

# Calcineurin Does Not Mediate Exercise-Induced Increase in Muscle GLUT4

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Exercise induces a rapid increase in expression of the GLUT4 isoform of the glucose transporter in skeletal muscle. One of the signals responsible for this adaptation appears to be an increase in cytosolic  $\text{Ca}^{2+}$ . Myocyte enhancer factor 2A (MEF2A) is a transcription factor that is involved in the regulation of GLUT4 expression. It has been reported that the  $\text{Ca}^{2+}$ -regulated phosphatase calcineurin mediates the activation of MEF2 by exercise. It has also been shown that the expression of activated calcineurin in mouse skeletal muscle results in an increase in GLUT4. These findings suggest that increases in cytosolic  $\text{Ca}^{2+}$  induce increased GLUT4 expression by activating calcineurin. However, we have obtained evidence that this response is mediated by a  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase. The purpose of this study was to test the hypothesis that calcineurin is involved in mediating exercise-induced increases in GLUT4. Rats were exercised on 5 successive days using a swimming protocol. One group of swimmers was given 20 mg/kg body weight of cyclosporin, a calcineurin inhibitor, 2 h before exercise. A second group was given vehicle. GLUT4 protein was increased ~80%, GLUT4 mRNA was increased ~2.5-fold, MEF2A protein was increased twofold, and hexokinase II protein was increased ~2.5-fold 18 h after the last exercise bout. The cyclosporin treatment completely inhibited calcineurin activity but did not affect the adaptive increases in GLUT4, MEF2A, or hexokinase expression. We conclude that calcineurin activation does not mediate the adaptive increase in GLUT4 expression induced in skeletal muscle by exercise. *Diabetes* 54:624–628, 2005

**E**xercise induces a rapid increase in the expression of the GLUT4 isoform of the glucose transporter in skeletal muscle (1). This is one of the more important health benefits of exercise, as it counters insulin resistance by increasing the responsiveness of the glucose transport process in muscle to insulin

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CAMK,  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase; CsA, cyclosporin A; ECL, enhanced chemiluminescence; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; NRF-1, nuclear respiratory factor 1.

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(2). Increases in cytosolic  $\text{Ca}^{2+}$  can induce an increase in GLUT4 expression in muscle cells, and it seems likely that  $\text{Ca}^{2+}$  is one of the signals that activate the pathway(s) by which exercise increases GLUT4 expression in skeletal muscle (3). It has been reported that the calcium-regulated phosphatase calcineurin mediates the exercise-induced activation of MEF2 in muscle (4). Myocyte enhancer factor 2A (MEF2A) is a transcription factor involved in the regulation of GLUT4 expression (5,6). It has also been shown that the expression of activated calcineurin in mouse skeletal muscle results in increased expression of GLUT4 (7).

These findings suggest that increases in cytosolic  $\text{Ca}^{2+}$  mediate increased expression of GLUT4 in muscle by activating calcineurin. In contrast, studies in which cytosolic  $\text{Ca}^{2+}$  levels were raised by exposing L6 myotubes or rat skeletal muscles to caffeine or ionomycin have provided evidence that the induction of increased GLUT4 expression by  $\text{Ca}^{2+}$  is mediated by activation of  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase (CAMK) (3). In light of this apparent discrepancy, the purpose of the present study was to evaluate the role of calcineurin in mediating exercise-induced increases in GLUT4 expression. To this end, we determined the effect of the calcineurin inhibitor cyclosporin on the adaptive response of muscle GLUT4 to exercise.

## RESEARCH DESIGN AND METHODS

Sandimmune (cyclosporin A; CsA) was purchased from Novartis (Cambridge, MA). A Biomol Green Cellular Calcineurin Assay Kit PLUS was purchased from Biomol (Plymouth Meeting, PA). A polyclonal antibody specific for GLUT4 was the generous gift of Dr. Mike Mueckler (Washington University, St. Louis, MO). The goat anti-human hexokinase II and rabbit anti-human MEF2A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and donkey anti-goat IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham (Arlington Heights, IL). TRIZOL reagent for isolation of RNA was purchased from Gibco-BRL (Grand Island, NY). Reagents for isolation of mRNA were obtained from Ambion (Austin, TX). The ImProm-II Reverse Transcription System and PCR Master Mix were purchased from Promega (Madison, WI). All other reagents were obtained from Sigma (St. Louis, MO).

This research was approved by the Animal Studies Committee of the Washington University School of Medicine (St. Louis, MO). Male Wistar rats (body mass 180–200 g) were obtained from Charles River (Wilmington, MA) and maintained on a diet of Purina chow and water. Animals were randomly assigned to the exercise + CsA treatment group, exercise + vehicle group, sedentary + vehicle group, or sedentary + CsA group. Rats in the exercise groups were accustomed to swimming for 10 min/day for 2 days. They were then exercised on 5 successive days using a previously described swimming protocol (2) that involves two 3-h swimming sessions separated by a 45-min rest period during which the rats are kept warm and given food and water. After completing the swimming on the 5th day, the animals were fasted overnight.

Animals were injected once daily subcutaneously between the shoulder

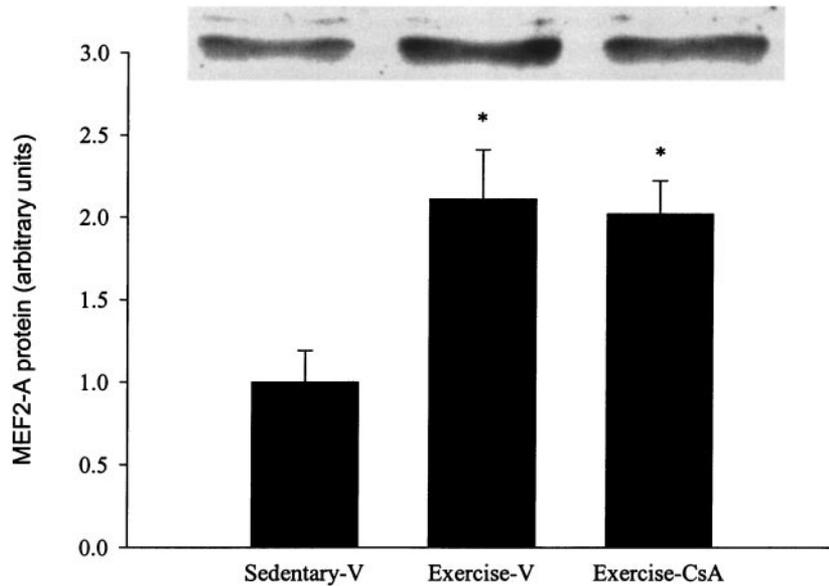


FIG. 1. MEF2A protein level is increased in epitrochlearis muscles of rats after five daily bouts of swimming. The sedentary control rats and the animals in one group of swimmers were given an injection of vehicle (V), and the rats in the other group of swimmers were given cyclosporin A (CsA) injections 2 h prior to each exercise session. Muscles were harvested 18 h after the last exercise session. The bars represent means  $\pm$  SE for five rats per group. \* $P < 0.05$ .

blades with CsA (20 mg/kg body mass) or an equal volume of vehicle (Cremophor EL-650 mg/ml and 32.9% ethanol) 2 days before starting the exercise (8). The animals that were not given cyclosporin had their food intake decreased so that they gained weight at the same rate as the animals given cyclosporin. During the exercise training period, the CsA injections were given 2 h before exercise. Fasted rats were killed 18 h after the last exercise bout and 2–3 h after the last injection of CsA or vehicle. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body mass), the epitrochlearis and triceps muscles were dissected out, and a blood sample was drawn from the aorta.

**Calcineurin activity.** A Biomol Green Cellular Calcineurin Assay Kit PLUS was used to measure calcineurin (protein phosphatase 2B) activity in skeletal muscle homogenates.

**Cyclosporin assay.** The cyclosporin concentration in blood was measured using the Dade Dimension CsA immunoassay, which was performed using a Flex reagent cartridge (Dade Behring, Newark, DE) (9).

**Western blot analysis.** Muscles were homogenized in ice-cold 250 mmol/l sucrose containing 20 mmol/l HEPES and 1 mmol/l EDTA (pH 7.4). The protein concentration was measured by the method of Lowry et al. (10). Aliquots of homogenate were solubilized in Laemmli buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS containing 0.1% Tween 20. The membranes were probed with the following primary antibodies: anti-GLUT4, anti-hexokinase II, and anti-MEF2A, and incubated with 1% nonfat milk in PBS containing 0.1% Tween 20 overnight at 4°C. The blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary anti-immunoglobulin G antibody. Antibody bound protein was detected by ECL.

**GLUT4 mRNA assay.** GLUT4 mRNA was measured using competitive RT-PCR, as previously described (11).

**Statistics.** Data are expressed as means  $\pm$  SE. Statistically significant differences were determined using unpaired Student's *t* tests.

## RESULTS

**Food intake and body weights.** Cyclosporin treatment decreased rats' food intake to  $\sim$ 16 g per day compared with  $\sim$ 19 g per day for the vehicle-treated rats. We, therefore, reduced the food intake of the vehicle-treated rats to keep their rate of weight gain similar to that of the cyclosporin-treated animals. Final body weights averaged  $224 \pm 5$  g for the vehicle-treated swimmers,  $225 \pm 4$  g for the cyclosporin-treated swimmers,  $221 \pm 3$  g for the vehicle-treated sedentary controls, and  $220 \pm 4$  g for the cyclosporin-treated sedentary rats.

**Inhibition of calcineurin.** The cyclosporin treatment resulted in a serum cyclosporin concentration of  $>4.5$

$\mu$ g/ml, measured 2–3 h after the final injection of cyclosporin, when the animals were killed. Calcineurin activity averaged  $0.95 \pm 0.11$  nmol  $\cdot$  min $^{-1}$   $\cdot$  mg muscle protein $^{-1}$  in the triceps muscle of the vehicle-injected rats and was undetectable in muscle from cyclosporin-treated rats. Further evidence that the cyclosporin treatment was effective in inhibiting calcineurin was provided by the finding that an exercise-induced increase in cytochrome oxidase subunit 1, which averaged  $\sim$ 150% in the vehicle-treated swimmers, was completely blocked in the cyclosporin-treated swimmers (data not shown).

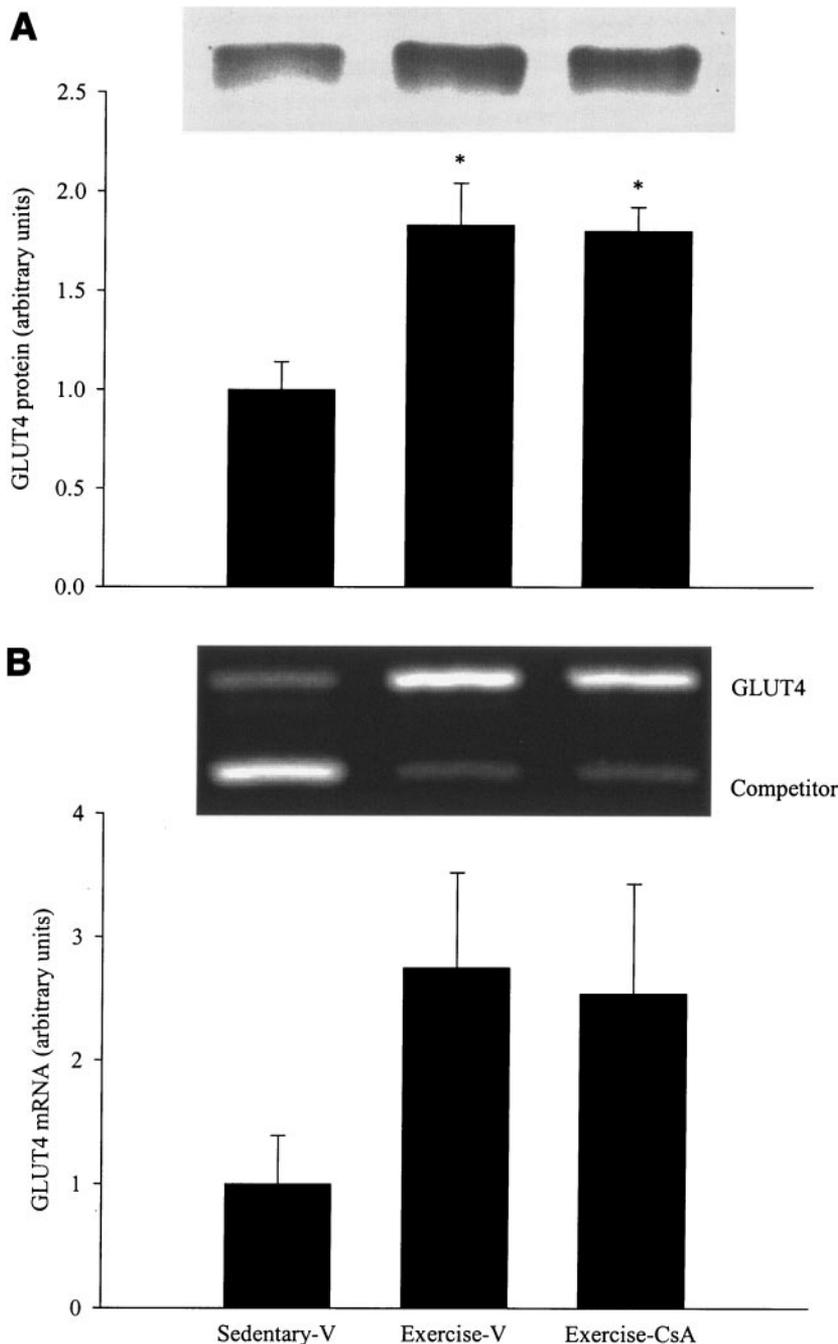
**Adaptive responses in gene expression.** The findings in the sedentary + vehicle and sedentary + CsA groups were similar, so only the data on the vehicle-treated sedentary control group are shown in the figures. As seen in Fig. 1, the expression of MEF2A protein was increased approximately twofold in epitrochlearis muscle 18 h after the last exercise bout. MEF2A is a transcription factor involved in the induction of GLUT4 expression (5,6,12). The inhibition of calcineurin had no effect on the adaptive increase in MEF2A protein.

GLUT4 protein in the epitrochlearis muscle was increased  $\sim$ 80% (Fig. 2A) and GLUT4 mRNA was increased  $\sim$ 2.5-fold (Fig. 2B) 18 h after the last exercise bout. Inhibition of calcineurin did not affect the adaptive increases of GLUT4 mRNA or protein.

Hexokinase expression appears to be regulated in parallel with GLUT4 expression in skeletal muscle (1). As shown in Fig. 3, hexokinase II protein expression was increased  $\sim$ 2.5-fold in epitrochlearis muscle of the exercised animals. Inhibition of calcineurin had no effect on the adaptive increase in hexokinase.

## DISCUSSION

The results of this study show that the inhibition of calcineurin does not prevent the adaptive increases in GLUT4, MEF2A, or hexokinase induced in skeletal muscle by exercise. The exercise-induced increases in the expression of GLUT4 and MEF2A are mimicked by raising cytosolic Ca $^{2+}$  in L6 myotubes or epitrochlearis muscles (3). A number of studies have shown that activation of



**FIG. 2.** Effects of five daily bouts of swimming on GLUT4 protein level (*A*) in epitrochlearis muscles and GLUT4 mRNA level (*B*) in triceps muscles. Rats were treated with cyclosporin A (CsA) or vehicle (V) as described in Fig. 1. The bars in *A* represent means  $\pm$  SE for six rats in the Sedentary-V and Exercise-V groups and nine rats in the Exercise-CsA group. \* $P < 0.01$  vs. Sedentary-V. The bars in *B* represent means  $\pm$  SE for six rats per group.

calcineurin induces the slow, oxidative muscle fiber gene regulatory program in skeletal muscle (13–15). It has also been reported that activation of MEF2 by muscle contractions is mediated by dephosphorylation of MEF2 by calcineurin (4,15). On the basis of these studies, it has been concluded that there is a direct correlation between calcineurin activity and expression of the genes that are transcriptionally activated by muscle contractions, and that calcineurin mediates this effect by activating MEF2 (4).

MEF2A regulates the expression of GLUT4 (5,6). Furthermore, expression of a constitutively active form of calcineurin in mouse skeletal muscle results in increased expression of GLUT4 (7). In light of the evidence that calcineurin activates MEF2 and increases GLUT4 expression, our finding that the inhibition of calcineurin does not

prevent the exercise-induced increase in GLUT4 may seem surprising. However, we have found that the CAMK inhibitor KN93 completely blocks the increase in GLUT4 expression induced by raising cytosolic  $Ca^{2+}$  in myocytes (3). This finding provided evidence that the increase in myocyte GLUT4 induced by  $Ca^{2+}$  is mediated by CAMK, not calcineurin, and thus fits with the present results showing that inhibition of calcineurin does not prevent exercise-induced increases in GLUT4. Although the effect of inhibiting CAMK was evaluated using L6 myotubes (3), it has also been shown that contractions result in increased phosphorylation (on thr 287) of CAMK-II, the CAMK isoform found in skeletal muscle (16), and that the CAMK inhibitor KN62 completely blocks activation of CAMK-II in rat epitrochlearis muscles (17). Calcineurin

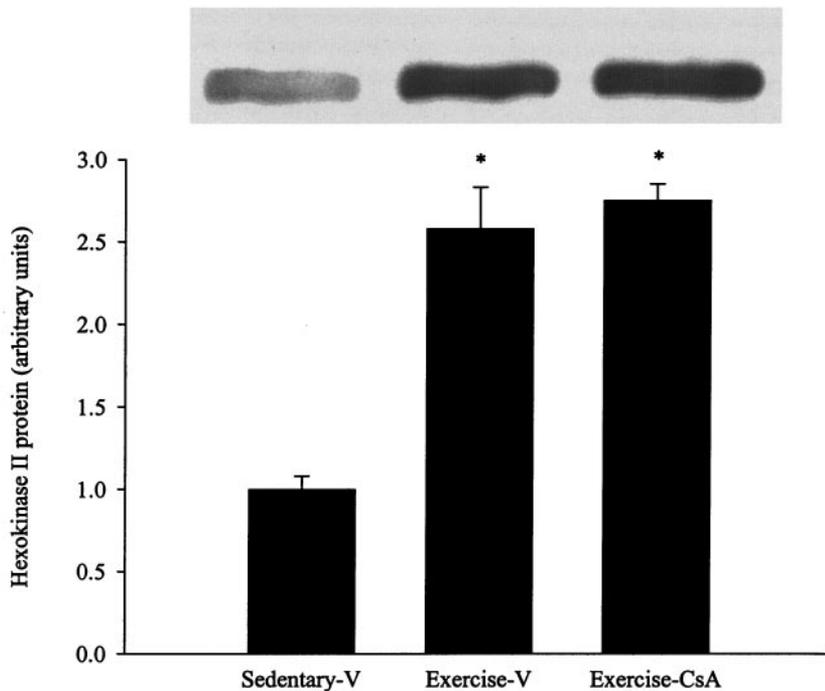


FIG. 3. Hexokinase II protein level is increased in epitrochlearis muscles of rats after five daily bouts of swimming. Rats were treated with cyclosporin A (CsA) or vehicle (V) as described in Fig. 1. The bars represent the means  $\pm$  SE for six rats in the Sedentary-V and Exercise-V groups and nine rats in the Exercise-CsA group. \* $P < 0.001$ .

mediates conversion of fast-twitch to slow-twitch skeletal muscle fibers (13–15). Short-term endurance exercise does not cause conversion of fast to slow fibers (18), so this mechanism is not responsible for the exercise-induced increase in GLUT4 expression. However, it seems likely that fast to slow fiber conversion mediates the increase in GLUT4 in muscles of mice overexpressing calcineurin.

Wu et al. (4) have reported that exercise training does not result in an increase in the abundance of MEF2A in skeletal muscle. However, we found that exercise does induce increased expression of MEF2A in muscle (Fig. 1). This finding is in keeping with our previous observation that raising cytosolic  $Ca^{2+}$  in L6 myotubes or epitrochlearis muscle also induces an increase in MEF2A protein (3). Recent studies have provided insights regarding a possible mechanism that may explain this increase in MEF2A. Exercise induces an increase in the expression and DNA binding activity of the transcription activator nuclear respiratory factor 1 (NRF-1) in muscle (19,20). We have also found that overexpression of NRF-1 in skeletal muscle of mice results in increased expression of MEF2A and GLUT4 (21). At that time, it was not known whether NRF-1 activated transcription of MEF2A, so it was not clear whether the increased expression of MEF2A was mediated directly by NRF-1 or was a secondary consequence of another effect of NRF-1. However, Gulick (22) has recently shown that the MEF2A promoter has an NRF-1 binding site. Furthermore, MEF2A promoter-reporters transfected into myocytes demonstrate NRF-1 element-dependent promoter activity (22).

It seems likely that the increase in MEF2A expression plays a role in maintaining the exercise-induced increase in GLUT4 between exercise sessions. However, because the time course of the increases in MEF2A and GLUT4 proteins after a bout of exercise appear to be similar (P.M.G.R. and J.O.H., unpublished observations), it does not seem possible that the increased expression of MEF2A could mediate the initial increase in GLUT4 transcription

induced by exercise/ $Ca^{2+}$ . Instead, it is probable that activation of preexisting MEF2A is responsible for inducing the initial increase in GLUT4 transcription during exercise.

In light of our finding that inhibition of calcineurin does not affect the exercise-induced increase in GLUT4, we suggest the following alternative mechanism. The transcriptional activity of MEF2A is repressed by the binding of class II histone deacetylases (HDACs) to its DNA binding domain (21). CAMK phosphorylates HDACs, causing dissociation of MEF2A from HDACs with an unmasking of MEF2's transcriptional activity (23). MEF2A is further activated through phosphorylation by the p38 mitogen-activated protein kinase (MAPK) (24,25). Direct evidence in support of this mechanism was provided by a recent study on contracting human skeletal muscle by McGee and Hargreaves (26). Regarding the roles of CAMK and p38 MAPK, it is of interest that Enslin et al. (27) have shown that p38 MAPK can be activated via a pathway in which p38 MAPK lies downstream of CAMK.

In conclusion, it appears that activation of calcineurin in muscle during exercise is not involved in mediating the exercise-induced increase in GLUT4. This conclusion is based on our finding that inhibition of calcineurin does not prevent the increase in GLUT4. McGee and Hargreaves (26) arrived at the same conclusion on the basis of their finding that exercise does not result in nuclear translocation of nuclear factor of activated T-cells. Calcineurin is involved in mediating the conversion of fast- to slow-twitch muscle fibers (14).

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