the respective age-matched sedentary rats (4 wk: 1.4 ± 0.1 vs. 1.4 ± 0.1; 8-wk: 1.6 ± 0.1 vs. 1.5 ± 0.1; 12-wk: 1.9 ± 0.1 vs. 1.9 ± 0.1 g; trained vs. sedentary, respectively). But, when the left ventricular weight-to-brain weight ratio was calculated, 12-wk exercise-trained rats exhibited a higher value of left ventricular weight-to-brain weight ratio (data not shown). Based on the above findings, the present results...
indicate that rats trained for 12 wk underwent physiological cardiac adaptation.

Although mechanical stretch is an integral component that activates cardiac hypertrophic signaling pathways, specifically MAPK, a number of additional factors, such as adrenergic signaling (2) and emotional stress (41), may also contribute to the activation of cardiac MAPK. In the present study, a single bout of exercise and exercise training using treadmill might cause the induction of other factors, i.e., factors that help the rats to run, such as shock grid. Thus we cannot rule out the possibility of the involvement of these factors other than exercise in the alteration of MAPK in rat heart. But one should keep in mind that, when the rats receive exercise training for several weeks, the rats may get familiar with the training device, which would, in turn, reduce the possibility of the involvement of the emotional stress in such an experimental setting. Further experiments using a free-wheel running exercise may help to clarify the role of emotional stress in activation of cardiac MAPK by a bout of exercise and exercise training. Alternatively, the determination of stress marker may justify and validate the data in the present study. Second, we here demonstrate loss of activation of multiple MAPK signaling pathways in the training-induced hypertrophic heart by a single bout of acute exercise in vivo. However, a report by Korzick et al. (20) showed enhanced activation of ERK through protein kinase C signaling when the perfused heart of exercise-trained rats was exposed to α-adrenergic stimulation. The present study cannot at this point exclude possible activation of MAPK in exercise training-induced hypertrophied heart in response to an α-adrenergic stimulation. Future studies
should clarify this issue and examine the other cardiac hypertrophic signaling pathways, such as inflammatory cytokine-induced activation of janus kinase/signal transducers, activators of transcription (JAK/STAT) (21), and calcium-induced activation of calcineurin (25) in the current experimental setting. Third, although the present study showed no difference in the activation of MAPK in the 12-wk-trained heart after a single bout of exercise and the 24-h postexercise period compared with the age-matched resting rats, it is difficult to detect any significant differences between the experimental groups, because the present study used a small sample size (n = 2) to investigate the MAPK activation pattern in the rat heart after 12 wk of exercise training. To draw a convincing conclusion, more animals should receive 12 wk of exercise training and be evaluated for the cardiac activation pattern of MAPK. The present study used constant exercise intensity, i.e., a treadmill speed of 30 m/min, throughout the study period, irrespective of the different time period of exercise training. The rats after a certain time period of exercise training may get accustomed to exercise training, which ultimately may cause a difference in the aerobic capacity and finally may have some additional influences on the parameters of cardiac physiological adaptation (42). It would be important to use a relative exercise intensity, depending on their oxygen consumption in the present experimental setting. Thus the comparison of the absolute vs. relative intensity of exercise training in the present design may explore further new information concerning the MAPK activation pattern as well as the induction of hypertrophic change in the rat heart after different time periods of exercise.

In summary, the present study provides the first comprehensive analysis of cardiac activation pattern of multiple MAPKs (ERK1/2, JNK1/2, and p38) in rats in response to an acute bout of exercise either in trained or untrained rats. We also here demonstrate that, in the heart of untrained rats, a single bout of acute exercise induces time-dependent transient activation of multiple MAPKs and MAPKKs (MEK1/2, SEK, and MKK3/6) and expression of immediate early gene mRNA (c-fos, c-jun, and c-myc). The exercise-induced activation of multiple cardiac MAPK pathways is gradually diminished with the development of exercise training-induced cardiac hypertrophy. Thus the time-course alteration of myocardial MAPK signaling pathways to a single bout of exercise provides insights that may help extend our knowledge and understanding of molecular signaling underlying exercise training-induced cardiac adaptation.

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