Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 56 (2007) 1192 – 1200
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Evidence for the regulation of contraction-induced fatty acid oxidation via extracellular signal-regulated kinase 1/2 activation independent of changes in fatty acid uptake

Marcella A. Raney, Lorraine P. Turcotte*

Departments of Kinesiology and Biological Sciences, College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA 90089-0652, USA

Received 10 November 2006; accepted 10 April 2007

Abstract

Data show that extracellular signal-regulated kinase 1/2 (ERK1/2) may be involved in the regulation of fatty acid (FA) uptake during muscle contraction via stimulation of CD36 translocation to the plasma membrane. The perfused hind limb model was used to determine (1) the importance of ERK1/2 signaling on contraction-induced FA uptake and (2) the effect of ERK1/2-mediated FA uptake on contraction-induced FA oxidation. We perfused rat hind limbs with 8 mmol/L glucose, 550 μmol/L palmitate, and no insulin at rest in the absence of inhibitor and during moderate-intensity electrical stimulation and dose-dependent pharmacologic inhibition of ERK1/2 using increasing concentrations of PD98059 (P1 = none, P2 = 10 μmol/L, P3 = 20 μmol/L, P4 = 50 μmol/L). Increasing PD98059 concentration resulted in a gradual decrease in contraction-induced ERK1/2 phosphorylation, and this was accompanied by a decrease in contraction-induced FA uptake (concentration required for 50% inhibition [IC_{50}] = 15.8 ± 1.6 μmol/L) and in plasma membrane CD36 content (IC_{50} = 8.7 ± 0.3 μmol/L) ($P < .05$). Percent FA oxidation was significantly lower in P3 and P4 compared with P1 and P2. Based on IC_{50} values, FA oxidation demonstrated a greater sensitivity than FA uptake to changes in ERK1/2 phosphorylation (IC_{50} = 5.4 ± 0.3 μmol/L) ($P < .05$). A positive correlation was found between FA uptake and plasma membrane CD36 content (R^2 = 0.85, $P < .05$). Plasma membrane CD36 content, FA uptake, and FA oxidation each shared a positive correlation with ERK1/2 phosphorylation (R^2 = 0.64, 0.66, and 0.71, respectively; $P < .05$). These results suggest that during moderate-intensity muscle contraction, ERK1/2 phosphorylation is required for translocation of CD36 to the plasma membrane and the subsequent increase in FA uptake. In addition, these data suggest that ERK1/2 signaling may be involved in the regulation of FA oxidation independently of its effects on FA uptake.

1. Introduction

Muscle contraction increases fatty acid (FA) uptake and oxidation in skeletal muscle [1-4]. However, the signaling mechanisms regulating the contraction-induced increase in FA metabolism are relatively unknown. It is now generally accepted that contraction-induced FA uptake is mediated in part by the translocation of the FA transporter FAT/CD36 from intracellular stores to the plasma membrane [1,5]. Contraction-induced FA oxidation is also mediated by a transporter system at the mitochondrial membrane, which includes transport of long-chain fatty-acyl-coenzyme A (CoA) into the mitochondria via carnitine palmitoyltransferase 1. However, the intracellular signaling mechanisms regulating CD36 translocation to the plasma membrane and increased transport of fatty-acyl-CoA across the mitochondrial membrane during contraction in skeletal muscle are not completely defined.

Evidence shows that the signaling molecule extracellular signal-regulated kinase (ERK1/2) may be involved in the regulation of various aspects of contraction-induced FA metabolism [4,6]. Indeed, our laboratory has shown that inhibition of contraction-induced ERK1/2 phosphorylation with the mitogen-activated protein (MAP)/ERK kinase 1/2 (MEK1/2) inhibitor PD98059 prevents the contraction-induced increase in plasma membrane CD36 content and FA uptake during moderate-intensity muscle contraction [4]. These results suggest that ERK1/2 signaling is involved in

* Corresponding author. Tel.: +1 213 740 8588; fax: +1 213 740 7909.
E-mail address: turcotte@usc.edu (L.P. Turcotte).

0026-0495/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2007.04.014

the regulation of FA uptake during moderate-intensity muscle contraction via an increase in CD36 translocation to the plasma membrane. However, to determine the relative importance of ERK1/2 signaling in the regulation of contraction-induced FA uptake during moderate-intensity muscle contraction, a significant linear relationship must be established between ERK1/2 activation and FA uptake as well as with CD36 plasma membrane content. Furthermore, in the above experiments, the effects of ERK1/2 inhibition and the subsequent decrease in FA uptake on contraction-induced FA oxidation were not measured.

Data from recent experiments demonstrate that FA uptake and oxidation as well as ERK1/2 phosphorylation increase from rest to moderate-intensity muscle contraction [7]. Percent FA oxidation was not changed by contraction intensity, suggesting that total FA oxidation was increased in response to the increase in FA uptake that may have been regulated by ERK1/2 activation [7]. In this scenario of cellular signaling, it might be expected that a decrease in ERK1/2 activation during moderate-intensity muscle contraction would be accompanied by similar relative decreases in FA uptake and total FA oxidation. However, we have shown repeatedly that during different experimental conditions, changes in total FA oxidation do not coincide with changes in FA uptake [3,8,9]. This indicates that factors other than FA uptake mediate FA oxidation and that these factors may mediate these changes in FA oxidation independently of FA uptake. In line with this argument, manipulation of FA uptake via different levels of ERK1/2 activation when contraction intensity and other intensity-dependent factors are held constant allows for the evaluation of the role of FA uptake in the regulation of FA oxidation.

Thus, the purpose of this study was to determine the effect of dose-dependent pharmacologic inhibition of ERK1/2 phosphorylation on contraction-induced FA uptake and oxidation to determine (1) whether the relationship between ERK1/2 phosphorylation and FA uptake is linear across different levels of ERK1/2 activation and (2) whether the sequential decrease in ERK1/2 phosphorylation level would be associated with a sequential decrease in total FA oxidation during moderate-intensity muscle contraction. Dose-dependent inhibition of contraction-induced ERK1/2 phosphorylation has been previously achieved with increasing concentrations of the MEK1/2-specific inhibitor PD98059 in a study performed in rat skeletal muscle [10], which made this inhibitor an obvious choice to achieve our purpose. We hypothesized that during moderate-intensity muscle contraction, dose-dependent inhibition of ERK1/2 would lead to a dose-dependent decrease in FA uptake demonstrating the existence of a linear relationship between the 2 variables. We further hypothesized that during moderate-intensity muscle contraction, dose-dependent inhibition of ERK1/2 would not be associated with a dose-dependent decrease in FA oxidation, providing evidence for the notion that factors other than FA uptake regulate FA oxidation.

2. Materials and methods

2.1. Animal preparation

Male Wistar rats (~285-330 g; N = 38) were housed in pairs and kept on a 12:12-hour light-dark cycle. They received standard rat chow and water ad libitum. Rats were randomly assigned to 1 of 4 groups whose hind limbs were perfused with dimethyl sulfoxide and increasing concentrations of PD98059, an inhibitor of the ERK1/2 upstream kinase MEK1/2 [11,12], during moderate-intensity electrical stimulation: 0.0 μmol/L (P1; n = 8), 10 μmol/L (P2; n = 7), 20 μmol/L (P3; n = 7), 50 μmol/L (P4; n = 8). An additional group of animals was perfused at rest in the absence of the inhibitor to serve as control (R; n = 8). All experiments were performed in the early morning during the postabsorptive phase. Ethical approval for the present study was obtained from the Institutional Animal Care and Use Committee at the University of Southern California, Los Angeles.

2.2. Hind limb perfusion

On the day of the experiment, rats were anesthetized and prepared surgically for hind limb perfusion as previously described [7,13]. Before the perfusion, catheters were inserted, and heparin (150 IU) was administered into the inferior vena cava. The rats were killed with an intracardial injection of pentobarbital sodium (0.4 mg/g body weight), and arterial and venous catheters were inserted immediately into the descending aorta and ascending vena cava. The preparation was placed in a perfusion apparatus where the left iliac vessels were tied off and a clamp was fixed tightly around the proximal part of the leg to prevent bleeding [13].

The initial perfusate (250 mL) consisted of Krebs-Henseleit solution, 5% bovine serum albumin (Bovuminar, pH 7; Serologicals, Norcross, GA), 550 μmol/L albumin-bound palmitate, 8 μCi of albumin-bound [1-^{14}C] palmitic acid (MP Biomedicals, Irvine, CA), 8 mmol/L glucose, and vehicle only (dimethyl sulfoxide) or 10, 20, or 50 μmol/L PD98059 dissolved in dimethyl sulfoxide. The inhibitor was added directly to the perfusate with the assumption that this would be the effective inhibitor concentration despite the fact that measurements of inhibitor uptake into the hind limb were not made. Because insulin has been shown to activate the ERK1/2 pathway [10,14], insulin was not included to isolate contraction effects on ERK1/2 phosphorylation and muscle metabolism. The perfusate (37°C) was continuously gassed with a mixture of 95% O_2 and 5% CO_2, which yielded arterial pH values of 7.4 to 7.6 and arterial P_{O_2} and P_{CO_2} values that were typically 320 to 450 and 35 to 52 mm Hg, respectively. Mean perfusion pressures were not affected by PD98059 and averaged 111.5 ± 18.3, 120.3 ± 13.3, 122.6 ± 16.2, 131.0 ± 17.1, and 119.3 ± 22.8 mm Hg in the R, P1, P2, P3, and P4 groups, respectively ($P > .05$).

Hind limbs were equilibrated for 20 minutes and then perfused for an additional 20 minutes at a perfusate flow of 15 mL/min (average for all groups, 0.85 ± 0.01 mL · min^{-1} ·

g^{-1} perfused muscle). Animals were prepared for electrical stimulation as previously described [7]. Isometric muscle contractions of moderate intensity were induced by stimulating the sciatic nerve electrically with supramaximal 15-V trains of 100 Hz with impulse duration of 1 millisecond, delivered every 2 seconds and lasting 50 milliseconds [15]. In previous studies, it has been demonstrated that moderate-intensity muscle contraction results in the greatest increase in both FA uptake and oxidation when compared to a range of contraction intensities [7]. It has also been demonstrated that contraction-induced FA uptake is prevented with treatment of 50 μmol/L PD98059 during moderate intensity [4]. Therefore, to maximize conditions, which would provide evidence for the presence or absence of a relationship between ERK1/2 phosphorylation and FA metabolism, we chose to use a moderate-intensity contraction protocol for our current study. During the 20-minute muscle stimulation, the tension developed by the gastrocnemius-soleus-plantaris muscle group was recorded with a modular chart recorder (Cole Parmer, Vernon Hills, IL). The decrease in tension development over the stimulation period was used as an indicator of performance.

Post-equilibration, arterial and venous perfusate samples were taken at 5, 10, 15, and 20 minutes for analysis of [^{14}C]FA and $^{14}CO_2$ radioactivities, as well as FA, glucose, and lactate concentrations. Arterial and venous perfusate samples for determinations of P_{CO_2}, P_{O_2}, and pH were taken at 10 and 20 minutes. At the end of the 20-minute experimental period, the gastrocnemius-soleus-plantaris muscle group of the right leg was freeze-clamped in situ with aluminum clamps precooled in liquid nitrogen, taken out, and stored for later analysis.

2.3. Blood sample analyses

YSI-1500 was used to analyze both plasma glucose and lactate concentrations (Yellow Springs Instrument, Yellow Springs, OH). Plasma FA concentrations were determined spectrophotometrically using the WAKO NEFA-C test (Biochemical Diagnostics, Edgewood, NY). Plasma [^{14}C] FA and $^{14}CO_2$ radioactivities were determined in duplicate as previously described in detail [2,13,16]. P_{CO_2}, P_{O_2}, and pH were measured with an ABL-5 analyzer (Radiometer America, Westlake, OH).

2.4. Muscle sample analyses

Muscle samples (~90 mg) were powdered under liquid nitrogen, homogenized with 1 mL of ice-cold radioimmunoprecipitation assay (RIPA) buffer, and prepared for Western blot analysis as previously described [17] to quantify dual phosphorylation of ERK1/2. For adenosine monophosphate–activated protein kinase (AMPK) activity determinations, powdered muscle (~400 mg) was homogenized in AMPK buffer (210 mmol/L sucrose, 1 mmol/L EDTA, 5 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L DTT, 2 mmol/L PMSF, 50 mmol/L HEPES, pH 7.4) and centrifuged for 45 seconds at 15 000 *g*. The supernatant was used to determine total AMPK activity, whereas isoform-specific AMPK activity was determined in immunoprecipitates from 200 μg of supernatant protein after overnight incubation at 4°C with ~1.5 μg of affinity-purified, isoform-specific goat immunoglobulin G against α_2-AMPK in 20 μL of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). ^{32}P-adenosine triphosphate incorporation into SAMS peptide was used to measure total and α_2-AMPK activity from the respective preparations [18]. Plasma membranes were isolated as previously described in detail [4,19] and plasma membrane CD36 content determined by Western blot analysis with mouse monoclonal anti-CD36 antibody (1:2000, Cascade Biosciences, Winchester, MA) as previously described [4].

2.5. Calculations and statistics

Fractional uptake was calculated as the difference in radioactivity between the arterial and venous perfusate samples divided by the radioactivity in the arterial sample [2,13]. Palmitate delivery was calculated by multiplying perfusate plasma flow by the arterial perfusate plasma palmitate concentration [2,13]. Palmitate uptake was calculated by multiplying plasma delivery by the fractional uptake [2,13]. Percent palmitate oxidation was calculated by dividing the total amount of radioactivity recovered as $^{14}CO_2$ by the total amount of radioactivity that was taken up by the muscles [2,13]. Total palmitate oxidation was calculated by multiplying palmitate uptake by the percent oxidation [2,13]. Both percent and total palmitate oxidation values were corrected for label fixation by using previously calculated acetate correction factors [13]. Oxygen and glucose uptake as well as lactate release were calculated as previously described [13] and are expressed per gram of perfused muscle, which was previously determined to be 5.6% of body weight for unilateral hind limb perfusion [9]. At rest and during muscle contraction, time effects for glucose, lactate, and FA concentrations and kinetic data were analyzed using 1-way analysis of variance (StatSoft Statistica v 5.0, Tulsa, OK) with repeated measures. Because there was no significant difference in values measured after 10, 15, and 20 minutes of perfusion, average values were used for each animal in subsequent analyses. The effects of PD98059 concentration on the same variables as well as on muscle ERK1/2 phosphorylation, CD36 plasma membrane content, and AMPK activities were analyzed using 1-way analysis of variance. IC_{50}, defined as the PD98059 concentration required for 50% inhibition of contraction-induced ERK1/2 phosphorylation, FA uptake, plasma membrane CD36 content, and total FA oxidation, was calculated from plots of the respective variable vs log PD98059 concentration [20]. Each plot contained data from body weight–matched animals belonging to the P1, P2, P3, and P4 groups. Sheffé's test for post hoc multiple comparisons was performed when appropriate. The square of Pearson product moment coefficient was used to

determine the significance of correlation between ERK1/2 phosphorylation and plasma membrane CD36 content, FA uptake, and FA oxidation as well as between plasma membrane CD36 content and FA uptake. A significance level of .05 was chosen for all statistical methods except in correlational analysis where different P values are given.

3. Results

3.1. Muscle performance and oxygen uptake

Oxygen uptake did not vary over time in any of the groups ($P > .05$). Muscle contraction increased oxygen uptake by 62.6% ($P < .05$). PD98059 had no effect on oxygen uptake during moderate-intensity muscle contraction and averaged 18.6 ± 0.6 μmol · h^{-1} · g^{-1} for all contraction groups ($P > .05$) (Table 1). The initial amount of tension developed by the contracting muscles was also not affected by ERK1/2 inhibition and averaged 770.8 ± 32.8 g for all groups ($P > .05$) (Table 1). Muscle tension development decreased during the first 10 to 15 minutes of electrical stimulation. After 10 minutes of electrical stimulation, muscle tension development had decreased to 62% to 70% of initial tension development in all groups.

3.2. Palmitate metabolism

The arterial specific activity for palmitate did not vary over time and was not significantly different between groups averaging 42.6 ± 1.8, 42.9 ± 1.0, 42.7 ± 1.4, 43.5 ± 1.0, 44.8 ± 1.5 μCi/mmol in the R, P1, P2, P3, and P4 groups, respectively ($P > .05$). Perfusate palmitate concentration and palmitate delivery did not change during the 20-minute experimental period for any group ($P > .05$) and, as dictated by the protocol, were not significantly different between groups ($P > .05$) (Table 1). As expected, moderate-intensity muscle contraction increased FA uptake and plasma membrane CD36 content by 91.6% and 59.2%, respectively ($P < .05$) (Fig. 1A and B). During moderate-intensity muscle contraction, fractional (P1, 0.041 ± 0.002; P2, 0.034 ± 0.002; P3, 0.032 ± 0.003; P4, 0.023 ± 0.003; $P < .05$) and total FA uptake (P1, 17.1 ± 0.6; P2, 14.7 ± 0.3; P3, 13.4 ± 0.5; P4, 10.0 ± 0.6 nmol · min^{-1} · g^{-1}; $P < .05$) decreased in a linear manner with increasing concentration of PD98059 resulting in an IC_{50} value of 15.8 ± 1.6 μmol/L for the effect of PD98059 on total FA uptake (Fig. 1A). Plasma membrane CD36 content decreased in a similar fashion (P1, 147.3 ± 5.5; P2, 115.9 ± 3.8; P3, 105.9 ± 3.7; P4, 80.8 ± 2.4 relative density units; $P < .05$) but had a significantly lower IC_{50} value (IC_{50} = 8.7 ± 0.3 μmol/L, $P < .05$) (Fig. 1B). As shown previously [3,7], muscle contraction did not change the percentage of FA oxidized (12.6 ± 0.7 vs 14.0 ± 0.8, $P > .05$) but increased total FA oxidation by 80.7% ($P < .05$) (Fig. 1C). During moderate-intensity muscle contraction, percent FA oxidation was significantly greater in P1 and P2 compared with P3 and P4 groups (14.0 ± 0.8 and 12.8 ± 0.9 vs 10.1 ± 0.6 and 9.6 ± 0.6, $P < .05$). Total FA oxidation decreased with increasing concentrations of PD98059 reaching resting rates when contracting hind limbs were perfused with 20 and 50 μmol/L PD98059 (P1, 2.5 ± 0.1; P2, 1.8 ± 0.1; P3, 1.4 ± 0.1; P4, 1.3 ± 0.1 nmol · min^{-1} · g^{-1}, $P < .05$) and resulting in an IC_{50} value of 5.4 ± 0.3 μmol/L for the effect of PD98059 on total FA oxidation (Fig. 1C). This IC_{50} value was significantly lower than that for the effect of PD98059 on total FA uptake ($P < .05$). Resting values for fractional FA uptake (R, 0.020 ± 0.001), total FA uptake (R, 8.9 ± 0.5 nmol · min^{-1} · g^{-1}), plasma membrane CD36 content (R, 92.5 ± 7.0 relative density), and total FA oxidation (R, 1.4 ± 0.1 nmol · min^{-1} · g^{-1}) were not significantly different from values calculated during treatment of contracting hind limbs with 50 μmol/L PD98059 (P4) ($P > .05$).

3.3. Substrate exchange across the hindquarter

Arterial perfusate glucose concentration did not change during the 20-minute experimental period for any group ($P > .05$). Similarly, average glucose concentration was not significantly different between groups (R, 7.3 ± 0.8; P1, 7.5 ± 0.2; P2, 7.8 ± 0.4; P3, 8.0 ± 0.2; P4, 7.9 ± 0.2 mmol/L; $P > .05$). Glucose uptake did not change over time in any of the groups ($P > .05$). Muscle contraction increased glucose uptake 1.6-fold ($P < .05$) (Fig. 2). Contraction-induced glucose uptake was not affected by treatment with low doses (10 and 20 μmol/L) of PD98059 but was significantly decreased during treatment with 50 μmol/L PD98059 (R, 5.6 ± 1.1; P1, 14.4 ± 0.6; P2, 14.1 ± 0.7; P3, 13.1 ±

Table 1
Perfusion characteristics of rat hind limbs perfused at rest or during moderate-intensity electrical stimulation with increasing concentration of PD98059

	R (n = 8)	P1 (n = 8)	P2 (n = 7)	P3 (n = 7)	4 (n = 8)
Oxygen uptake (μmol · h^{-1} · g^{-1})	11.5 ± 0.6	18.7 ± 1.5 *	18.9 ± 2.0 *	18.6 ± 1.2 *	18.1 ± 0.4 *
Tension development (g)	NA	797.7 ± 38.3	785.9 ± 40.4	755.0 ± 54.4	790.0 ± 60.6
FA concentration (μmol/L)	540.2 ± 11.6	552.4 ± 19.9	537.6 ± 15.0	553.2 ± 16.3	535.0 ± 17.3
FA delivery (nmol · min^{-1} · g^{-1})	470.2 ± 31.3	424.1 ± 24.7	446.8 ± 26.5	467.6 ± 15.9	472.8 ± 28.7
Initial lactate concentration (mmol/L)	0.45 ± 0.04	0.46 ± 0.08	0.48 ± 0.08	0.46 ± 0.07	0.48 ± 0.05
Final lactate concentration (mmol/L)	0.49 ± 0.07	1.24 ± 0.09 *	1.33 ± 0.05 *	1.61 ± 0.13*,†	1.77 ± 0.03*,†
Lactate release (μmol · h^{-1} · g^{-1})	16.3 ± 1.3	29.9 ± 2.0 *	37.5 ± 4.2 *	39.3 ± 2.1*,†	39.8 ± 3.5*,†

Values are expressed as means ± SE. R indicates rest; P1, none; P2, 10 μmol/L; P3, 20 μmol/L; P4, 50 μmol/L; NA, not applicable.
* $P < .05$ compared to R.
† $P < .05$ compared to P1.

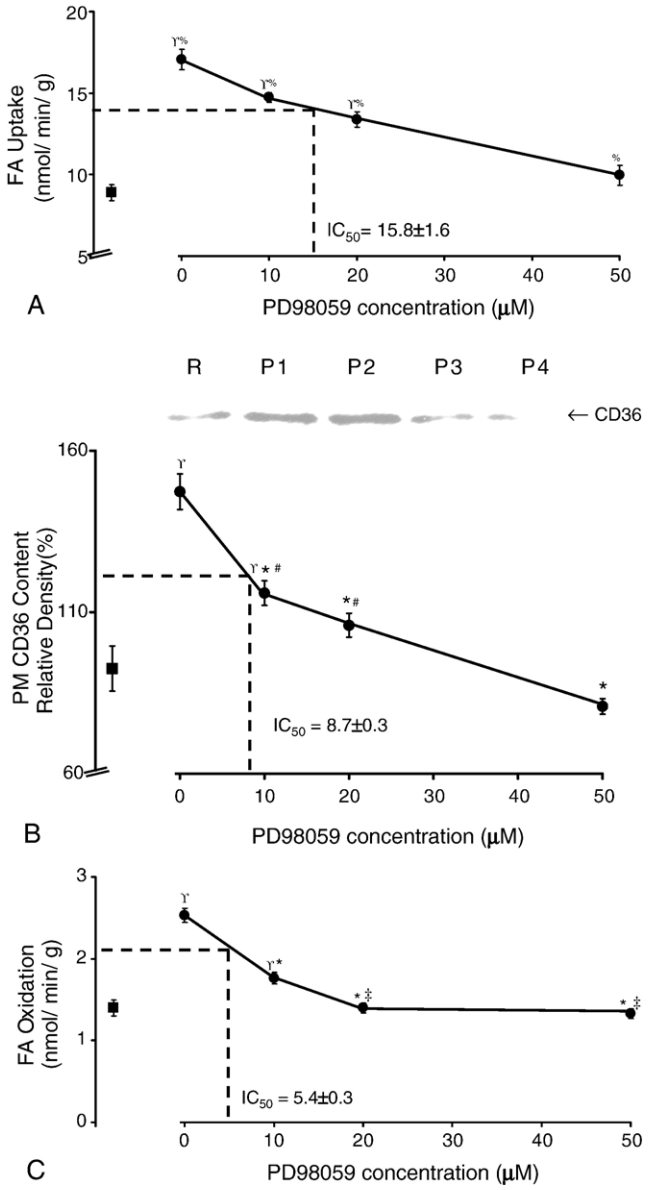

Fig. 1. Effect of increasing concentration of PD98059 on FA uptake (A), plasma membrane CD36 content (B), and total FA oxidation (C) in rat hind limbs perfused at rest (■) or during moderate-intensity muscle contraction (●). Values are means ± SE. Because there was no significant difference in values measured after 10, 15, and 20 minutes of perfusion, average values were used for each animal. Total palmitate oxidation was corrected for label fixation, as described in Materials and methods. $^{\Upsilon}P < .05$ compared with R animals; $^{*}P < .05$ compared with 0.0 μmol/L PD98059; $^{\#}P < .05$ compared with 50 μmol/L PD98059; $^{\ddagger}P < .05$ compared with 10 μmol/L PD98059; $^{\%}P < .05$ compared with all contraction groups.

0.5 vs P4, 10.8 ± 0.5 $\mu mol \cdot h^{-1} \cdot g^{-1}$; $P < .05$) (Fig. 2). Initial arterial perfusate lactate concentration was not significantly different between groups ($P > .05$) (Table 1). At the conclusion of the perfusion, arterial lactate concentration had increased 1.7-fold in the P1 and P2 groups and 2.7-fold in the P3 and P4 groups ($P < .05$) compared to initial arterial lactate concentration. Lactate release did not vary significantly over time in any of the groups ($P > .05$) but was

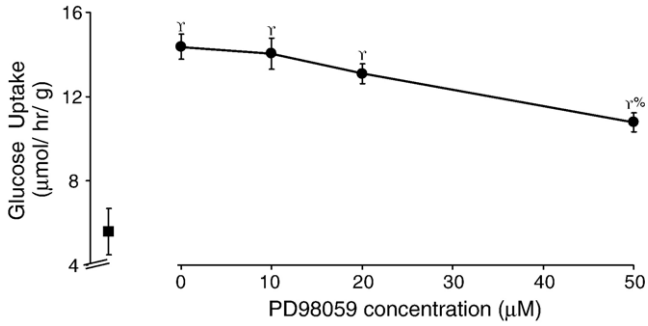

Fig. 2. Effect of increasing concentration of PD98059 on glucose uptake in rat hind limbs perfused at rest (■) or during moderate-intensity muscle contraction (●). Values are means ± SE. Because there was no significant difference in values measured after 10, 15, and 20 minutes of perfusion, average values were used for each animal. $^{\Upsilon}P < .05$ compared with R animals; $^{\%}P < .05$ compared with all contraction groups.

significantly greater in contracting compared with resting muscle ($P < .05$). Lactate release was significantly greater in contracting muscle perfused in the presence of 20 and

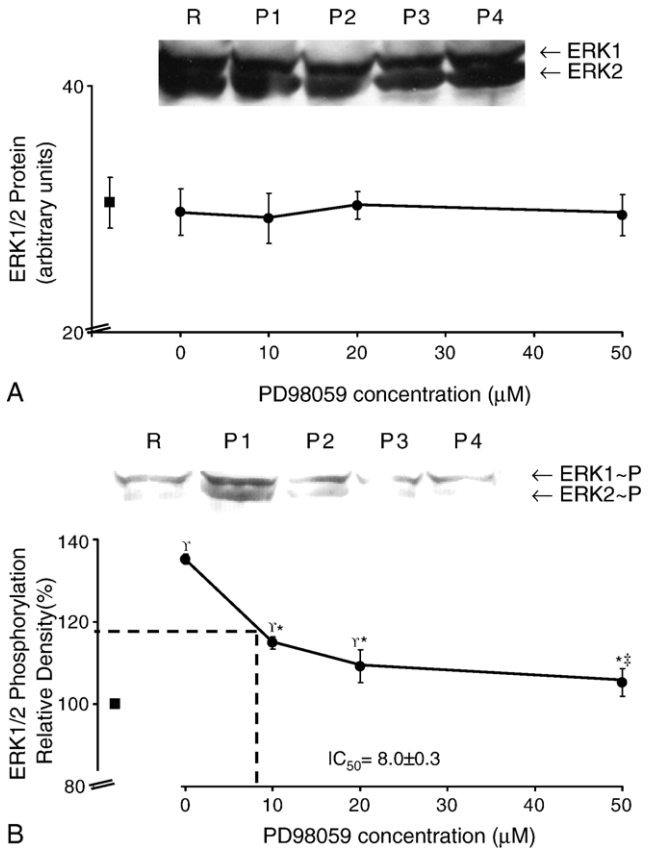

Fig. 3. Effect of increasing concentration of PD98059 on ERK1/2 total protein concentration (A) and phosphorylation (B) in rat hind limbs perfused at rest (■) or during moderate-intensity muscle contraction (●). Values are means ± SE. ERK1/2 protein results are expressed after standardization with the protein content present in a nonperfused gastrocnemius muscle standard. ERK1/2 phosphorylation is calculated as a percentage of phosphorylation measured at rest. $^{\Upsilon}P < .05$ compared with R animals; $^{*}P < .05$ compared with 0.0 μmol/L PD98059; $^{\ddagger}P < .05$ compared with 10 μmol/L PD98059.

50 μmol/L PD98059 when compared with contracting muscle perfused in the absence of PD98059 ($P < .05$) (Table 1).

3.4. Enzyme activities

ERK1/2 protein expression was not different between groups ($P > .05$) (Fig. 3A). ERK1/2 phosphorylation (relative density units) was significantly greater during moderate-intensity muscle contraction than at rest and decreased gradually with increasing concentration of PD98059 (R, 100; P1, 135.2 ± 1.2; P2, 114.9 ± 1.5; P3, 109.2 ± 4.0; P4, 105.3 ± 3.4, $P < .05$) resulting in an IC_{50} value of 8.0 ± 0.3 μmol/L for the effect of PD98059 on ERK1/2 phosphorylation (Fig. 3B). ERK1 and ERK2 phosphorylation corresponded to 51% to 56% and 44% to 49% of total ERK1/2 phosphorylation, respectively. ERK2 phosphorylation demonstrated a gradual decrease with increasing concentration of PD98059 paralleling the decrease in total ERK1/2 phosphorylation ($P < .05$), whereas ERK1 phosphorylation decreased to a lesser extent and was not significantly lower during treatment with 50 μmol/L PD98059 compared with 20 μmol/L PD98059 ($P > .05$). Muscle contraction increased total and α_2-AMPK activity by 77% and 40%, respectively. ERK1/2 inhibition had no effect on either total or α_2-AMPK activity during muscle contraction ($P > .05$) (Fig. 4).

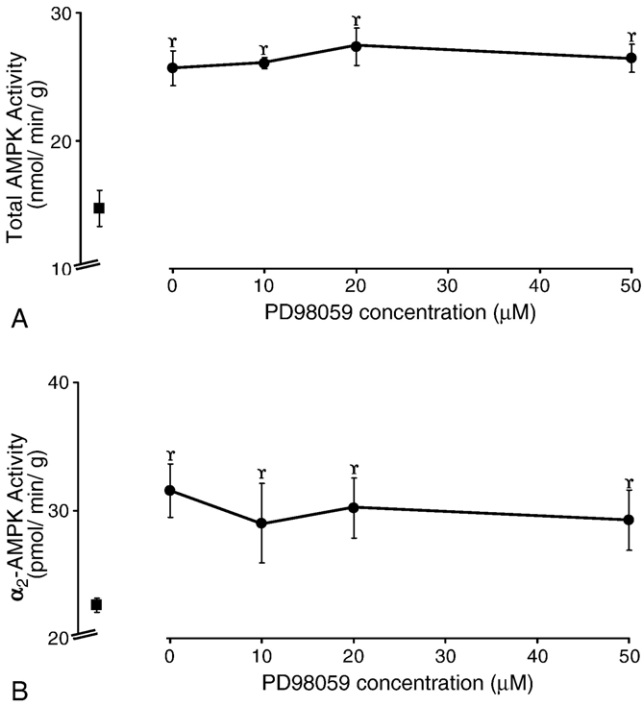

Fig. 4. Effect of increasing concentration of PD98059 on total (A) and α_2 (B) AMPK activity in rat hind limbs perfused at rest (■) or during moderate-intensity muscle contraction (●). Values are means ± SE. Adenosine monophosphate–activated protein kinase activity is calculated per gram of protein present in the assay preparation. $^{\Upsilon}P < .05$ compared with R animals. No significant difference was found between contraction groups ($P > .05$).

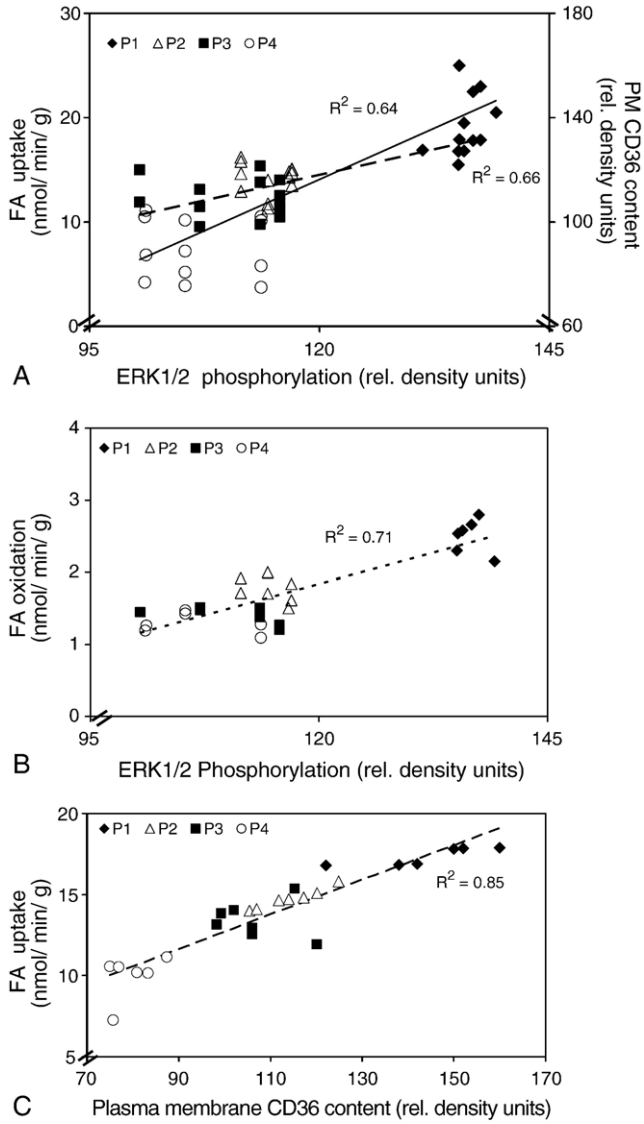

Fig. 5. Correlation analysis between ERK1/2 phosphorylation and FA uptake and plasma membrane (PM) CD36 content (A); ERK1/2 phosphorylation and total FA oxidation (B); and plasma membrane CD36 content and FA uptake (C) in rat hind limbs perfused during moderate-intensity muscle contraction in the presence of increasing PD98059 concentration. Closed diamonds indicate P1 (n = 8); open triangles, P2 (n = 7); closed squares, P3 (n = 7); open circles, P4 (n = 8). In A, dashed and straight lines represent correlation between ERK1/2 phosphorylation and FA uptake ($R^2 = 0.66$; $y = 0.198 \times -2.3$, $P < .001$) and PM CD36 content ($R^2 = 0.64$; $y = 1.58 \times -72.7$, $P < .001$), respectively. In B, dotted line represents correlation between ERK1/2 phosphorylation and total FA oxidation ($R^2 = 0.71$; $y = 0.036 \times -1.2$, $P < .0005$), respectively. In C, dashed line represents correlation between PM CD36 content and FA uptake ($R^2 = 0.85$; $y = 0.120 \times +5.3$, $P < .0005$).

3.5. Correlational analysis

Fractional ($R^2 = 0.68$, $P < .001$) and total FA uptake ($R^2 = 0.66$, $P < .001$), plasma membrane CD36 content ($R^2 = 0.64$, $P < .001$), and total FA oxidation ($R^2 = 0.71$, $P < .0005$) each shared a significant positive correlation with ERK1/2 phosphorylation during moderate-intensity muscle

contraction (Fig. 5A and B). Total FA uptake also shared a strong positive correlation with plasma membrane CD36 content during moderate-intensity muscle contraction (R^2 = 0.85, $P < .0005$) (Fig. 5C).

4. Discussion

This study provides new evidence in support of the hypothesis that ERK1/2 activation is required to measure an increase in FA uptake during moderate-intensity muscle contraction. This evidence includes a simultaneous decrease in ERK1/2 phosphorylation and FA uptake with increasing PD98059 concentration. In addition, because the decrease in FA uptake (14%-42%) was accompanied by a similar relative decrease in plasma membrane CD36 content (21%-45%), our results support the notion that CD36 is involved in the molecular mechanism mediating FA entry into skeletal muscle. Our study also provides new evidence for the notion that factors other than FA uptake regulate FA oxidation during muscle contraction. Indeed, the differences in IC_{50} values and correlational coefficients between FA uptake or FA oxidation and PD98059 concentration and ERK1/2 phosphorylation, respectively, indicate that FA uptake and oxidation are regulated differently by changes in ERK1/2 activation. The significantly lower IC_{50} value suggests that FA oxidation is more sensitive to changes in ERK1/2 activation and this is corroborated by the stronger correlation between ERK1/2 phosphorylation and FA oxidation. Thus, ERK1/2 activation may play a crucial role in the regulation of contraction-induced FA oxidation independently of its effects on FA uptake during moderate-intensity muscle contraction.

Recently, our laboratory showed that treatment with PD98059 abolished both contraction-induced ERK1/2 phosphorylation and FA uptake during moderate-intensity muscle contraction [4] and that ERK1/2 phosphorylation and FA uptake shared a significant positive correlation when contraction intensity was increased from rest to moderate [7]. In combination with these results, our current study, which demonstrates that dose-dependent inhibition of contraction-induced ERK1/2 phosphorylation during moderate-intensity muscle contraction is paralleled by a similar decrease in contraction-induced FA uptake, suggests that ERK1/2 signaling plays a significant role in the regulation of FA uptake under these contraction conditions. Our study also provides insight into the mechanism that mediates the effects of ERK1/2 inhibition on contraction-induced FA uptake. Previous studies performed in giant sarcolemmal vesicles with the CD36 inhibitor sulfo-*N*-succinimidyl oleate and with transgenic mice have shown that contraction-induced FA uptake occurs in large part via translocation of CD36 from intracellular pools to the plasma membrane [1,21,22]. The strong positive correlation between FA uptake and plasma membrane CD36 content (R^2 = 0.85) and between ERK1/2 phosphorylation and plasma membrane CD36 content (R^2 = 0.64) found in this study supports the model proposed by other laboratories and us that describes the transport role of CD36 in the molecular mechanism that allows FA to enter skeletal muscle during muscle contraction [1,4,5]. Therefore, the mechanism by which ERK1/2 regulates contraction-induced FA uptake likely includes regulation of CD36 translocation to the plasma membrane.

The dramatic effect that ERK1/2 inhibition has on contraction-induced FA uptake may help to explain some of the decrease in FA oxidation measured in our study. In the past, it has been suggested that total FA oxidation is primarily a function of FA uptake [21,22]. If FA uptake is the only factor responsible for changes in total FA oxidation, we would have expected the change in total FA oxidation to parallel the linear decrease in FA uptake with increasing concentration of PD98059. However, as illustrated in Fig. 1A and C, lower concentrations of PD98059 and thus smaller decreases in ERK1/2 activation were required to achieve maximal inhibition of contraction-induced FA oxidation, bringing FA oxidation rates down to resting level when compared with the PD98059 concentrations required for similar changes in FA uptake. In addition, as stated in the Results, we measured a significant decrease in the percent FA oxidation in P3 and P4 compared with P1 and P2 demonstrating a shift in utilization of FA once they have entered the cell dependent on ERK1/2 phosphorylation. Together, these results indicate that cellular mechanism(s) other than intracellular FA uptake regulate contraction-induced FA oxidation and that these mechanisms are more sensitive to changes in ERK1/2 activation than mechanisms regulating FA uptake.

Thus, as we have shown repeatedly, under different experimental conditions, the magnitude and direction of the changes in FA uptake and oxidation do not always coincide, indicating that cellular signaling intermediates impact FA uptake and oxidation in independent ways [3,8,9]. The mechanism by which ERK1/2 inhibition might regulate FA oxidation cannot be determined from our results. Although IC_{50} values, correlational coefficients, and percent FA oxidation values suggest ERK1/2 regulates FA uptake and oxidation independently of one another, additional studies must be performed to verify the direct involvement of ERK1/2 in the regulation of FA oxidation. Evidence collected in hepatocytes suggests that regulation of FA oxidation may occur in part via ERK1/2-dependent activation of AMP-activated protein kinase (AMPK) [23]. However, as our results show, changes in ERK1/2 phosphorylation are not associated with changes in AMPK activity, suggesting that AMPK may lie upstream of ERK1/2 or be part of a distinct signaling pathway during moderate-intensity muscle contraction in skeletal muscle. Other studies have suggested that CD36 translocates from the cytosol to the mitochondrial membrane during muscle contraction and that it acts downstream of carnitine palmitoyltransferase 1 to facilitate long-chain fatty-acyl-CoA entry into the mitochondria for oxidation [24,25]. It is therefore possible that upon activation

by muscle contraction, ERK1/2 mediates translocation of CD36 to both the plasma and mitochondrial membranes resulting in an increase in FA uptake and FA oxidation, respectively. Additional studies must be performed to determine whether the decrease in FA oxidation in our study would have been accompanied by a decrease in CD36 content in the mitochondrial membrane and whether this would have been achieved independently of the simultaneous decrease in FA uptake and intracellular FA availability.

As opposed to the effect of ERK1/2 inhibition on FA metabolism, we did not measure a dose-dependent decrease in glucose uptake, which is in agreement with previous studies performed in isolated and perfused muscle [10,26]. Glucose uptake was slightly decreased, however, with maximal ERK1/2 inhibition that is also in agreement with the tendency for blunted glucose transport in perfused red and white muscles during treatment with 50 μmol/L PD98059 as shown by Wojtaszewski et al [10]. The mechanism by which this concentration of PD98059 may affect glucose uptake is not known. In a previous study performed in our laboratory, no significant decrease in contraction-induced glucose uptake was measured in hind limbs perfused with 50 μmol/L PD98059 and contracting at an intensity higher than the one used in this study [4,15]. This indicates that stimulation intensity may play a role in determining whether or not ERK1/2 inhibition will affect glucose uptake during muscle contraction. This possibility has yet to be confirmed. As opposed to the lack of a dose-dependent decrease in glucose uptake, increasing PD98059 concentrations were accompanied by an increase in lactate release. Given that lactate release can be used as an index of glycogen utilization [27], our results agree with other data and show that when a fuel supply is limited during muscle contraction, skeletal muscle compensates by increasing the contribution of other available fuel sources to total energy expenditure [28-31].

It has been debated that ERK1/2 phosphorylation is not a valid indicator of ERK1/2 activity based on results from a study performed in humans [32]. However, in this study, it was demonstrated that the relative increase in ERK1/2 phosphorylation and ERK1/2 activity were similar during low- to moderate-intensity muscle contraction [32], suggesting that either measurement is an adequate representation of in vivo activation. Because the stimulation protocol chosen for our study has been described as moderate intensity [15], we feel confident that ERK1/2 phosphorylation is an accurate indication of in vivo ERK1/2 activity during treatment with increasing concentrations of PD98059.

The signaling mechanisms regulating contraction-induced FA metabolism are far from being fully delineated. The results from this study establish a key role for ERK1/2 activation in the regulation of contraction-induced FA uptake during moderate-intensity muscle contraction. Furthermore, evidence suggests that ERK1/2 signaling increases FA uptake via stimulation of CD36 translocation to the plasma membrane. We also show for the first time that an ERK1/2-dependent signaling mechanism may also regulate contraction-induced FA oxidation independently of its effects on FA uptake.

Acknowledgment

The present study was supported by grants from the University of Southern California Women in Science and Engineering program.

References

[1] Bonen A, Luiken JJFP, Arumugam Y, Glatz JFC, Tandon NN. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. J Biol Chem 2000;275:14501-8.

[2] Turcotte LP, Petry C, Kiens B, Richter EA. Contraction-induced increase in Vmax of palmitate uptake and oxidation in perfused skeletal muscle. J Appl Physiol 1998;84:1788-94.

[3] Raney MA, Yee AJ, Todd MK, Turcotte LP. AMPK activation is not critical in the regulation of muscle FA uptake and oxidation during low-intensity muscle contraction. Am J Physiol Endocrinol Metab 2005;288:E592-8.

[4] Turcotte LP, Raney MA, Todd MK. ERK1/2 inhibition prevents contraction-induced increase in plasma membrane CD36 content and FA uptake in rodent muscle. Acta Physiol Scand 2005;184:131-9.

[5] Bonen A, Dyck DJ, Ibrahimi A, Abumrad N. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. Am J Physiol Endocrinol Metab 1999;276:E642-9.

[6] Donsmark M, Langfort J, Holm C, Ploug T, Galbo H. Contractions activate hormone-sensitive lipase in rat muscle by protein kinase C and mitogen-activated protein kinase. J Physiol 2003;550:845-54.

[7] Raney MA, Turcotte LP. Regulation of contraction-induced FA uptake and oxidation by AMPK and ERK1/2 is intensity-dependent in rodent muscle. Am J Physiol Endocrinol Metab 2006;291:E1220-7.

[8] Tucker MZ, Turcotte LP. Brief food restriction in old animals decreases triglyceride content and insulin-stimulated triglyceride synthesis. J Gerontol 2005;60:157-64.

[9] Turcotte LP, Swenberger JR, Yee AJ. High carbohydrate availability increases LCFA uptake and decreases LCFA oxidation in perfused muscle. Am J Physiol Endocrinol Metab 2002;282:E177-83.

[10] Wojtaszewski JFP, Lynge J, Jakobsen AB, Goodyear LJ, Richter EA. Differential regulation of MAP kinase by contraction and insulin in skeletal muscle: metabolic implications. Am J Physiol Endocrinol Metab 1999;277:E724-32.

[11] Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 1995;270:27489-94.

[12] Ryder JW, Fahlman R, Wallberg-Henriksson H, Alessi DR, Krook A, Zierath JR. Effect of contraction on mitogen-activated protein kinase signal transduction in skeletal muscle. Involvement of the mitogen- and stress-activated protein kinase. J Biol Chem 2000;275:1457-62.

[13] Turcotte LP, Swenberger JR, Tucker MZ, Yee AJ. Training-induced elevation in FABPpm is associated with increased palmitate use in contracting muscle. J Appl Physiol 1999;87:285-93.

[14] Leng Y, Steiler TL, Zierath JR. Effects of insulin, contraction, and phorbol esters on mitogen-activated protein kinase signaling in skeletal muscle from lean and *ob*/*ob* mice. Diabetes 2004;53:1436-44.

[15] Kim J, Solis RS, Arias EB, Cartee GD. Post-contraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5′ AMP-activated protein kinase phosphorylation. J Appl Physiol 2004;96:575-83.

[16] Turcotte LP, Richter EA, Kiens B. Increased plasma FFA uptake and oxidation during prolonged exercise in trained vs untrained humans. Am J Physiol 1992;262:E791-9.

[17] Martineau L, Gardiner P. Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. J Appl Physiol 2001;91:693-702.

[18] Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol 1996;270:E299-304.

[19] Turcotte LP, Swenberger JR, Tucker MZ, Yee AJ, Trump G, Luiken JJFP, et al. Muscle palmitate uptake and binding are saturable and inhibited by antibodies to FABPPM. Mol Cell Biochem 2002;210: 53-63.

[20] Starritt EC, Howlett RA, Heigenhauser GJF, Spriet LL. Sensitivity of CPT I to malonyl-CoA in trained and untrained human skeletal muscle. Am J Physiol 2000;278:E462-8.

[21] Coburn CT, Knapp Jr FF, Febbraio M, Beets AL, Silverstein RL, Abumrad NA. Defective uptake and utilization of long chain fatty acids in muscle and adipose tissue of CD36 knockout mice. J Biol Chem 2000;275:32523-9.

[22] Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, et al. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. J Biol Chem 1999; 274:26761-6.

[23] Brusq JM, Ancellin N, Grondin P, Guillard R, Martin S, Saintillan Y, et al. Inhibition of lipid synthesis through activation of AMP-kinase: an additional mechanism for the hypolipidemic effects of berberine. J Lipid Res 2006;47:1281-8.

[24] Campbell SE, Tandon NN, Woldegiorgis G, Luiken JJFP, Glatz JFC, Bonen A. A novel function for fatty acid translocase (FAT/CD36). Involvement in long chain fatty acid transfer into the mitochondria. J Biol Chem 2004;279:36235-41.

[25] Bezaire V, Bruce CR, Heigenhauser GJF, Tandon NN, Glatz JFC, Luiken JJFP, et al. Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. Am J Physiol Endocrinol Metab 2006;290: 509-15.

[26] Hayashi T, Hirshman MF, Dufresne SD, Goodyear LJ. Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. Am J Physiol Cell Physiol 1999;277:C701-7.

[27] Young ME, Radda GK, Leighton B. Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR —an activator of AMP-activated protein kinase. FEBS Lett 1996;382:43-7.

[28] Romijn JA, Coyle EF, Sidossis LS, Rodenblatt J, Wolfe RR. Substrate metabolism during different exercise intensities in endurance-trained women. J Appl Physiol 2000;88:1707-14.

[29] Wolfe RR, Klein S, Carraro F, Weber J-M. Role of triglyceride–fatty acid cycle in controlling fat metabolism in humans during and after exercise. Am J Physiol 1990;258:E382-9.

[30] Helge JW, Ayre KJ, Chaunchaiyakul S, Hulbert AJ, Kiens B, Storlien LH. Endurance in high fat fed rats: effects of carbohydrate content and fatty acid profile. J Appl Physiol 1998;85:1342-8.

[31] Coyle EF, Jeukendrup AE, Oseto MC, Hodgkinson BJ, Zderic TW. Low-fat diet alters intramuscular substrates and reduces lipolysis and fat oxidation during exercise. Am J Physiol Endocrinol Metab 2001;280:E391-8.

[32] Richter EA, Vistisen B, Maarbjerg SJ, Sajan M, Farese RV, Kiens B. Differential effect of bicycling exercise intensity on activity and phosphorylation of atypical protein kinase C and extracellular signal-regulated protein kinase in skeletal muscle. J Physiol 2004;560: 909-18.