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Regulatory Effects of Exercise and Dietary Intervention in Mitogen Activated Protein Kinase Signaling Pathways in Rats

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As a central component of a novel protein kinase cascade, the activation of the mitogen-activated protein (MAP) kinase cascade has attracted considerable attention. We sought to determine the effect of exercise and diet on the activation of the extracellular-signal regulated protein kinase (ERK) 1/2 and the p38 MAP kinase pathways in rat soleus muscle. Forty-eight Sprague-Dawley rats were assigned to one of two dietary conditions: high-carbohydrate (CHO) or high-fat (FAT). Animals having each dietary condition were further divided into one of three subgroups: a sedentary control group that did not exercise (NT), a group that performed 8 weeks of treadmill running and was sacrificed 48 h after their final treadmill run (CE), and a group that was sacrificed immediately after their final routine exercise training (AE). A high-fat diet did not have any significant effect on phosphorylated and total forms of ERK 1/2 or p38 MAP kinase. In chronically trained muscle that was taken 48 h after the last training, phosphorylated ERK 1/2 significantly increased only in the FAT but not in the CHO groups. In the case of total ERK 1/2, it increased significantly for both groups. In contrast, both phosphorylated and total forms of p38 MAP kinase decreased markedly compared to sedentary muscle. In muscle that was taken immediately after a last bout of exercise, phosphorylated ERK 1/2 increased in both groups but statistical significance was seen only in the CHO group. Total ERK 1/2 in acutely stimulated muscle increased only in the CHO-AE group even though the degree was much lower than the phosphorylated status. Muscle that was taken immediately after the routine training increased in phosphorylation status of p38 MAP kinase for both dietary conditions. However, statistical significance was seen only in the CHO group owing to a large variation with FAT. In conclusion, a high-fat diet *per se* did not have any notable effect versus a high-carbohydrate diet on MAP kinase pathways. However, when diet (either CHO or FAT) was combined with exercise and/or training, there was differentiated protein expression in MAP kinase pathways. This indicates MAP kinase pathways have diverse control mechanisms in slow-twitch fibers.

Key words : Exercise training, Diet, ERK 1/2, p38 MAP kinase, Skeletal muscle

INTRODUCTION

Physical exercise and dietary manipulations are major factors that induce significant metabolic, biochemical and cellular changes in skeletal muscle. Physical exercise *per se* has crucial effects on a variety of metabolic and cellular processes in skeletal muscle[1,2]. Most of these functional adaptations result from the cumulative effects of repeated bouts of exercise on the expression of key muscle proteins[3,4], although a single exercise session can induce transient increases in skeletal muscle gene transcription[5]. A dietary regimen, independent from exercise, also has significant effects on substrate and cellular and/or genetic levels in molecular events[6,7]. Despite considerable progress in identifying a variety of mammalian signaling cascades over the past decade, the precise intracellular signaling mechanisms occurring through exercise and/or diet manipulation that lead to alterations in the expression of muscle proteins are not completely understood.

It has been established that contractile activity (induced by physical activity or electrical stimulation) and dietary intervention (high-carbohydrate or fat-feeding) are potent stimulators of glucose transport and uptake[8,9] and several other cellular growth and metabolic processes in skeletal muscle[10,11]. However, evidence indicates that these two stimulators use different signaling mechanisms in the stimulation of glucose transport[12,13]. It is commonly agreed that diet-induced glucose transport to the skeletal muscle occurs by an insulin-dependent signaling pathway, including insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol (PI) 3-kinase and perhaps an Akt-dependent signaling pathway[14,15].

It is generally accepted that glucose transport is impaired in insulin resistance, which plays an important role in the development of hyperinsulinaemia and hyperglycaemia associated with obesity[16] and non-insulin-dependent diabetes[17]. Studies have shown that a high-fat diet induces a state of insulin resistance and impairs glucose transport to working muscle in

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rats[18-21]. Reduced glucose transport in skeletal muscle, however has not been shown to match with diminished glucose transporter (GLUT4)[22-25]. Thus, the insulin resistance that develops in response to a high-fat diet in the skeletal muscle could result from impaired insulin signaling cascade. In fact, there are some studies showing that proteins in IRS-1 associated PI 3-kinase signaling pathways decreased significantly with a high-fat diet[26,27]. Although possible mechanisms, including down-regulation of IRS-1 and PI 3-kinase in muscle in response to a high-fat diet, have been proposed for insulin resistance condition, the role of other signaling pathways, such as MAP kinase, also have to be elucidated.

Although recent studies have shown that a "physiological" bout of exercise activity and/or electrical-induced muscle contraction activates the MAP kinase signaling cascades in both rat[28-31] and human[32-34] skeletal muscle, the mechanisms behind muscle contraction-induced glucose transport and uptake remain largely unresolved. Moreover, the interaction of diet and exercise (or chronic training) on MAP kinase signaling pathways has not been examined. Therefore, we first determined whether a high-fat diet *per se* also alters (i.e., down-regulates) major proteins in the MAP kinase pathways. Second, whether any interactive relationship in MAP kinase cascades occurs between exercise (both chronic training and a single acute bout of exercise) and dietary intervention. Improving our knowledge of the impact of changes in diet and regular exercise is important for determining the regulation of key metabolic, mitogenic and genetic events in skeletal muscle as they might relate to health and disease states.

MATERIALS AND METHODS

1. Materials

Total ERK1/2 rabbit polyclonal IgG-antibody was purchased from Santa Cruz Biotechnology (catalogue no. Sc-93; Santa Cruz, CA, USA). Phospho-ERK 1/2 (Thr202/Tyr204, #9101S), p38 MAPK (no. 9212) and phospho-p38 MAPK (Thr180/Tyr182; no. 9211) antibodies were purchased from New England Biolabs (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (no. 7071-1) was also purchased from New England Biolabs. Reagents for enhanced chemiluminescence were purchased from NEN Life Science Products (Boston, MA, USA; no. NEL104). All other reagents were of analytical grade (Sigma, Castle Hill, NSW, Australia).

2. Animal Care

Forty-eight female Sprague-Dawley rats [initial body mass (BM) 90 ~ 100g] were obtained from Monash University Animals Services (Melbourne, Victoria, Australia) and were housed two per cage in an environmentally controlled laboratory (temperature 22±1℃, and relative humidity 50±2%) with a 12-h light-dark cycle (lights 07.00-19.00). For the 7 days prior to the experimental interventions, animals were fed standard rodent chow (Barastock, Victoria, Australia), given *ad libitum* access to water and were familiarized to laboratory conditions by performing 10 min·d-^{1} on three separate occasions.

3. Animal Training Program and Dietary Treatments

After 1 week, animals were randomly assigned to one of two dietary conditions: high-carbohydrate (CHO; n=24) in which animals were fed with standard rodent chow (16 E% fat, 20 E% protein, and 64% carbohydrate) or high-fat (FAT; n=24), in which animals were fed 78 E% fat and 22 E% protein. The high-fat diet was freshly prepared every 2 weeks and stored at 4℃.

Animals in each dietary condition (n=24) were further divided into one of three subgroups; (i) a sedentary control group that did not exercise (NT; n= 8), (ii) a group that undertook 8 weeks of treadmill running and was sacrificed 48 h after their final treadmill run (CT; n=8), and (iii) a group that was sacrificed immediately after their final routine exercise training (AE; n=8). All animals that undertook treadmill running trained 4 times·wk^{-1} for the 8 wk period. The training duration intensity was progressively increased over the initial 4 weeks so that all animals could complete a 1000 m treadmill run at a speed of 28 m·min^{-1} without the use of an electric shock grid or other external motivation. It is important to stress that this velocity was the highest voluntary running speed that all animals could attain after the first 4 weeks of training without electrical stimulation. This latter aspect is critical to subsequent interpretation of the results, because the MAPK signaling pathways are stimulated by a variety of environmental and physical stressors[32,35].

Once animals had attained their final exercise prescription, training was maintained for a further 4 weeks. At the completion of their final training session, half the animals in each training group were immediately sacrificed. The remaining animals were rested for 48 h before being sacrificed. The training program was chosen because previous investigations have shown this speed to elicit 75 ~ 80% of maximal oxygen uptake (VO_2max) in rats[36,37]. Animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg·g^{-1} BM). Once anaesthesia took effect (2-3 min), hind limb muscles from the right leg were exposed and the soleus muscle (89% type I fibres)[38] was dissected out and rapidly frozen in liquid nitrogen. Muscle samples were stored at 80℃ until subsequent analysis.

4. Muscle Homogenization

Portions of the soleus muscle (approximately 15 mg) were homogenized on ice in 1.5 mL lysis buffer (containing 25 mmol/L Tris-HCl, pH 6.8, 1 % sodium dodecyl sulphate (SDS), 5 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L sodium vanadate, 10% glycerol, 17.4 μg/mL phenylmethylsulphonyl

fluoride (PMSF), 10 μg/mL leupeptin and 1 μg/mL aprotinin). Homogenates heated for 10 min at 90℃ were vortexed and spun at 7000*g* for 10 min at 4℃. Supernatants were removed and protein content was determined using a commercially available kit (Micro BCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA). The supernatant was stored at -80℃ until subsequent analysis.

5. Western Blot Analysis

Aliquots of muscle homogenates containing 30 μg of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% resolving gel). A standard from the rat aorta was loaded in parallel with the muscle samples to identify ERK1/2 and p38 MAP kinase. However, the aorta showed only ERK1/2 but not p38 MAPK expression. Accordingly, all data were normalized against the corresponding sedentary value. Resolved proteins were transferred to polyvinylidenedifluoride membranes (MSI, 0.45mm; Osmonics) and blocked for 2 h with 5% non-fat milk. Membranes were incubated in the appropriate primary antibody specific for phospho-ERK1/2 (1:2000 dilution) and p38 MAP kinase (1:1000 dilution) overnight at 4℃. Membranes were washed in 0.05% Tween-20, incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase at a dilution of 1:7500 and washed in 0.05% Tween-20 again. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry. After immunoblotting with phospho-specific ERK1/2 or p38 MAPK antibodies, membranes were stripped for 30 min in buffer containing 62.5 mmol/L Tris-HCl (pH 6.7) 100 mmol/L β-mercaptoethanol and 2% SDS and were immunoblotted with antibodies specific for total ERK1/2 (1:10000 dilution) and p38 MAP kinase (1:2000 dilution).

6. Statistical Analysis

Data for the chronic exercise training and dietary intervention were analyzed using a two-way analysis of variance (2 x 2 factorial analysis of variance) with diet and training as fixed factors. To look at the effect of acute exercise and compare with either chronic exercise training or dietary intervention, one-way analysis of variance (ANOVA) was used. Where ANOVA revealed a significant difference, a Tukey's *post hoc* test was administered to locate the difference. Significance was accepted when $P<0.05$. All data are presented as mean±SEM.

RESULTS

1. Food Consumption, Energy Intake and Weight Gain

Table 1 shows food consumption and energy intake among the experimental groups. Estimated food consumption and energy intake were significantly higher in the CHO groups than in the FAT groups ($P<0.01$). At the end of the 8-week experimental period, there were no differences in the final BM of animals from any of the six groups (248±11, 256±8 and 255±7 g in the CHO-NT, CHO-CT, and CHO-AE groups, and 272±10, 258±8, 255±7 g in the FAT-NT, FAT-CT, and FAT-AE groups, respectively).

Table 1. Estimated daily food consumption and energy intake among experimental groups

	NT	CT	AE	Diet effect
Food consumption($g \cdot d^{-1}$)				
CHO	18.62±0.25	17.89±0.31	18.20±0.29	
				P<0.01
FAT	11.38±0.14	10.65±0.16	11.10±0.18	
Energy intake ($kcal \cdot d^{-1}$)				
CHO	68.85±1.52	66.15±1.60	67.30±1.49	
				P<0.01
FAT	60.91±0.96	57.95±0.92	60.40±0.84	

NT, no training; CT, chronic training; AE, acute exercise; CHO, high-carbohydrate diet; FAT, high-fat diet. Values are means±S.E.M. n=8 animals per group.

2. Total ERK 1/2 Expression

Figure 1 shows the interaction of exercise (training) and diet on total ERK 1/2 expression. Diet *per se* had no effect on ERK 1/2 expression or total ERK 1/2 expressions. Total ERK 1/2 expressions changed markedly in both the CHO and FAT groups when sampled 48 h after final training [Figure. 1; CHO-CT: 3.44 ±0.84-fold; FAT-CT: 2.19 ± 0.50 ($P<0.05$)]. Interestingly, there was only a significant increase in total ERK 1/2 in the CHO groups [CHO-AE; 1.27 ± 0.05, ($P<0.05$)], but not in the FAT groups [FAT-AE; 1.09 ± 0.07, ($P>0.05$)] that were taken immediately after their routine training.

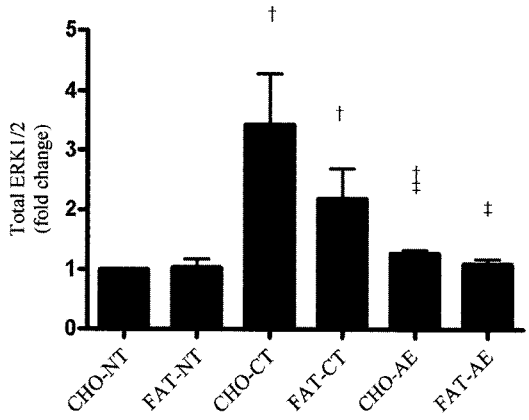

Fig. 1 Effects of exercise and diet on total extracellular signal regulated kinase (ERK) 1/2 expression in rat skeletal muscle. CHO, high carbohydrate diet. FAT, high fat diet, NT, sedentary; AE, acute exercise; CT, chronic training. All data were normalized against the CHO-NT value.
† $P<0.05$compared with NT when dietary conditions were same. ‡ $P<0.05$ compared with chronic training.

3. Phosphorylated ERK1/2 Expression

Figure 2 displays the interaction of exercise (training) and diet on phosphorylated ERK 1/2 expression. Similar to the total ERK 1/2 expression, diet *per se* had no effect on ERK 1/2 expression in phosphorylated forms of ERK 1/2 expression. Although phosphorylated ERK 1/2 was not different (P>0.05) from sedentary values in the CHO groups, it was significantly different (P<0.05) in trained muscles in the FAT groups that were sampled 48 h after final training (Figure. 2). In contrast, phosphorylated ERK 1/2 in the muscle of rats in the CHO groups showed a significant increase compared to their sedentary values (CHO-AE; 2.07 ± 0.46; P<0.05), but not in the muscle of rats in the FAT groups (FAT-AE; 1.53 ± 0.33; P>0.05) that were sampled immediately after their routine bout of training. In addition, phosphorylated ERK1/2 was not significantly different between CHO-AE and FAT-AE (P>0.05).

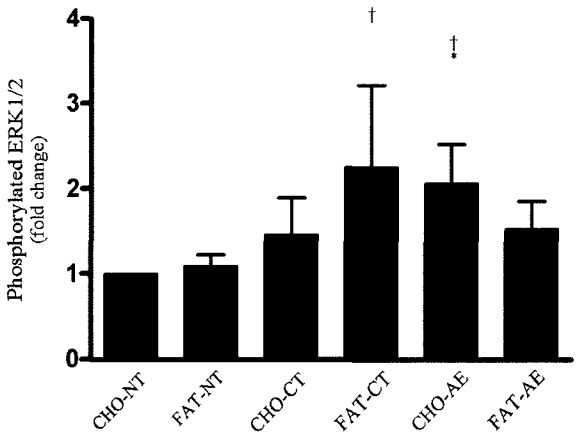

Fig. 2 Effects of exercise and diet on phosphorylated extracellular signal regulated kinase (ERK) 1/2 expression in rat skeletal muscle.

CHO, high carbohydrate diet; FAT, high fat diet; NT, sedentary; AE, acute exercise; CT, chronic training. All data were normalized against the CHO-NT value.

† P<0.05 compared with NT when dietary conditions were same. *P<0.05 compared with FAT when exercise/training conditions were same.

4. Total p38 MAP Kinase Expression

Figure 3 shows the interaction of exercise (training) and diet on total p38 MAP kinase expression. Diet *per se* had no effect on p38 MAP kinase expression. Total forms of p38 MAP kinase decreased significantly regardless of dietary conditions in muscles that were sampled 48 h after the final training bout compared to sedentary muscles (CHO-CT; 0.10 ± 0.03-fold, FAT-CT; 0.24 ± 0.09-fold, P<0.05, Fig. 3). Comparisons between chronically trained muscles (CHO-CT vs. FAT-CT) demonstrated a significant difference in total p38 MAP kinase expression. The muscles taken from rats immediately after the routine bout of training increased in total status for both dietary conditions (CHO-AE; 1.44 ± 0.11, P<0.05; FAT-AE; 1.39 ± 0.14, P<0.05).

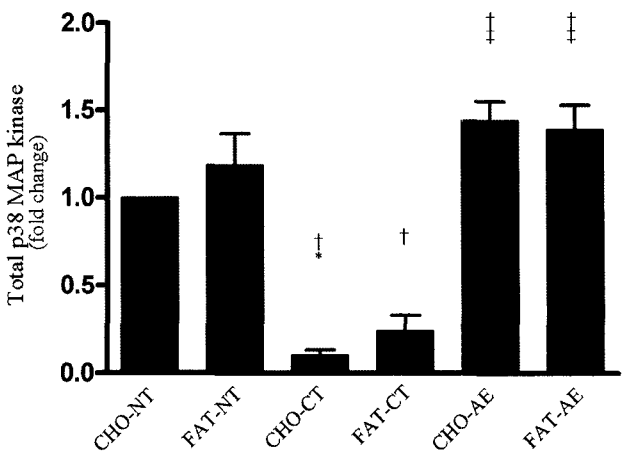

Fig. 3 Effects of exercise and diet on total p38 mitogen activated protein (p38 MAP) kinase expression in rat skeletal muscle.

CHO, high carbohydrate diet; FAT, high fat diet; NT, sedentary; AE, acute exercise; CT, chronic training. All data were normalized against the CHO-NT value.

† P<0.05 compared with NT when dietary conditions were same. ‡ P<0.05 compared with chronic training. * P<0.05 compared with FAT when exercise/training conditions were same.

5. Phosphorylated p38 MAP Kinase Expression

Figure 4 shows the interaction of exercise (training) and diet on phosphorylated p38 MAP kinase expression. Diet *per se* had no effect on p38 MAP kinase expression. In this study, the most striking finding was that phosphorylation of p38 MAP kinase markedly decreased regardless of dietary conditions in muscles that were sampled 48 h after the final training bout compared to sedentary muscles (CHO-CT; 0.28 ± 0.08-fold, FAT-CT; 0.22 ± 0.09-fold, P<0.05, Fig. 4). The muscles of rats that were sampled immediately after their one exercise training bout increased in phosphorylation

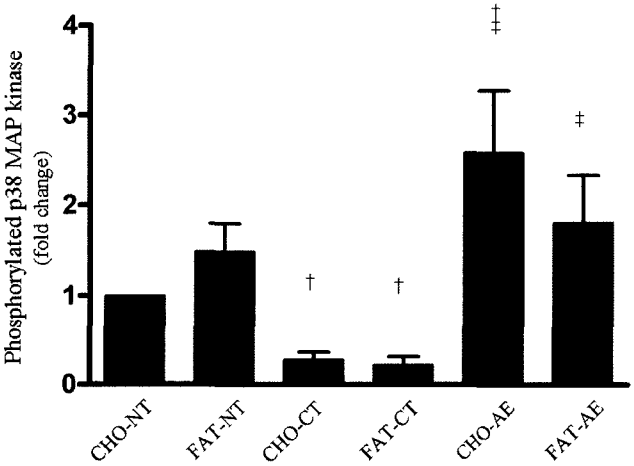

Fig. 4 Effects of exercise and diet on phosphorylated p38 mitogen activated protein (p38 MAP) kinase expression in rat skeletal muscle.

CHO, high carbohydrate diet; FAT, high fat diet; NT, sedentary; AE, acute exercise; CT, chronic training. All data were normalized against the CHO-NT value.

† P<0.05 compared with NT when dietary conditions were same. ‡ P<0.05 compared with chronic training.

status for both dietary conditions. However, statistical significance was reached only in the CHO groups owing to a large variation with the FAT groups [CHO-AE: 2.59 ± 0.69, (P<0.05); FAT-AE: 1.81 ± 0.53, (P>0.05)].

DISCUSSION

Physical activity and dietary manipulation is a crucial regulator of protein synthesis[39] and gene transcription[40] in skeletal muscle. Regular exercise (or muscle contraction) or dietary intervention leads to structural remodeling of muscle, resulting in profound effects on the functional capacity of the tissue[2]. To determine the underlying molecular mechanisms through which contraction causes these important changes in skeletal muscle, the signaling molecules that convert mechanical/biochemical contraction stimulus into intracellular responses must be defined. The transmission of extracellular signals to their intracellular targets is mediated by a network of interacting proteins that governs a large number of cellular processes[35]. As a central component of a novel protein kinase cascade, the activation of the MAP kinase cascade has attracted considerable attention. This pathway is extensively used for transcytoplasmic signaling to the nucleus and is involved in a wide variety of cellular responses to external stimuli[41]. In addition, recent investigations have shown that a "physiological" bout of exercise activates the MAP kinase signaling cascades in both rat[28-31] and human[32-34] skeletal muscle. However, no relationship has been found between muscle contractile (i.e. physical exercise) and dietary intervention on MAP kinase signaling pathways. Therefore, we first determined whether a high-fat diet *per se* alters (i.e., down-regulates) major proteins in MAPK pathways. Second, whether any interactive relationship in MAPK cascades occurs between exercise (both chronic training and a single acute bout of exercise) and dietary intervention.

In the present study, notable stimulatory effects occurred from either chronic or acute exercise whereas no significant unique dietary effect was revealed in proteins involved in the MAP kinase pathways. This indicates that the inhibition of insulin-stimulated signaling pathways is not associated with MAP kinase pathways. Thus, high-fat diet-derived glucose transport impairment does not appear to have any connections with MAP kinase intracellular signaling pathways. This confirms, as reported in earlier studies (Cheatham & Kahn, 1995; Virkamki et al., 1999), that high-fat diet-induced inhibition in cellular signaling cascades is mostly related to insulin-dependent signaling pathways, including insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol (PI) 3-kinase and perhaps an Akt-dependent signaling pathway.

Although recent studies have shown that a "physiological" bout of exercise activity and/or electrical-induced muscle contraction activates the MAPK signaling cascades in both rat (Goodyear et al., 1996; Hayashi et al., 1999; Lee et al., 2002; Nader & Esser, 2001) and human (Aronson et al., 1997; Krook et al., 2000; Widegren et al., 2000) skeletal muscle, the mechanisms behind muscle contraction-induced glucose transport and uptake remain largely unresolved. Moreover, no one has examined the interaction of diet and exercise (or chronic training) on the MAPK signaling pathways. Improving our knowledge of the impact of changes in diet and regular exercise are important for determining the regulation of key metabolic, mitogenic and genetic events in skeletal muscle as they might relate to health and disease states. In addition to this, the current findings demonstrated that after acute exercise in trained rats, the increase in ERK1/2 and p38 MAP kinase in both its phosphorylated and total forms occurred only in the muscles of rats in the CHO groups and not in the FAT groups. This indicates that an independent (or separate) mechanism for the activation of MAP kinase pathways during exercise operates when animals adapt to different diets. It has been extensively proposed that the activation of MAP kinase pathways induced by physical exercise or electrical muscle contraction uses independent pathways from those of insulin mediated signaling pathways[42,43]. Inhibited activation in MAP kinase pathways after a long-term high-fat diet, however, has to be elucidated by looking at more small adaptor proteins (i.e., mSOS, Shc, Grb2, etc) that exert their unique and complex effects on molecular signaling events.

Another important finding of the present study was that the pattern of post-exercise phosphorylation of ERK1/2 (figure 2) and p38 MAP kinase (figure 4) was different. Whereas ERK1/2 phosphorylation had returned to baseline (sedentary) values 48h after the last bout of exercise, p38 was marginally lower than resting levels at this time. Widegren *et al.*[44] reported that phosphorylation of the p42/44 MAP kinase was rapid, confined to the working muscle and that it returned to basal levels after the cessation of exercise. In contrast, p38 MAP kinase phosphorylation was induced more slowly and was not restricted to the exercising muscle[44], suggesting that systemic factors have a role in the activation of this kinase. In a cross-sectional study, Yu et al.[45] reported that ERK1/2 expression increased in the muscle of moderately trained subjects, but p38 MAP kinase expression decreased compared to that of healthy but untrained individuals. They proposed that regular exercise training was associated with down-regulation of some of the early components of contraction-induced signaling cascades and that such a feedback mechanism may serve to balance protein degradation with protein synthesis in skeletal muscle[45]. Thus, it would appear that the mechanisms by which cells transduce external stimuli into intracellular adaptations are likely to involve intricate regulation of a number of (independent) signaling cascades. Furthermore,

the integration of these various control mechanisms may represent a general adaptive response for control of mitochondrial protein expression during aerobic training of skeletal muscle.

Because ERK1/2 protein expression increased in trained muscle but p38 MAP kinase expression decreased, it would appear that components of the mitogenic signaling cascade play specialized roles in modulating exercise-induced adaptations on gene expression, as has been suggested recently[31,45,46]. In this regard, Wretman *et al.*[46] have recently hypothesized that ERK1/2 is activated chiefly by metabolic events, whereas activation of p38 MAP kinase is elicited more in response to mechanical "stress" overloading. Our findings of increased p38 MAP kinase expression after an acute bout of intense treadmill running are in accord with such a hypothesis. These data point to a very rapid (translational) control of protein expression. However, the approximate halving of total ERK1/2 over the same period is difficult to explain. Because ERK1/2 has been shown to phosphorylate and activate nuclear proteins[47-48] then contraction-induced activation of the MAP kinase signaling cascade may lead to divergent alterations in the biochemical profile of skeletal muscle that occur as a result of the cumulative effect of repeated bouts of exercise. Activation of the ERK signaling pathway is not thought to be crucial for acute metabolic regulation in skeletal muscle, such as glucose and amino acid transport or glycogen synthesis[29,43]. Accordingly, it is possible that ERK activation is involved in adaptive cellular changes at the level of mRNA and protein expression in response to changes in muscle contractile activity: the ERK proteins are assumed to translocate to the cell nucleus, causing phosphorylation of transcription factors and thereby increasing the rate of gene transcription[35]. Although it is likely that a major consequence of the exercise-induced activation of the MAP kinase pathway is gene transcription[28], such a link remains to be proven experimentally.

CONCLUSIONS

In conclusion, this is the first study to show the interaction of exercise and diet on the MAP kinase signaling cascade. An important finding from this study is that a high-fat diet *per se* did not have any significant effect on MAP kinase pathways. However, when diet (either CHO or FAT) was combined with exercise and/or training, there was differentiated protein expression in MAP kinase pathways. This indicates that MAP kinase pathways have diverse control mechanisms in slow-twitch fibers. Improving our understanding of the impact of changes in diet and the effects of regular exercise is important for determining the regulation of key metabolic, mitogenic and genetic events in skeletal muscle as they might relate to health and disease states.

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