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Palmitate movement across red and white muscle membranes of rainbow trout

Jeff G. Richards,[1] Arend Bonen,[2] George J. F. Heigenhauser,[3] and Chris M. Wood[1]

Departments of [1]Biology and [3]Medicine, McMaster University, Hamilton, Ontario L8S 4K1; and [2]Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Richards, Jeff G., Arend Bonen, George J. F. Heigenhauser, and Chris M. Wood. Palmitate movement across red and white muscle membranes of rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 286: R46–R53, 2004. First published September 11, 2003; 10.1152/ajpregu.00319.2003.—We examined the movement of [^{3}H]palmitate across giant sarcolemmal vesicles prepared from red and white muscle of rainbow trout (*Oncorhynchus mykiss*). Red and white muscle fatty acid carriers have similar affinities for palmitate (apparent K_m = 26 ± 6 and 33 ± 8 nM, respectively); however, red muscle has a higher maximal uptake compared with white muscle (V_{max} = 476 ± 41 vs. 229 ± 23 pmol·mg protein^{-1}·s^{-1}, respectively). Phloretin (250 μM) inhibited palmitate influx in red and white muscle vesicles by ~40%, $HgCl_2$ (2.5 mM) inhibited palmitate uptake by 20–30%, and the anion-exchange inhibitor DIDS (250 μM) inhibited palmitate influx in red and white muscle vesicles by ~15 and 30%, respectively. Western blot analysis of red and white muscle vesicles did not detect a mammalian-type fatty acid transporter (FAT); however, preincubation of vesicles with sulfo-*N*-succinimidyloleate, a specific inhibitor of FAT in rats, reduced palmitate uptake in red and white muscle vesicles by ~15 and 25%, respectively. A mammalian-type plasma membrane fatty acid-binding protein was identified in trout muscle using Western blotting, but the protein differed in size between red and white muscle. At low concentrations of free palmitate (2.5 nM), addition of high concentrations (111 μM total) of oleate (18:0) caused ~50% reduction in palmitate uptake by red and white muscle vesicles, but high concentrations (100 μM) of octanoate (8:0) caused no inhibition of uptake. Five days of aerobic swimming at ~2 body lengths/s and 9 days of chronic cortisol elevation in vivo, both of which stimulate lipid metabolism, had no effect on the rate of palmitate movement in red or white muscle vesicles.

long-chain fatty acid; transport; rainbow trout (*Oncorhynchus mykiss*), sarcolemmal vesicle

RAINBOW TROUT ARE KNOWN TO rely heavily on fatty acid oxidation in red muscle during periods of sustained "aerobic" swimming (26, 37) and in white muscle during recovery from high-intensity exercise (32, 36, 45). Lipid, usually in the form of triacylglycerol (TAG), is stored in, and mobilized from, visceral adipose tissue, liver, and muscle; however, the relative contributions of each of these lipid pools in supplying substrate for muscle during exercise and recovery are not known (33). Rates of TAG-fatty acid cycling in trout are high compared with other vertebrates (5), suggesting that there is a high rate of fatty acid exchange between tissues of trout, either for reesterification or oxidation. Fatty acids are moved between tissues in plasma bound to albumin and high-density lipoproteins (14). Intracellular fatty acid-binding proteins have been identified in teleost fish (40) and act to transport fatty acids from the point of cellular entry to the mitochondria for oxidation, or to sites of intracellular esterification and storage. However, no information is available on the mechanism of fatty acid movement across muscle cell membranes in fish.

Considerable controversy surrounds the mechanism of cellular fatty acid uptake in mammals. Investigations using artificial, protein-free, phospholipid bilayers suggest that simple "flip-flop" diffusion of fatty acids across cell membranes is rapid and can account for rates of fatty acid uptake by cells observed in vivo (18, 20). However, at physiological pH, long-chain fatty acids (LCFA) exist as an ionized anionic molecule. It has been argued that, in true biological membranes, with membrane-bound, anionic proteins, fatty acids cannot interact with the phospholipid membrane and thus diffusion of fatty acids into cells is limited. Furthermore, the rate of dissociation of LCFA from plasma carrier proteins (albumin) is thought to be insufficient for diffusion to explain the observed rates of fatty acid uptake (4). In light of these kinetic constraints on diffusion as a means for fatty acids to transverse cell membranes, considerable effort has focused on the potential involvement of specific proteins in facilitating fatty acid sequestration from the plasma and fatty acid movement across cell membranes (8, 24, 44).

There is a growing body of literature suggesting that specific membrane-bound proteins facilitate fatty acid movement across mammalian muscle cell membranes (24, 29). Abumrad et al. (3) first demonstrated that LCFA permeation in isolated adipocytes was via a mechanism that displayed saturation kinetics and that could be inhibited by protein-modifying drugs (phloretin, DIDS; see Ref. 2) and competing LCFA. Recently, similar biochemical indexes of protein-mediated uptake have been demonstrated in mammalian muscle using isolated giant sarcolemmal vesicles (10, 28, 30). Many proteins implicated in fatty acid movement across cell membranes have been cloned in mammals (1, 28). Specifically, proteins involved in sequestering fatty acids from the plasma ($FABP_{pm}$) and proteins possibly involved in the translocation of fatty acids across the membrane (FAT/CD36; see Ref. 8) have been identified, and the quantity of these proteins in membrane vesicles positively correlates with the rate of fatty acid uptake (30). Antibodies to these proteins are effective at blocking LCFA uptake by isolated muscle vesicles (10, 30). Cellular redistribution of FAT/CD36 from intracellular stores to the sarcolemma has been implicated as a mechanism to rapidly change the rate of fatty acid uptake by rat muscle during exercise (9).

Address for reprint requests and other correspondence: J. G. Richards, Dept. of Zoology, The Univ. of British Columbia, 6270 Univ. Blvd., Vancouver, British Columbia, Canada V6T 1Z4 (E-mail: jrichard@zoology.ubc.ca).

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The primary objective of the present study was to characterize the movement of LCFA across red and white muscle cell membranes from trout. We hypothesized that movement of LCFA across red and white muscle cells would occur via a facilitated-transport mechanism. Furthermore, insights into the importance of membrane transport in regulating the rate of lipid oxidation in muscle were gained by examining the effects of long-term exercise and chronic cortisol elevations, both known to stimulate lipid oxidation in fish, on LCFA uptake by muscle. To accomplish these goals, we examined palmitate movement across giant sarcolemmal vesicles isolated from red and white muscle of trout. Giant sarcolemmal vesicles have previously been used in trout to examine the kinetics of lactate and glucose movement across white muscle membranes of trout (25, 27). Sarcolemmal vesicles are ideal for investigating membrane transport processes because they are right-side-out (34) and are devoid of metabolic machinery (i.e., mitochondria; see Refs. 10 and 24). Therefore, metabolite transport can be examined in the absence of metabolism.

MATERIALS AND METHODS

Animal care. Adult rainbow trout (*Oncorhynchus mykiss*, Walbaum; 300–450 g) were purchased from Humber Springs Trout Hatchery (Orangeville, Ontario, Canada). Trout were transported to our freshwater holding facility and held in 800-liter tanks supplied with aerated, dechlorinated city of Hamilton tapwater at 12°C for at least 1 mo before experimentation. Fish were fed to satiation every other day with commercial trout pellets. Before tissue sampling, trout were carefully netted from their tank with minimal disturbance and killed by concussion. All experimental procedures fully comply with Canadian Council of Animal Care guidelines.

Giant sarcolemmal vesicles. Vesicles were isolated from red and white muscle using a modified protocol from Labree and Milligan (25). Briefly, freshly killed fish were skinned along the lateral surface, and red muscle (~10 g) was dissected from both sides of a trout. White muscle (50–60 g) was taken from the dorsal epaxial muscle mass. Both the red and white muscles were placed in separate petri dishes containing 30 ml filtered vesicle preparation buffer (140 mM KCl, 5 mM MOPS, 3.5 mM PMSF with 0.25% DMSO, pH 7.4). Muscle was scored extensively perpendicular to the myosepta with a scalpel blade, and the muscle pieces were transferred to a flask containing ~1:1 wt/vol of vesicle preparation buffer containing 8,000 units of type IV collagenase (Worthington Biochemical), 1 mM $CaCl_2$, and 0.3 mg/ml aprotinin. The flask was shaken gently in a water bath at 37°C for 1 h. After the incubation, the muscle pieces were allowed to settle, and the supernatant was filtered through four layers of cheesecloth in a graduated cylinder. The remaining muscle pieces were washed with one-third of the volume of the supernatant with 10 mM EDTA in vesicle preparation buffer. The muscle pieces were allowed to settle, and the supernatant was filtered and combined with the previous supernatant. For the red muscle, the supernatant was placed in a 15-ml conical vial and centrifuged for 10 min at 50 *g*. This centrifugation step caused endogenous lipid to accumulate at the top of the muscle supernatant, which was gently removed by aspiration. For the white muscle preparation, centrifugation was not necessary. The supernatants were then added to Percoll (Sigma) and 1.4 M KCl in a ratio of 1:0.33:0.0575, and the resulting solution was bottom loaded in a round-bottom, 14-ml centrifuge tube containing 3 ml of 5.75% 5(*N*-2,3-dihydroxypropylacetoamido)-2,4,6-triiodo-*N*,*N*′-bis(2,3-dihydroxypropyl)-isophthalamide (Nycodenz) in vesicle preparation buffer. The tubes were topped with 2 ml vesicle preparation buffer and centrifuged at 200 *g* for 45 min with a slow acceleration and deceleration (Beckmann Allegra 21R; Beckman Coulter Canada, Mississauga, Ontario). The vesicles floated through the Percoll and Nycodenz layers and accumulated at the interface between the Nycodenz layer and vesicle preparation buffer. Vesicles were removed gently with a transfer pipette, combined in 15-ml conical vials, and centrifuged at 2,000 *g* for 30 min. The supernatant was removed by careful aspiration, and the pellet was suspended in a known volume of buffer containing 140 mM KCl and 5 mM MOPS, pH 7.4, to yield a protein concentration of ~1 μg/μl. Vesicle protein was determined using the method of Bradford (12) with BSA standards (Sigma). Each vesicle preparation was visually examined using a microscope to ensure the formation of vesicles. Vesicles were sized with a hemocytometer.

Fatty acid transport. Palmitate uptake was measured using a reaction mixture containing 14.1 μM unlabeled palmitate, 0.3 μCi 9,10-[^{3}H]palmitate (American Radiolabeled Chemicals, St. Louis, MO), and 0.05 μCi [^{14}C]polyethylene glycol (PEG-4000; mol wt 4,000 g/mol; American Radiolabeled Chemicals) in a solution containing BSA (fraction V, fatty acid free; Boehringer Mannheim, Montreal, Quebec, Canada), 140 mM KCl, and 5 mM MOPS, pH 7.4. [^{14}C]PEG-4000 served as an extravesicular space marker. The concentration of BSA was adjusted to give molar ratios of fatty acid to BSA from 0.2 to 5, which resulted in a free palmitate concentration ranging from 1.1 to 95.9 nM (38). Transport studies were carried out in 1.7-ml polyethylene microcentrifuge tubes containing 50 μl of vesicle suspension (~50 μg protein). Palmitate uptake was initiated by the addition of 50 μl fatty acid reaction mixture to the vesicle suspension and mixing by gentle tituration. We estimate the extravesicular space to exceed the intravesicular space by > 50-fold. The assay temperature was 20°C. The uptake was stopped by the addition of 1.4 ml ice-cold stop solution containing 2.5 mM $HgCl_2$, 0.1% BSA, 140 mM KCl, and 5 mM MOPS, pH 7.4. The centrifuge tube was then centrifuged at 12,000 *g* for 1 min at 20°C, and the supernatant was gently aspirated away from the vesicle pellet. The sides of the centrifuge tube were cleared of any droplets, and the tip of the centrifuge tube containing the vesicle pellet was cleaved into a 20-ml glass scintillation vial using a pair of pet-nail clippers. A 10-μl sample of the reaction mixture was also taken and added to a scintillation vial. Scintillation cocktail (5 ml; ACS; Amersham) was added to each vial, and the vials were shaken and allowed to stand in the dark for 1 h before counting. The vials were counted on a LKB scintillation counter (Rackbeta 1217) using a double-label counting program. The amount of [^{3}H]palmitate in the extravesicular space was calculated as described in Labree and Milligan (25). Net [^{3}H]palmitate uptake was calculated from the radioactivity in the vesicle pellet minus the extravesicular radioactivity. Net [^{3}H]palmitate uptake was converted to picomole palmitate uptake using the specific activity (μCi/mmol) and is expressed per milligram protein.

To validate the use of KCl/MOPS as a transport medium, we monitored [^{3}H]palmitate uptake by trout muscle vesicles in the presence of 140 mM KCl/5 mM MOPS or 140 mM NaCl/5 mM MOPS. The choice of KCl or NaCl in the transport medium did not affect palmitate uptake by red and white muscle vesicles. Red muscle vesicles incubated with KCl and NaCl had palmitate uptake rates of 51.6 ± 3.5 and 50.5 ± 5.7 pmol · mg protein^{-1} · s^{-1}, respectively (n = 3 for each), and white muscle vesicles incubated with KCl and NaCl had palmitate uptake rates of 17.0 ± 2.0 and 17.3 ± 2.1 pmol · mg protein^{-1} · s^{-1}, respectively (n = 3 for each). Therefore, we elected to use 140 mM KCl/5 mM MOPS as our transport medium.

A time course for palmitate uptake into red and white muscle vesicles at 20°C was determined by incubating vesicles for up to 30 s with either 4.8 or 33.9 nM free palmitate (14.1 μM total palmitate, fatty acid-to-BSA ratio = 0.93 and 3.78, respectively; see Ref. 38). Unless otherwise stated, a transport period of 10 s was used in all subsequent transport studies. To examine the concentration dependence of palmitate uptake by red and white muscle vesicles, the fatty acid-to-BSA ratio in the reaction mixtures was adjusted to 0.2, 0.4, 0.9, 1.9, 3.0, 3.7, 4.2, and 5.0, which yielded free palmitate concentrations of 1.1, 2.0, 4.8, 10.2, 20.5, 33.9, 50.2, and 95.9 nM, respectively (38). In these solutions, total unlabeled palmitate and

[^{3}H]palmitate remained constant; thus, specific activity (μCi/mmol) remained constant. The effects of temperature on palmitate uptake were examined in red and white muscle vesicles equilibrated to 20, 10, and 0°C using free palmitate concentrations of 4.8 nM.

The effects of known pharmacological blockers of fatty acid transport on palmitate uptake by red and white muscle vesicles were determined in the presence of 14.1 μM total palmitate. Vesicles were preincubated with either 250 μM phloretin in 0.3% DMSO; 250 μM DIDS in 0.3% DMSO; 2.5 mM $HgCl_2$; and 50 μM sulfo-*N*-succinimidyloleate (SSO) in 0.2% DMSO in the dark for 0.5 h. SSO is a nonreversible blocker of FAT/CD36 (13); therefore, before transport studies were carried out, the SSO was removed from the vesicles by centrifugation and replaced by KCl/MOPS buffer. Transport assays were carried out in the presence of the other inhibitors. Transport controls (i.e., vesicle preparation − inhibitor) were run concurrently with experimental treatments and contained an equivalent amount of any vehicle (e.g., DMSO).

The effects of phloretin, DIDS, and $HgCl_2$ competition with palmitate for binding to BSA were assessed using heptane-aqueous partitioning of bound and unbound palmitate in our solutions. Similar procedures have been used to characterize the basic kinetics of fatty acid interactions with albumin (e.g., see Ref. 39). Briefly, an aliquot (50 μl) of reaction mixture containing experimental concentrations of the pharmacological agent and appropriate vehicle was added to 450 μl of 140 mM KCl and 5 mM MOPS, pH 7.4, in a siliconized borosilicate vial. To the top of the aqueous solution, 500 μl *n*-heptane were added, and the tubes were tightly sealed with Teflon-lined caps and allowed to incubate stirred overnight at 25°C. Fifty microliters of the aqueous phase and heptane phase were then counted for [^{3}H]palmitate. The reaction media used to construct a concentration-dependence relationship were also run in a similar manner to the above reaction media. These provided a standard curve to which our unknown solutions were compared to determine the effects of pharmacological agents on free palmitate concentrations.

To examine the effects of other competing fatty acids on palmitate uptake by vesicles, transport studies were conducted in the presence of excess oleate (18:0; 111 μM) and octanoate (8:0; 100 μM). Reaction mixtures contained 7.4 μM total palmitate in 0.1% fatty acid-free BSA (fatty acid-to-BSA ratio = ~0.5), which resulted in a low free palmitate concentration of 2.5 nM (38). To this reaction medium, high concentrations of competing fatty acid were added. To determine the interactions between palmitate and the competing fatty acid for binding to albumin, we used the calculations outlined by Abumrad et al. (2). Expected changes in palmitate uptake by the muscle vesicles were assessed by comparing the predicted change in free palmitate with the known responses with the vesicles to changes in free palmitate determined above (see Fig. 2). Octanoate does not compete with long-chain fatty acids for binding to albumin; therefore, no correction was required for changes in free palmitate in the presence of excess octanoate (10).

To determine if palmitate movement into muscle was affected by long-term exercise, trout (389 ± 44 g; 32.2 ± 0.4 cm, n = 12) were swum at 60 cm/s (1.9 ± 0.1 body lengths/s) for 5 days in a darkened, 156-liter, Beamish-style swim tunnel (37) served with a flow-through (200 ml/min) of fresh water. Nonexercised fish were held in a darkened Plexiglas box of approximately the same volume as the swimming chamber of the swim tunnel served with a similar flow-through (200 ml/min) of fresh water. Fish were checked eight times daily, and any fish that was not swimming for the entire period was discarded. At the end of the 5 days, fish were removed from the fish box or tunnel and killed by concussion; vesicles were prepared from their red and white muscle as described above, and transport studies were conducted at a free palmitate concentration of 4.8 nM.

Chronic elevations in plasma cortisol in trout have been reported to stimulate lipid metabolism (15). To determine if chronic cortisol treatment affected palmitate movement in red and white muscle, trout were fitted with coconut oil implants containing cortisol (17). Briefly, adult trout (364 ± 21 g, n = 8) were anesthetized with MS-222 anesthetic and injected peritoneally with 10 μl/g body wt of liquid coconut oil at 25°C containing cortisol to yield a dose of 250 mg cortisol/kg fish (cortisol = 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; Sigma; see Ref. 17). A second group of fish (sham; 348 ± 30 g, n = 8) was treated identically to the cortisol-implanted fish, but were injected with coconut oil that did not contain cortisol. The coconut oil quickly solidifies at 12°C, forming a solid implant that slowly releases cortisol to the circulation. Fish were revived, and the cortisol and sham fish were placed in separate 200-liter tanks for 9 days. At the end of 9 days, fish were killed quickly by concussion, blood samples were taken for cortisol determination, vesicles were prepared from their red and white muscle, and transport studies were conducted at 4.8 nM free palmitate. Blood cells were immediately separated from the plasma by centrifugation and frozen in liquid N_2. Cortisol concentrations were determined on thawed plasma using a ^{125}I-labeled RIA (ICN Biochemicals, Costa Mesa, CA).

Western blotting. Vesicles from rat heart and red and white gastrocnemius muscle (positive controls) and trout red and white muscle were separated on 10% SDS-polyacrylamide gels (150 V for 1 h). Proteins were then transferred to Immobilon polyvinylidene difluoride membranes (100 V for 90 min) and shaken gently for ~16 h in *buffer A* [20 mM Tris-base, 137 mM NaCl, 0.1 mM HCl, pH 7.5, 0.1% (vol/vol) Tween 20, and 10% (wt/vol) nonfat dry milk] at room temperature. Subsequently, membranes were incubated for 1 h with either a monoclonal antibody to CD36 (1:500; Cedarlane Laboratories, Hornby, Ontario) or a polyclonal antiserum against $FABP_{pm}$ (1:2,500; rabbit antibody raised against rat hepatic membrane fatty acid-binding protein; see Ref. 41) in *buffer A*, followed by three washes in *buffer A*. Membranes were then incubated for 1 h with rat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:7,000). Membranes were washed with *buffer A*. Detection occurred with an enhanced chemiluminescence detection method by exposing the membranes to film (Hyperfilm-ECL) at room temperature according to the instructions of the manufacturer. Film was developed and fixed in GXB fixer/replenisher.

Data presentation and statistical analysis. All data are presented as means ± SE (n = no. of fish). A nonlinear regression model was used to describe the effects of changes in free palmitate concentration on palmitate uptake by the red and white muscle vesicles and to determine the K_m and V_{max} constants of transport (SigmaPlot2000; SPSS). Significant differences between treatments and paired controls were determined using a two-tailed paired Student's *t*-test. One-way ANOVA followed by a Least-Significant Difference method of pairwise multiple comparisons was used to evaluate the effects of temperature on palmitate uptake. Results were considered significant at $P < 0.05$.

RESULTS

Vesicles. Sarcolemmal vesicles isolated from trout red and white muscle were spherical in shape with a diameter of 14.0 ± 0.8 μm (n = 84; size range 5–25 μm) and 13.8 ± 0.9 μm (n = 112; size range 5–55 μm), respectively.

Palmitate transport. Palmitate uptake by red and white muscle vesicles at 20°C was linear for at least 30 and 15 s, respectively, when incubated with 4.8 nM free palmitate (Fig. 1*A*). In vesicles incubated with 33.9 nM free palmitate, palmitate uptake by red and white muscle vesicles was linear over 20 s (Fig. 1*B*). At all time points, red muscle vesicles accumulated between 2.3 to 3.6 times more palmitate than white muscle vesicles.

Palmitate uptake by red and white muscle vesicles at 20°C rapidly increased and saturated as external free palmitate was increased up to 95 nM (Fig. 2). Uptake of palmitate saturated at approximately two times the rate in red muscle vesicles

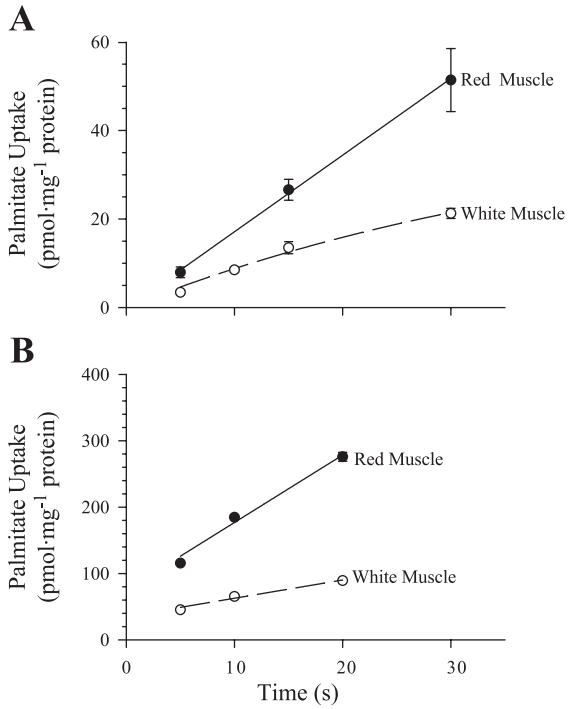

Fig. 1. Time course at 20°C of palmitate uptake by sarcolemmal vesicles isolated from trout red and white muscle during exposure to 4.8 (*A*) or 33.9 (*B*) nM free palmitate. Data are means ± SE. In A, $n = 4$ fish for each and in B, $n = 2$ fish for each group.

compared with white muscle vesicles. Red and white muscle vesicles exhibited similar affinities (K_m) for palmitate uptake, but the maximum transport capacity (V_{max}) was 2.1-fold higher in red muscle vesicles than in white muscle vesicles (Table 1).

The addition of 250 μM phloretin and 250 μM DIDS in 0.3% DMSO to the transport medium caused 1.9- and 2.0-fold increases in free palmitate concentrations, respectively, compared with the addition of 0.3% DMSO alone (Table 2). The addition of 2.5 mM $HgCl_2$ to the transport medium caused a 1.8-fold increase in free palmitate compared with controls. Taking these changes in free palmitate into account, palmitate

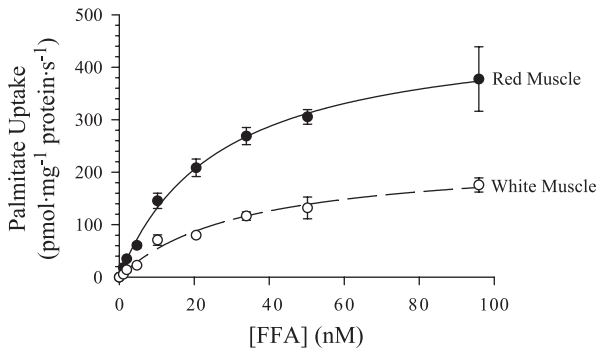

Fig. 2. Concentration dependence of palmitate uptake at 20°C by sarcolemmal vesicles isolated from trout red and white muscle using increasing concentrations of free palmitate. The transport period was 10 s. Free palmitate concentrations were calculated according to Richieri et al. (38). Data are means ± SE. From *left* to *right*, $n = 6, 6, 6, 5, 6, 5, 6$, and 5 for red muscle vesicles and $n = 5, 5, 5, 5, 4, 5, 5$, and 5 for white muscles.

Table 1. *K_m and V_{max} for palmitate uptake in vesicles isolated from red and white muscle*

	K_m, nM free palmitate	V_{max}, pmol·mg protein^{-1}·s^{-1}
Red muscle	26±6	476±41
White muscle	33±8	229±23
Significance	NS	$P < 0.05$

Values are means ± SE; $n = 9$ fish in each group. NS, not significant.

uptake in both red and white muscle vesicles was inhibited by ~40% with the addition of 250 μM phloretin (Fig. 3, *A* and *B*). The addition of 250 μM DIDS to the transport medium decreased palmitate uptake in red and white muscle vesicles by 15 and 20%, respectively (Fig. 3, *A* and *B*). The addition of 2.5 mM $HgCl_2$ reduced palmitate uptake by 21 and 29% in red and white muscle vesicles, respectively (Fig. 3, *A* and *B*). Preincubation of vesicles with SSO in 0.2% DMSO decreased palmitate uptake by red and white muscle vesicles by 15 and 25%, respectively (Fig. 3, *A* and *B*).

Palmitate uptake by red and white muscle vesicles was inhibited by 49 and 48%, respectively, in the presence of 111 μM oleate (18:0; Fig. 4, *A* and *B*). However, 100 μM octanoate (8:0) did not significantly affect palmitate uptake by either red or white muscle vesicles. Decreases in temperature from 20°C to 10 and to 0°C decreased palmitate uptake by red muscle vesicles by 60 and 85%, respectively (Fig. 5*A*), yielding Q_{10} values between 2.5 and 3.3. The same stepwise decreases in temperature decreased palmitate uptake by white muscle vesicles by 49 and 77% (Fig. 5*B*), yielding Q_{10} values between 1.9 and 2.2.

Western blotting. Western blot analysis for FAT/CD36 detected protein in rat red muscle, white gastrocnemius muscle, and heart but did not detect a homologous protein in trout red or white muscle (Fig. 6*A*). Western blots probed with antibodies against rat hepatic $FABP_{pm}$ revealed a single immunoreactive band of the appropriate size (~43 kDa) in trout red muscle vesicles but revealed two bands in the trout white muscle (Fig. 6*B*), one faint band of the expected size and a darker band of higher molecular weight.

Long-term swimming and cortisol implants. Five days of exercise at 1.9 body lengths/s did not affect palmitate uptake by red or white muscle vesicles (Fig. 7). Furthermore, fish implanted with coconut oil implants containing cortisol (yielding plasma cortisol concentrations of 238 ± 110 ng/ml; $n = 6$) did not exhibit different palmitate uptake rates in either red or

Table 2. *Effects of phloretin, DIDS, and $HgCl_2$ on free palmitate concentration during transport*

	Free Palmitate, nM
Control	4.65±0.53
DMSO (0.3%)	4.74±0.04
Phloretin 250 μM	9.03±0.53
DIDS (250 μM)	9.39±0.09
$HgCl_2$ (2.5 mM)	8.55±0.68

Values are means ± SE. Control represents measurements done in the absence of a pharmacological agent and without carrier. Phloretin and DIDS were dissolved in 0.3% DMSO, and therefore their carrier control is 0.3% DMSO.

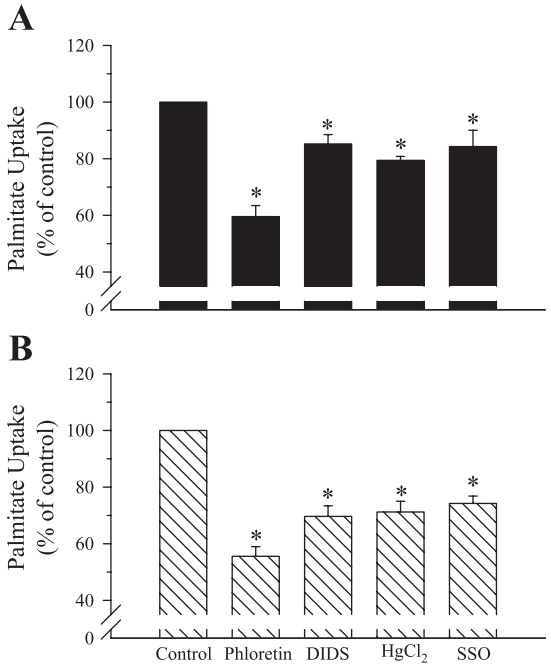

Fig. 3. Inhibition of palmitate uptake by 250 μM phloretin, 250 μM DIDS, 2.5 mM $HgCl_2$, and 50 μM sulfo-*N*-succinimidyloleate (SSO) in sarcolemmal vesicles isolated from trout red (*A*) and white (*B*) muscle. Data are means ± SE and are expressed relative to untreated control (100%); $n = 5$ for each bar. *Significant difference compared with controls ($P < 0.05$).

white muscle vesicles compared with sham-implanted fish (coconut oil with no cortisol; plasma cortisol = 14.8 ± 7.2 ng/ml; $n = 6$; data not shown).

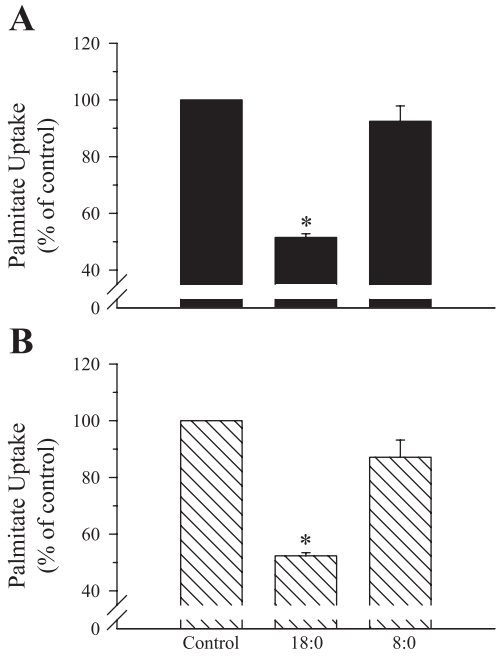

Fig. 4. Competition for palmitate uptake by sarcolemmal vesicles isolated from trout red (*A*) and white (*B*) muscle with 100 μM oleate (18:0) and 100 μM octanoate (8:0). Data are means ± SE and are expressed relative to untreated control (100%); $n = 5$ for each bar. *Significant difference compared with controls ($P < 0.05$).

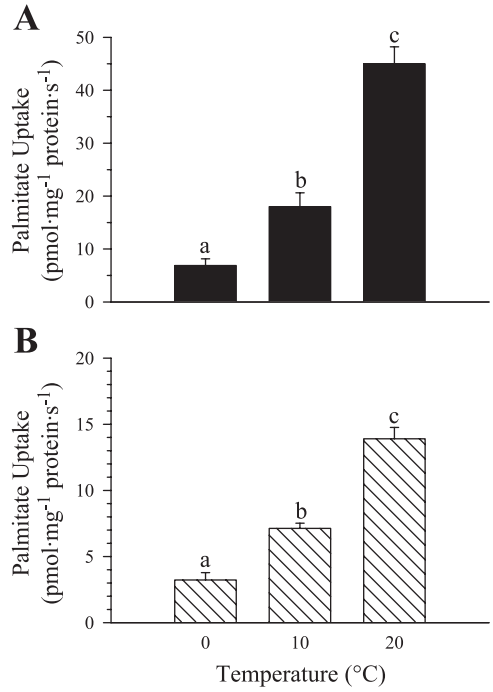

Fig. 5. Effects of temperature on palmitate uptake by sarcolemmal vesicles isolated from trout red (*A*) and white (*B*) muscle. Vesicles were incubated with 4.8 nM free palmitate. Data are means ± SE; $n = 5$ for each bar. Bars with different letters are significantly different ($P < 0.05$).

DISCUSSION

Sarcolemmal vesicles. Giant sarcolemmal vesicles have become a powerful tool in elucidating the biochemical and molecular mechanisms by which lactate (23, 34), glucose (35), and fatty acids (10, 16, 24, 42) move across muscle cell membranes in mammals. Giant sarcolemmal vesicles have also been used to examine the kinetics and pharmacology of lactate retention in rainbow trout white muscle (25) and to characterize the kinetics of glucose movement across the white muscle of trout, American eel (*Anguilla rostrata*), and black bullhead catfish (*Ameiurus melas*; see Ref. 27). Giant sarcolemmal vesicles are advantageous for examining metabolite transport across cell membranes because the vesicles are oriented right-side-out (34), they contain intracellular fluid (8), and transport processes are divorced from subsequent metabolism (6, 24).

In particular, the presence of intracellular fluid within these vesicles makes them ideal for examining fatty acid movement

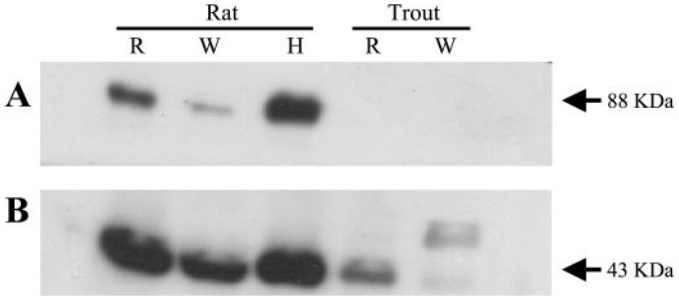

Fig. 6. Western blot analysis for fatty acid translocase (*A*) and fatty acid-binding protein from plasma membrane (*B*) in vesicles prepared from rat red (R) and white (W) gastrocnemius muscle and heart (H; positive control) and in vesicles prepared from trout red (R) and white muscle (W).

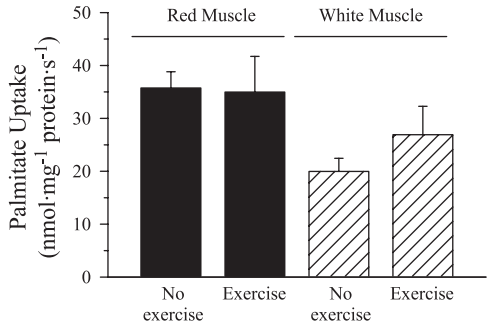

Fig. 7. Effects of 5 days of exercise at 1.9 body lengths/s on palmitate uptake by sarcolemmal vesicles isolated from trout red and white muscle. Vesicles were incubated with 4.8 nM free palmitate. Data are means ± SE; $n = 6$ for each bar.

across membranes because of the presence of cytoplasmic fatty acid-binding protein ($FABP_{cyt}$) within the vesicle. Bonen et al. (10) measured > 13-fold excess $FABP_{cyt}$ in vesicles prepared from rat muscle and demonstrated that this intracellular protein acts as a sink for fatty acid binding and provides the gradient for fatty acid uptake in the myocytes (8). Without intravesicular $FABP_{cyt}$, fatty acid uptake would be severely limited and dependent on the fatty acid solubility in an aqueous medium. In the present study, vesicles prepared from red and white muscle of trout were spherical in shape and were roughly the same size (~14 μm). Red and white muscle vesicles accumulated palmitate in a linear fashion over time (Fig. 1, *A* and *B*), indicating that during the short assay period used in the present study (10 s) the concentration of $FABP_{cyt}$ did not limit the rate of palmitate uptake.

Evidence for carrier-mediated uptake of fatty acids by trout muscle. The present study is the first to examine the movement of palmitate across giant sarcolemmal vesicles from red and white muscle of rainbow trout. Although considerable controversy surrounds the mechanisms involved in fatty acid movement across cell membranes (18, 19), we provide biochemical and pharmacological evidence to suggest that palmitate uptake by trout muscle occurs via a facilitated transport mechanism. Palmitate uptake by red and white muscle vesicles occurs via a saturable mechanism displaying the characteristics of a low-affinity, high-capacity carrier (Fig. 2 and Table 1). Saturation kinetics were only observed when fatty acid concentrations were expressed as free palmitate (38), indicating that it is the unbound fatty acid that is transported.

Inhibition of palmitate uptake in the presence of protein-modifying agents (phloretin, DIDS, $HgCl_2$, and SSO; Fig. 3) further suggests that membrane-bound proteins are involved in palmitate uptake. Phloretin is known to modify surface proteins and has proven effective in partially inhibiting fatty acid movement in other model systems (3, 10, 30). The addition of DIDS, a nonpermeable anion exchange inhibitor, to the vesicle suspension decreased palmitate uptake by red and white muscle vesicles (Fig. 3, *A* and *B*). Treatment of isolated rat adipocytes with 200 μM DIDS has been shown to cause strong, irreversible blockade of oleate uptake (2). $HgCl_2$, a nonspecific, membrane-permeable modifier of sulfhydryl groups on proteins, caused minor decreases in palmitate uptake by red and white muscle vesicles (Fig. 3, *A* and *B*). Phloretin, DIDS, and $HgCl_2$ were shown to interact with albumin and to cause an increase in free palmitate during the transport studies. Abumrad et al.(3) also demonstrated reduced binding of LCFA to albumin in the presence of phloretin. We further demonstrate that DIDS and $HgCl_2$ interact with albumin and cause an approximately twofold increase in free palmitate. Pretreatment of vesicles with SSO, a specific inhibitor of FAT/CD36 (13), decreased palmitate uptake by both red and white muscle vesicles. In rat heart and skeletal muscle, SSO inhibited LCFA uptake by ~50 and 75%, respectively (10, 30).

Competition between oleate and palmitate for uptake by red and white muscle vesicles further supports the involvement of specific sites for LCFA binding to the membranes for subsequent transport across red and white muscle vesicles (Fig. 4). Short-chain fatty acids (SCFA; e.g., octanoate) do not compete with palmitate for uptake, indicating that the transport mechanism shows specificity for LCFA (Fig. 4). The permeation of SCFA into vesicles is probably via another transport mechanism, although simple diffusion of SCFA has also been proposed (10). Decreases in incubation temperature from 20 to 0°C dramatically decreased palmitate uptake by red and white muscle vesicles and demonstrate maximum Q_{10} values of 3.3 and 2.2, respectively (Fig. 5). These high Q_{10} values suggest that temperature has an impact on palmitate transport rather than diffusion; however, changes in temperature will affect vesicle membrane fluidity and could also impact upon the flip-flop mechanism of diffusion.

Taken together, our biochemical and pharmacological results provide strong evidence suggesting that palmitate uptake in red and white muscle vesicles is via a carrier-mediated process. Recent evidence points to the involvement of several proteins in facilitating LCFA uptake in various cell types (2, 3, 8, 10, 11, 28). Putative fatty acid transport proteins (FATPs) have been identified in mammals, including a fatty acid-binding protein on or in the plasma membrane ($FABP_{pm}$), a fatty acid translocase (FAT/CD36), and a FATP (reviewed in Refs. 8 and 44). A mammalian-type $FABP_{pm}$ was identified in trout red and white muscle using Western blot analysis; however, the majority of the protein was larger in white muscle than red muscle, suggesting that the structure of $FABP_{pm}$ may differ in these two tissues (Fig. 6). Western blot analysis failed to demonstrate the presence of FAT/CD36 in trout red or white muscle vesicles; however, the specific inhibitor of FAT/CD36, SSO, caused a 15–25% decrease in palmitate uptake by trout vesicles. Based on the SSO inhibition of palmitate uptake by trout vesicles, it seems likely that a FAT/CD36-type protein is present in the membranes of trout muscle but is sufficiently different from the mammalian protein so as not to be immunoreactive in the Western blot analysis. Overall, it appears that LCFA uptake in trout red and white muscle probably occurs, at least in part, by a mechanism similar to that proposed for LCFA uptake in mammalian muscle.

In mammalian systems, the precise role of each protein and their potential interactions to ultimately facilitate fatty acid movement across membranes are not known. However, expression studies have demonstrated a clear correlative relationship between the expression pattern of $FABP_{pm}$ and FAT/CD36 and the rate of LCFA uptake by isolated vesicles (7, 8, 21, 30). Theoretically, coordination of these proteins to facilitate LCFA transport in cells involves the sequestering of fatty acids from the blood, either bound to albumin or as the free fatty acid, by $FABP_{pm}$. Fatty acids bound to $FABP_{pm}$ are probably then

donated to FAT/CD36, which may act to facilitate the translocation of fatty acids across the plasma membranes (8, 11).

Differences in transport between red muscle and white muscle. Vesicles prepared from red and white muscle of trout transport palmitate with the same affinity; however, red muscle vesicles transport palmitate at approximately two times the maximum rate as white muscle vesicles (Fig. 2 and Table 1). Red and white muscle vesicles made from trout were roughly the same size; thus, the differences in palmitate uptake by red and white muscle vesicles is not because of differences in surface-to-volume ratios. These differences in transport capacity (V_{max}) are probably the result of a greater number of transport proteins in the red muscle membranes compared with the white muscle membrane. The greater fatty acid transport capacity observed in red muscle compared with white muscle is probably related to the relative importance of lipid oxidation in each tissue. Red muscle of fish is known to have higher mitochondrial density compared with white muscle (citrate synthase activity 49 and 3.5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively; see Ref. 31), and red muscle has a higher capacity for LCFA oxidation. Furthermore, red muscle of trout is known to utilize lipid as a major substrate during prolonged periods of sustainable exercise [< critical swimming speed (U_{crit}); 26, 37]. In contrast, white muscle of fish only relies on oxidation of lipid fuels during postexercise recovery (36).

Despite the differential transport of palmitate into trout red and white muscle vesicles, the maximal transport capacity of trout red and white muscle vesicles is 600- and 750-fold higher, respectively, than that observed in similarly sized red and white muscle vesicles from rats (10). These higher transport capacities attest to the importance of lipid as a substrate for metabolic energy production in fish (5, 26, 33, 36, 37).

Influence of experimental treatments on fatty acid transport. Long periods of sustained swimming have been shown to increase the oxidative capacity of white and red muscle of trout (22). Furthermore, sustained swimming in trout has been characterized to rely heavily on fatty acid oxidation during swimming at speeds up to 60% U_{crit} (26, 37). Indeed, fatty acids have now been accepted as the major fuel for sustained exercise in teleost fish (33). However, debate exists as to the source of lipid for oxidation (exogenous fatty acids or endogenous TAG), and also how the rate of lipid oxidation is regulated in fish muscle (33). In mammals, the rate of fatty acid oxidation is, in part, regulated by the rate of fatty acid movement in the muscle cell. Chronic electrical stimulation of muscle (7 days at 10 Hz) in rats has been shown to increase the expression of FAT/CD36 and to result in an increase in palmitate uptake compared with nonstimulated muscle (7, 11). Furthermore, LCFA uptake by rat muscle can by enhanced on an acute time scale by the cellular redistribution of FAT/CD36 from intracellular stores to the sarcolemma (9). In the present study, prolonged exercise (5 days at 1.9 body lengths/s; ~60% U_{crit}) did not enhance palmitate movement in either red or white muscle vesicles. These results are in agreement with the results of Bernard et al. (5), who demonstrated that prolonged swimming did not cause a change in TAG/fatty acid cycling in trout. It should be noted that, during prolonged exercise, the training effects will only occur in the small proportion of muscle used to power the exercise. However, in the preparation of the muscle vesicles, large masses of muscle are required; therefore, the lack of observed response may also be because of a dilution effect. Chronic cortisol elevation, which is known to increase lipid oxidation in trout (15), similarly had no effect on palmitate uptake rates. Together, these data suggest that fatty acid transport in trout muscle may be regulated differently than in mammalian muscle.

Richards et al. (36, 37) postulated that the rate of fatty acid oxidation in trout muscle is probably regulated by carnitine palmitoyltransferase-1 (CPT-I), the enzyme responsible for catalyzing the binding of fatty acids to carnitine for transport in the mitochondria for oxidation (43). CPT-I is regulated in vivo by changes in malonyl-CoA. Richards et al. (37) demonstrated decreases in red muscle malonyl-CoA concentrations during sustained exercise. Decreases in malonyl-CoA reduce the resting inhibition of CPT-I and thereby enhance lipid oxidation in mammals. However, detailed studies into the role of malonyl-CoA and CPT-I in regulating the rate of fatty acid oxidation in fish muscle have not been done.

In conclusion, we demonstrate that LCFA uptake by trout red and white muscle membrane vesicles occurs via a saturable mechanism that is sensitive to inhibition by phloretin, DIDS, $HgCl_2$, and SSO, is competitively inhibited by other LCFA, and has a Q_{10} for uptake of two to three. Taken together, these data provide strong evidence that fatty acid uptake in the red and white muscle of trout is via a carrier-mediated process. Western blot analysis and pharmacological manipulation suggest that mammalian-like proteins ($FABP_{pm}$ and FAT/CD36) may be involved in LCFA uptake in trout muscle. Palmitate uptake in red muscle has a maximal rate that is two times that of white muscle, supporting the importance of fatty acid oxidation in fueling red muscle contraction compared with white muscle contraction. Rates of fatty acid oxidation do not appear to be chronically regulated at the point of uptake in the muscle because long-term aerobic exercise and cortisol implantation, both of which have been demonstrated to enhance fatty acid oxidation, do not stimulate uptake.

Perspectives

How is lipid oxidation regulated in fish? In general, there has been little research on lipid metabolism in fish. Measurements of enzyme activity, rates of fatty acid oxidation in isolated mitochondria, whole organism respirometry, and biochemical profiling of muscle during exercise and recovery all suggest that lipids are an important fuel in muscle during exercise and recovery. However, little information is available on the mechanisms that regulate the rate of lipid oxidation in muscle. In contrast to mammalian systems, the rate of whole organism TAG/fatty acid cycling (5) and the transport process of LCFA across muscle membranes do not appear to contribute to the overall regulation of lipid oxidation by muscle. Clearly, future work should focus on isolating the biochemical steps that regulate the overall rate of lipid metabolism in fish muscle. The importance of CPT-I and the modulating effects of malonyl-CoA should be examined in detail in fish muscle.

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