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In Vivo, Fatty Acid Translocase (CD36) Critically Regulates Skeletal Muscle Fuel Selection, Exercise Performance, and Training-induced Adaptation of Fatty Acid Oxidation*

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Background: CD36-mediated lipid transport may regulate muscle fuel selection and adaptation.
Results: CD36 ablation impaired fatty acid oxidation and prevented its exercise training-induced up-regulation. Without altering mitochondrial content, CD36 overexpression mimicked exercise training effects on fatty acid oxidation.
Conclusion: CD36 contributes to regulating fatty acid oxidation and adaptation in a mitochondrion-independent manner.
Significance: This work identified another mechanism regulating muscle fatty acid oxidation.

For ~40 years it has been widely accepted that (i) the exercise-induced increase in muscle fatty acid oxidation (FAO) is dependent on the increased delivery of circulating fatty acids, and (ii) exercise training-induced FAO up-regulation is largely attributable to muscle mitochondrial biogenesis. These long standing concepts were developed prior to the recent recognition that fatty acid entry into muscle occurs via a regulatable sarcolemmal CD36-mediated mechanism. We examined the role of CD36 in muscle fuel selection under basal conditions, during a metabolic challenge (exercise), and after exercise training. We also investigated whether CD36 overexpression, independent of mitochondrial changes, mimicked exercise training-induced FAO up-regulation. Under basal conditions CD36-KO *versus* WT mice displayed reduced fatty acid transport (−21%) and oxidation (−25%), intramuscular lipids (less than or equal to −31%), and hepatic glycogen (−20%); but muscle glycogen, VO_{2max}, and mitochondrial content and enzymes did not differ. In acutely exercised (78% VO_{2max}) CD36-KO mice, fatty acid transport (−41%), oxidation (−37%), and exercise duration (−44%) were reduced, whereas muscle and hepatic glycogen depletions were accelerated by 27–55%, revealing 2-fold greater carbohydrate use. Exercise training increased mtDNA and β-hydroxyacyl-CoA dehydrogenase similarly in WT and CD36-KO muscles, but FAO was increased only in WT muscle (+90%). Comparable CD36 increases, induced by exercise training (+44%) or by CD36 overexpression (+41%), increased FAO similarly (84–90%), either when mitochondrial biogenesis and FAO enzymes were up-regulated (exercise training) or when these were unaltered (CD36 overexpression). Thus, sarcolemmal CD36 has a key role in muscle fuel selection, exercise performance, and training-induced muscle FAO adaptation, challenging long held views of mechanisms involved in acute and adaptive regulation of muscle FAO.

Skeletal muscle by virtue of its mass (~40% body weight), its highly variable metabolic rate, and its capacity for metabolic and mitochondrial adaptation is a key tissue contributing to metabolic homeostasis. *In vivo*, fuel selection by skeletal muscle is complex, as this is influenced by a number of factors, including nutritional status, obesity, diabetes, and aerobic fitness (*cf.* Refs. 1–3), conditions in which molecular metabolic profiles and/or biochemical regulation of selected metabolic pathways are modified.

Explanations for skeletal muscle fatty acid fuel selection and the adaptive changes in fatty acid oxidation have for many years been sought in the blood borne delivery of fatty acids and in the biochemical regulatory mechanisms at the level of mitochondria (4–6). More recently, the mechanistic basis for chronically increasing fatty acid oxidation in genetically modified and exercise-trained animals has been explained by the coordinate up-regulation of genes involved in mitochondrial biogenesis, oxidative phosphorylation, and fatty acid oxidation, changes that can be induced by a number of molecular mechanisms, including the transcriptional co-activator PGC-1α (7–10), the transcription factor PPARδ[6] (11–14), and estrogen-related receptor γ (15, 16). However, changes in the mitochondrial proteome are

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[6] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; RER, respiratory exchange ratio; β-HAD, β-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; AMPK, adenosine 5′-monophosphate-activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; FABPpm, plasma membrane fatty acid-binding protein.

not necessarily the only mechanisms involved in regulating skeletal muscle fuel selection.

Because skeletal muscle is metabolically promiscuous, fuel selection can also be altered independent of changes in mitochondrial density and enzymes, PGC-1α, PPARδ, and estrogen-related receptor γ. For example, altering the provision of fatty acids via the circulation will alter the rates of fatty acid oxidation and glycogen utilization in a reciprocal manner (3, 17, 18). Yet the intramuscular molecular mechanisms accounting for these changes in fuel selection are not fully understood. In recent years, the regulation of fatty acid oxidation was thought to be mediated via the activation of the AMPK/ACC2/CPT-I axis. However, this has recently been brought into question (19–21). Therefore, other molecular regulatory mechanisms may be essential for skeletal muscle fuel selection.

Recent studies have shown that fatty acid transport into muscle occurs via a regulatable protein-mediated mechanism (22) that influences fatty acid utilization. For example, acute metabolic challenges (muscle contraction, AICAR), as well as genetically induced changes in fatty acid transporters, especially CD36, alter the rates of fatty acid transport and oxidation coordinately (23–25). Importantly, these changes in fatty acid oxidation mediated via sarcolemmal CD36-facilitated fatty acid transport can occur independent of fatty acid delivery to muscle, muscle mitochondrial density, and the expression of oxidative phosphorylation and fatty acid oxidation-related genes (26), mechanisms that have conventionally been viewed as being essential for the regulation of fatty acid oxidation.

The plasma membrane fatty acid transporter CD36 appears to be a central component of the molecular machinery involved in regulating fatty acid oxidation, as acute physiologic stimuli induce the translocation of this transporter to the plasma membrane (27, 28), whereas chronic stimuli, including low frequency muscle stimulation (29), exercise training (30), pharmaceutical activation of PPARδ (14, 31), and PGC-1α overexpression (32–35), induce the expression of CD36 concurrently with the mitochondrial molecular machinery for fatty acid oxidation. This suggests that *in vivo* CD36-mediated fatty acid transport, analogous to GLUT4-mediated glucose transport, may be a vital molecular process that contributes to the following: (*a*) the regulation of muscle fuel selection during an acute metabolic challenge (exercise), and (*b*) to the adaptation of fatty acid oxidation in response to a chronic metabolic stimulus (aerobic training). These suggestions, if correct, would alter considerably our long held understanding of the critical mechanisms involved in up-regulating fatty acid oxidation in skeletal muscle.

Here, we have examined the function of CD36 *in vivo* as follows: (*a*) its role in regulating substrate metabolism at rest and during exercise, and (*b*) its contribution to the adaptation of fatty acid oxidation induced by exercise training. In addition, we overexpressed CD36 in sedentary muscle to the same level as was induced by exercise training, allowing us (*c*) to examine the effects of CD36 on fatty acid oxidation independent of changes in the mitochondrial machinery. Our results establish a key role for CD36 in muscle fuel selection, muscle endurance performance, and metabolic adaptation in muscle fatty acid oxidation. Hence, our work challenges the long held understandings of the molecular mechanisms involved in the acute regulation and adaptation of fatty acid oxidation in mammalian muscle *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

The following were purchased from commercial sources: [1-^{14}C]palmitate and 3-*O*-[^{3}H]methylglucose (PerkinElmer Life Sciences), [^{3}H]glucose (GE Healthcare), fatty acid kit (Wako Diagnostics, Richmond, VA), and insulin (Humulin-R, Lilly); antibodies were from the following: anti-AMPK and anti-phosphorylated AMPK (Thr-172), anti-ACC, anti-phosphorylated ACC (Cell Signaling Technology, Danvers, MA); anti-GLUT4 (Chemicon International, Temecula, CA); anti-COXIV (Molecular Probes, Eugene, OR); anti-CPT1 (Alpha Diagnostics San Antonio, TX); anti-CD36, anti-FATP1, and anti-FATP4 (Santa Cruz Biotechnology, Santa Cruz, CA), and FABPpm antisera (gift from Dr. J. Calles-Escandon (Wake Forest University)). Serum fatty acids were determined using a commercially available kit (Wako Diagnostics, Richmond, VA). All other reagents were obtained from Sigma.

Animals

Wild type (WT) and CD36 knock-out mice (KO) were kindly provided by Dr. M. Febbraio (Cleveland Clinic, Cleveland, OH). Mice were bred on site and were housed in controlled facilities (temperature 20 °C, 40% humidity, 12-h reverse light-dark cycle) and provided with water and chow *ad libitum*. Age-matched 12–18-week-old WT and CD36 KO mice were used in the present experiments. All procedures were approved by the University of Guelph animal care committee.

Characteristics of WT and CD36 KO Muscles

For descriptive purposes, we examined in WT and CD36 KO mice the following: circulating glucose and fatty acids, glucose tolerance, muscle fatty acid transporters, and muscle mitochondrial content and mitochondrial proteins involved in lipid utilization. Functional measurements of lipid utilization included determinations of fatty acid transport into giant vesicles, intramuscular lipid content, and fatty acid oxidation by perfused hindquarter muscles. Assessment of carbohydrate metabolism included determinations of basal and contraction- and insulin-stimulated glucose transport and hepatic and muscle glycogen.

Fatty Acids and Glucose Tolerance—Circulating fatty acids were measured with a commercially available kit (Wako Diagnostics), as reported previously (23). Glucose tolerance was determined via an intraperitoneal injection of glucose (1.0 g/kg body weight), and blood glucose was monitored at 0, 15, 30, 45, and 90 min using a glucose analyzer.

Fatty Acid Transporters, Mitochondrial Density, and Mitochondrial Proteins—Western blotting procedures were used to analyze proteins, in muscles harvested from anesthetized mice (sodium pentobarbital, Ceva (intraperitoneal 6.5 mg/100 g weight)). Muscle fatty acid transporters (CD36, FABPpm, FATP1, and FATP4), CPT1, and COXIV were determined from muscle lysates using Western blotting procedures and using

Ponceau staining to check for equal protein loading, procedures that we have previously reported (27, 28, 34). Citrate synthase and β-HAD activities and mtDNA were measured using standard procedures as we have reported previously (23, 33, 34).

Fatty Acid Transport, Esterification and Oxidation, and Intramuscular Lipids—The rates of palmitate transport were determined in giant sarcolemmal vesicles derived from hindlimb muscles. Because tissue requirements for transport studies are high, we pooled hindlimb muscles from 3 to 4 mice for each determination. Measurements were performed in five independently obtained pools of muscles. Giant sarcolemmal vesicles were obtained as we have described previously in detail (24, 27, 28, 36).

Palmitate oxidation and triacylglycerol synthesis were determined in perfused hindlimb muscles (Krebs-Henseleit buffer, 95% O_2 and 5% CO_2, pH 7.4, 37 °C, 8 mM glucose, 0.5 mM palmitate, [1-^{14}C]palmitate, 4% bovine serum albumin, flow rate 3 ml/min), as we have described previously in detail (23).

Intramuscular lipids (tri- and diacylglycerol and ceramide) were extracted from freeze-dried muscle using a modified Folch procedure and analyzed using gas-liquid chromatography (Hewlett-Packard 5890 Series II, Houston, TX) and flame-ionization detector (Agilent Technologies, Santa Clara, CA) as we have reported previously (33). For these purposes, it was necessary to pool 16 respective muscles for each independent determination.

Muscle and Hepatic Glycogen and Muscle Glucose Oxidation and Transport—We determined both pro- and macroglycogen depots in muscle and liver using analytic procedures described elsewhere (37, 38). Total glycogen was the sum of these two pools (37, 38).

In perfused hindlimb muscles, basal and insulin-stimulated (20 milliunits/ml) glucose (8 mM) oxidation rates were measured under euglycemic clamp conditions in perfused hindlimb muscles, in a similar manner to fatty acid oxidation, as we have described above and elsewhere (23). Similarly, basal, submaximal (150 micro-IU/ml) and maximal (20 milliunits/ml) insulin-stimulated rates of 3-*O*-[^{3}H]methylglucose (8 mM) transport were also examined under euglycemic clamp conditions in perfused hindlimb muscles as we have described previously (39, 40). We also examined, in a similar manner, 3-*O*-[^{3}H]methylglucose (8 mM) transport into contracting muscles, induced by sciatic nerve stimulation using repeated tetanic contractions. At the end of the perfusion experiments, muscles were excised, trimmed of connective tissue, and frozen in liquid N_2 for later determination of glucose transport rates.

Exercise Performance and Energy Expenditure, Fatty Acid Transport, and Substrate Utilization during Exercise

Treadmill exercise, at the same relative intensity (%VO_{2max}) in WT and CD36 KO mice was used as a physiologic stimulus to provide a metabolic challenge. Nonexercised WT and CD36 KO mice served as control.

Prior to the exercise studies, all WT and CD36 KO mice were familiarized with treadmill running over a 2-week period, including control mice. After this familiarization period, mice were randomly assigned to one of two groups, exercise or control (no exercise). In subgroups of WT and CD36 KO mice, we assessed lipid and carbohydrate metabolism from RER measurements, muscle and hepatic glycogen utilization during exercise, and the exercise-induced changes in fatty acid transport and plasma membrane fatty acid transporters. We compared aerobic exercise abilities from a timed treadmill run.

Maximal and Submaximal VO_2 and RER—Oxygen consumption was obtained during maximal and submaximal exercise using an enclosed treadmill system attached to a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH), as has been described elsewhere (8, 9). Peak oxygen consumption (VO_{2peak}) was determined using a progressively increased treadmill running workload. Submaximal oxygen consumption (VO_2) and RER were determined during treadmill running at 17 m/min, 0% grade. The relative intensity of exercise (%VO_{2peak}) was determined from these two measurements.

Aerobic Exercise Ability—The ability to perform aerobic exercise by WT and CD36 KO mice was determined from treadmill running (17 m/min, 0% grade) until the animals were fatigued. Criteria for volitional fatigue were those used previously by Spiegelman and co-workers for exercised PGC-1α transgenic (9) and KO mice (8).

Exercise Substrate Utilization during Fatiguing Exercise—At the end of exercise to volitional fatigue, mice were anesthetized, and muscle and liver samples were frozen (−80 °C) for later analyses of muscle and hepatic pro- and macro-glycogen depots (37, 38). Intramuscular triacylglycerol was determined as described above. Muscle samples were also analyzed, via Western blotting, for muscle AMPK and ACC and their phosphorylation states. Cardiac puncture was used to obtain blood samples for analyses of serum fatty acids (Wako Diagnostics, Richmond, VA). Nonexercised WT and CD36 KO mice were used as control.

Exercise-induced Fatty Acid Transport and Plasma Membrane Fatty Acid Transporters—To determine the effects of exercise on rates of fatty acid transport and plasma membrane fatty acid transporters, subgroups of WT and CD36 KO mice were each exercised for 45 min at 17 m/min, 0% grade. Plasma membrane fatty acid transporters and rates of fatty acid transport were determined immediately before and after exercise in giant sarcolemmal vesicles as we have described previously in detail (24, 28). To obtain sufficient tissue for these purposes, we pooled exercised lower hindlimb muscles of 3–4 mice for each of five independent experimental determinations.

Exercise Training-induced Adaptation of Fatty Acid Oxidation

To examine whether CD36 is required to observe metabolic adaptation in fatty acid oxidation, we trained WT and CD36 KO mice via treadmill running. We examined the training-induced effects on indices of mitochondrial biogenesis, fatty acid transport proteins, signaling proteins involved in fatty acid oxidation, and enzymes involved in mitochondrial fatty acid transport and oxidation. As well, we examined in isolated muscles the fatty acid oxidation under basal conditions and during a metabolic challenge.

Treadmill Training—At 8 weeks of age, mice were adapted to run on a treadmill. Thereafter, mice were randomly assigned to a control group (sedentary, no exercise) or to an exercise train-

ing group (5 days/week for 6 weeks). Our foregoing experiments revealed that although aerobic capacity in WT and CD36 KO mice were similar, the CD36 KO mice had a reduced ability for endurance running; therefore, we were careful to train both KO and WT mice at the same treadmill speed and for the same running time during each exercise session. The training was progressively increased over 4 weeks from 15 m/min, 0% grade for 45 min in week 1, to 21 m/min, 12.5% grade for 45 min at the end of week 4; conditions that were then maintained for weeks 5 and 6.

Training-induced Adaptation in Mitochondrial Biogenesis and Proteins of Fatty Acid Transport and Utilization—To assess muscle adaptation to exercise training, we compared in sedentary and trained WT and CD36 KO mice markers conventionally used for these purposes, including muscle mtDNA, COXIV via Western blotting, and the activity of citrate synthase (CS) (4, 33, 34, 41, 42).

Proteins involved in fatty acid transport (CD36, FABPpm, FATP1, and FATP4) and signaling of fatty acid oxidation (ACC, pACC, AMPK, and pAMPK) were measured using standard Western blotting procedures, in which protein loading was verified with Ponceau staining, as reported previously (24, 33, 34, 43). The activity of a marker enzyme of fatty acid oxidation, β-HAD activity, was measured using standard procedures (44). An index of mitochondrial fatty acid entry was provided by CPT-1 mRNA. This was measured in muscle homogenized in TRIzol (Invitrogen) from which RNA was isolated using RNeasy mini kit (Qiagen). Primers used to detect CPT1 mRNA (GenBank™ accession number NM-013495) were CPT1a-forward 5′-CCAGGCTACAGTGGGACATT-3′ and CPT1a-reverse 5′-AAGGAATGCAGGTCCACATC-3′. Real time-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG with a succinimidyl ester of carboxy-X-rhodamine (ROX) (Invitrogen) in an ABI 7500 thermocycler (Applied Biosystems).

Caffeine-stimulated Fatty Acid Transport and Oxidation in Skeletal Muscles and Mitochondrial Respiration—Recently, caffeine (3 mM) has been used to stimulate fatty acid oxidation in skeletal muscle (45). Therefore, as a prior step, we first assessed the metabolic effects of caffeine (3 mM) in subgroups of untrained WT and CD36 KO mice on whole muscle fatty acid oxidation (24, 43, 46), fatty acid transport into giant vesicles, plasma membrane CD36 and FABPpm (24, 27, 28), and mitochondrial state IV and III respiration in permeabilized muscles fibers (47). For whole muscle fatty acid oxidation, muscles were incubated, without or with caffeine (3 mM (45)) in pre-gassed (95% O_2, 5% CO_2) medium 199 (30 °C, pH 7.4) containing palmitate (1.0 μCi of [1-^{14}C]palmitate, 0.5 mM) complexed to 4% BSA (24, 43, 46). All of these procedures are routinely performed in our laboratory (23, 24, 27, 28, 33, 34, 43). Giant vesicles, fatty acid transport, and plasma membrane fatty acid transporters were determined as described above and elsewhere (24, 28).

For determination of mitochondrial respiration in permeabilized muscle fibers, we incubated soleus muscles from WT and CD36 KO mice in the presence or absence of 3 mM caffeine. Saponin-permeabilized fibers were prepared based on methods published previously (47, 48). Mitochondrial respiration was measured by high resolution respirometry (Oroboros Oxygraph-2k, Innsbruck, Austria) at 37 °C (room air saturation) and in the presence of 25 μM blebbistatin, a myosin ATPase inhibitor, as reported previously (49). Palmitate-supported respiration (1 mM ATP, 2 mM malate, 2 mM L-carnitine, 1 mM CoA, and 250 μM palmitate), pyruvate-supported respiration (5 mM pyruvate and 2 mM malate), and pyruvate (5 mM pyruvate and 2 mM malate) + succinate (10 mM)-supported respiration were determined in the absence (state IV) and presence (state III) of 5 mM ADP and in the absence or presence of 3 mM caffeine. Maximal electron transport chain activity was determined by the titration with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1 μM).

Adaptation of Fatty Acid Oxidation in Trained Muscle—To assess the adaptation in fatty acid oxidation after a period of exercise training, we compared in sedentary and exercise-trained WT and KO mice the rates of fatty acid oxidation under basal and stimulating conditions (caffeine, 3 mM) (45) in two types of isolated muscles, namely the slow-twitch oxidative soleus muscle and the fast twitch, oxidative-glycolytic extensor digitorum longus muscle, as we have described in detail previously (24, 43, 46). To avoid a possible influence of the last training session on muscle metabolism, these fatty acid oxidation studies in isolated muscles were performed 60 h after the last exercise training bout.

Overexpression of CD36 in Soleus Muscle—To examine the effects of CD36 on fatty acid muscle oxidation. we used an electrotransfection procedure to overexpress CD36 in soleus muscle, as we have reported in detail previously (24). Briefly, the cDNA for CD36 (a gift from Dr. N. Abumrad, Washington University School of Medicine, St Louis, MO) was used as a template to generate the expression plasmid. cDNAs were subcloned into the expression vector pcDNA3.1$^+$ for overexpression. Large scale stocks of the expression plasmids were isolated using the plasmid giga Kit (Machery-Nagel, MJS Biolynx Inc, Brockville, Ontario, Canada) following the manufacturer's instructions, as we have recently reported (24, 33, 34, 50). DNA concentration and integrity were verified by spectrophotometry and gel visualization of restriction digests.

While under anesthesia, the soleus muscle was exposed, and we injected 250 μg of plasmid DNA, dissolved in 200 μl of half-strength normal saline solution (0.45% NaCl). Empty pcDNA 3.1$^+$ plasmid was transfected into the contralateral muscle. Electrotransfection of the intact muscle *in vivo* was performed immediately thereafter (*i.e.* using Tweezertrodes (8 pulses, 200 V/cm, 1 Hz, 20 ms duration (ECM 830 Square Wave Electroporator; BTX, Harvard Apparatus, Holliston, MA) (24, 33, 34, 50). The overlying superficial muscle was then sutured, and the skin incision was closed, and animals were provided with analgesic (Temgesic). After 2 weeks, we determined the effect of CD36 overexpression on signaling protein (AMPK, pAMPK, ACC, and pACC)-selected enzymes involved in fatty acid oxidation (CPT1 and β-HAD) and markers of mitochondrial density (mtDNA and COXIV) (see above), as well as the rates of fatty acid oxidation in isolated soleus muscles under basal and stimulating conditions (3 mM caffeine).

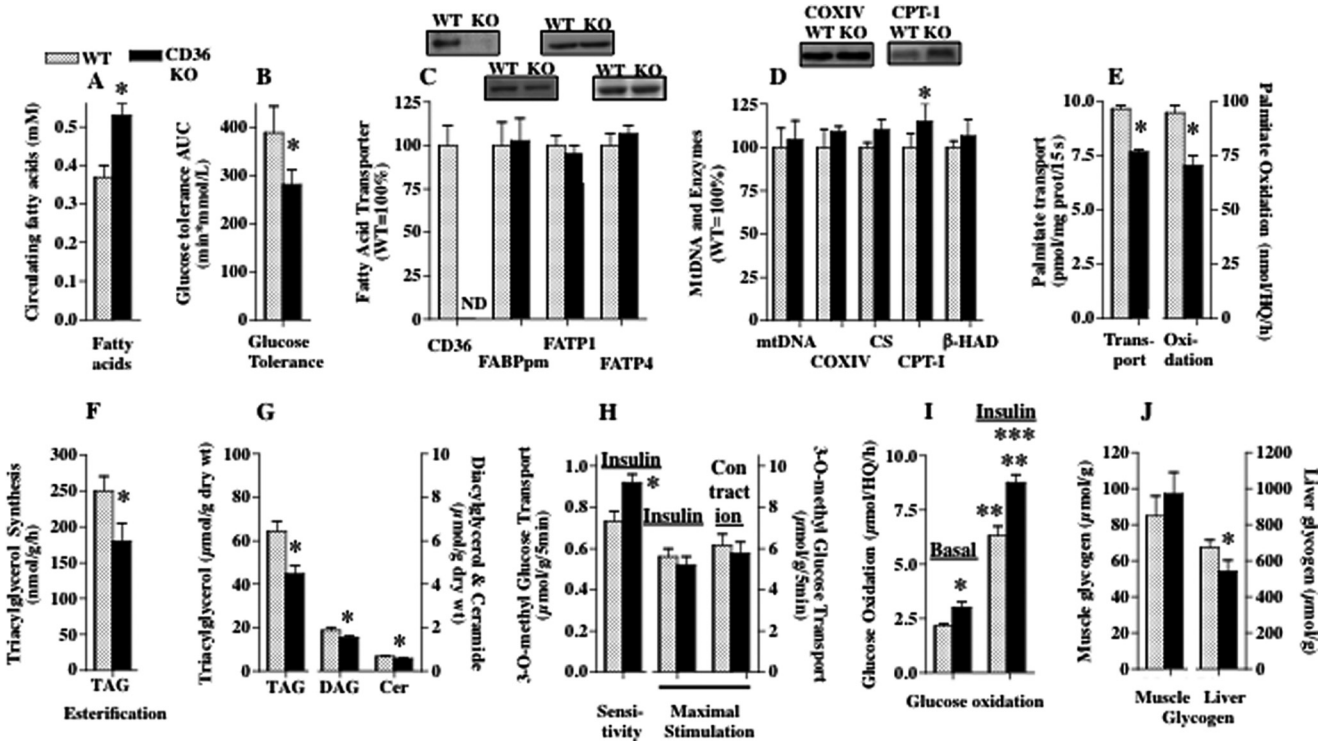

FIGURE 1. **Selected descriptive parameters in WT and CD36 KO mice.** *A,* circulating fatty acids. *B,* whole body glucose tolerance. *C,* muscle fatty acid transporters. *D,* muscle mitochondrial DNA and selected enzymes (COXIV and CPT-1 proteins; CS, and β-HAD activities). *E,* basal rates of palmitate transport into giant sarcolemmal vesicles and palmitate oxidation by perfused hindquarters. *F,* basal rates of muscle triacylglycerol (*TAG*) esterification. *G,* intramuscular lipid content (triacylglycerol (*TAG*), diacylglycerol (*DAG*), and ceramide (*Cer*)). *H,* insulin-stimulated glucose transport into muscle with submaximal (150 microunits/ml) and maximal insulin (20 milliunits/ml) concentrations, and maximal contraction-stimulated glucose transport (note the 10-fold differences in scale on *left* and *right ordinates*). *I,* basal and insulin-stimulated glucose oxidation in perfused hindquarters. *J,* total muscle and hepatic glycogen (total glycogen = proglycogen + macroglycogen) (note the differences in scale for muscle and hepatic glycogen). These various measurements are described under "Experimental Procedures." Means ± S.E. $n = 5–7$ independent determinations for each parameter, except fatty acid transport and intramuscular lipid content. For intramuscular lipid content determinations, 16 red gastrocnemius muscles were pooled for each of five independent measurements. For fatty acid transport determinations into giant sarcolemmal vesicles, lower hindlimb muscles from three animals were pooled for each determination. This was repeated for each of five independently determined experiments. For Western blotting, equal quantities of protein were loaded and confirmed with Ponceau staining. WT and KO muscles were loaded and analyzed on the same gels. Representative pairs of WT and KO Western blot data are shown in *C* and *D*. *, $p < 0.05$, CD36 KO *versus* WT. **, $p < 0.05$, insulin-stimulated *versus* basal in each of WT and FAT/CD36 KO. ***, $p < 0.05$, insulin-stimulated FAT/CD36 KO *versus* insulin-stimulated WT. *ND,* not detected.

Statistics—The data were analyzed using repeated measures analyses of variance or *t* tests, when appropriate. All data are reported as mean ± S.E.

RESULTS

Characterization of Skeletal Muscles in WT and CD36 KO Mice

Mice of the same age (12–18 weeks and the same weight (WT, 28.0 ± 0.6 g; CD36 KO, 28.3 ± 0.8 g) were used in this study. Although circulating glucose concentrations did not differ in WT and CD36 KO mice (data not shown and see Ref. 23), the concentrations of circulating fatty acids were higher in the CD36 KO mice (Fig. 1*A*) (23, 51). These KO mice also exhibited a better whole body glucose tolerance (Fig. 1*B*).

In muscles of WT and CD36 KO mice, we compared selected proteins, intramuscular substrate depots, hepatic glycogen, insulin sensitivity, muscle oxygen consumption, fatty acid oxidation, and mitochondrial content. The quantitative differences between WT and CD36 KO muscles were most evident in oxidative muscles (red gastrocnemius and soleus *versus* white gastrocnemius (data not shown)) in which fatty acids are primarily utilized. Therefore, the WT and CD36 KO muscle phenotypes are presented for oxidative muscles (red gastrocnemius, Fig. 1, *C, D, F, G,* and *H*), except where it was necessary to pool mixed hindlimb muscles to obtain sufficient giant sarcolemmal vesicles for fatty acid transport determinations (Fig. 1*E*, *left side*), and when fatty acid oxidation (Fig. 1*E*, *right side*) and glucose oxidation (Fig. 1*I*) were determined across a perfused hindlimb mixed muscle bed.

Protein Content in Muscle—In CD36 KO mice, the muscle content of other fatty acid transporters was unaltered (Fig. 1*C*), whereas the mitochondrial CPT-I was increased slightly (+18%, Fig. 1*D*). No differences were observed between WT and CD36 KO mice in markers of muscle mitochondrial content (mtDNA, CS, and COXIV) or fatty acid oxidation (β-HAD) (Fig. 1*D*).

Fatty Acid Transport, Esterification, and Oxidation—To determine the metabolic effects of CD36 ablation, we examined fatty acid transport and oxidation and intramuscular lipid storage. Ablation of CD36 impaired the basal rates of fatty acid transport (−21%, Fig. 1*E*, *left side*), fatty acid oxidation (−25%) (Fig. 1*E*, *right side*), and triacylglycerol esterification rate (−30%) (Fig. 1*F*). Intramuscular triacylglycerol (−31%), diacylglycerol (−18%), and ceramide (−15%) (Fig. 1*G*) content were also reduced in muscles of CD36 KO mice.

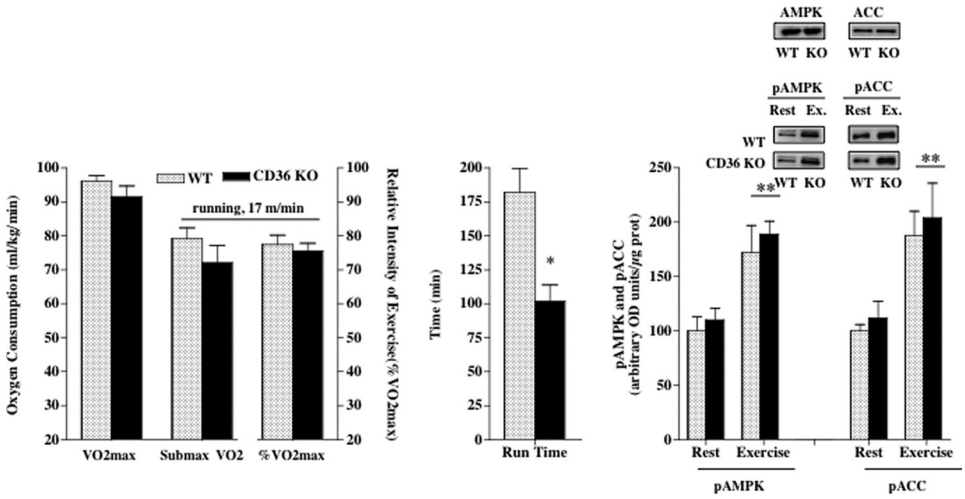

FIGURE 2. **Comparison of WT and CD36 KO mice with respect to maximal aerobic capacity (VO_{2max}) and submaximal VO_2 (submax VO_2) when running at 17 m/min, the relative intensity of submaximal exercise (%VO_{2max}) (*A*), exercise endurance to volitional fatigue (*B*), and muscle AMPK and pAMPK and ACC and pACC before and after exercise to volitional fatigue (*C*).** These various measurements are described under "Experimental Procedures." Mean ± S.E. $n = 5–7$ independent determinations for each parameter. For Western blotting in *C*, equal quantities of protein were loaded and confirmed with Ponceau staining. For each of AMPK, pAMPK, and pACC, WT and KO muscles were loaded and analyzed on the same gels. Separation of the pAMPK and pACC blots in *C* was done for clarity of the presentations to accompany the *bar graphs*. *, $p < 0.05$, CD36 KO *versus* WT. **, $p < 0.05$, exercise (*Ex.*) *versus* rest in both WT and KO mice.

Muscle Glucose Transport and Oxidation and Muscle and Hepatic Glycogen—In view of the reductions in intramuscular lipids and fatty acid oxidation in CD36 KO mice, it was of interest to determine whether muscle carbohydrate metabolism was concomitantly increased. For this purpose, muscle glucose transport and oxidation and intramuscular and hepatic glycogen depots were examined in WT and CD36 KO mice. As reported elsewhere (52), insulin-stimulated (150 microunits/ml) glucose transport into muscle was 26% greater in CD36 KO mice (Fig. 1*H*). However, no differences between genotypes were observed in maximal insulin-stimulated (20 milliunits/ml) or muscle contraction-induced glucose transport (Fig. 1*H*). Basal and insulin-stimulated glucose oxidation rates were also greater in CD36 KO mice (Fig. 1*I*). Muscle glycogen did not differ between WT and CD36 KO mice (Fig. 1*J*, *left side*), but hepatic glycogen was reduced (−20%) in CD36 KO mice (Fig. 1*J*, *right side*). Taken together, these observations suggest that under basal and insulin-stimulating conditions the CD36 KO animals are more reliant on glucose oxidation to meet their energy requirements.

Reduced Fatty Acid Utilization, Accelerated Glycogen Depletion, and Reduced Performance in Acutely Exercising CD36 KO Mice

Because neither the cage activity, nor resting whole body VO_2, nor VO_2 in an isolated muscle differed between WT and CD36 KO mice (data not shown), we compared the metabolic responses of WT and CD36 KO mice during a metabolic challenge, namely treadmill exercise. In particular, we speculated that the impaired fatty acid transport and oxidation rates observed under basal conditions would likely be further impaired in CD36 KO mice, resulting in larger differences in fuel selection between WT and CD36 KO mice, as well as possibly limiting the exercise performance of CD36 KO mice due to a more rapid depletion of hepatic and/or muscle glycogen depots.

Maximal and Submaximal VO_2, Exercise Intensity, and Endurance Performance—To compare exercise performance and fuel utilization in WT and CD36 KO mice, it is essential to exercise mice at the same relative intensity of exercise. The maximal oxygen consumption (VO_{2max}), a measure of aerobic endurance ability, was similar in the WT and CD36 KO mice (Fig. 2*A*), as was their submaximal oxygen consumption when running at 17 m/min (Fig. 2*A*). Therefore, the relative intensity of exercise (78% VO_{2max}) for treadmill running (17 m/min, 0% grade) did not differ between WT and CD36 KO mice (Fig. 2*A*). However, the ability to sustain exercise at this submaximal running speed was greatly reduced (−44%) in the CD36 KO mice (Fig. 2*B*). This was not attributable to differences in AMPK and ACC proteins (Fig. 2*C*) or their phosphorylations (Fig. 2*C*), which were similar in WT and CD36 KO mice at rest, and which were increased comparably by exercise (Fig. 2*C*).

Substrate Utilization during Exercise—At rest, the circulating fatty acid concentrations were higher in CD36 KO mice (Fig. 3*A*) (51, 53). As expected (54–56), during exercise circulating fatty acid availability to muscle was markedly increased in the WT mice (Fig. 3*A*). This effect was also observed in the CD36 KO mice (Fig. 3*A*). Although circulating fatty acids at the end of exercise were comparable in WT and CD36 KO mice (Fig. 3*A*, $p > 0.05$), the exercise RER was greater in the CD36 KO mice ($p < 0.05$, Fig. 3*B*), indicating that during exercise the CD36 KO mice relied more on carbohydrate metabolism and less on lipid metabolism than the WT mice. Specifically, during exercise, the WT mice derived 71 and 29% of their energy from lipids and carbohydrates, respectively (Fig. 3*C*). In contrast, in CD36 KO mice, exercised at the same relative intensity, carbohydrate use was almost doubled, as the CD36 KO mice derived 55% of their energy from carbohydrates and 45% from lipids (Fig. 3*C*). Thus, despite similar concentrations of circulating fatty acids during exercise and similar muscle mitochondrial content and enzymes in WT and CD36 KO mice, lipid oxida-

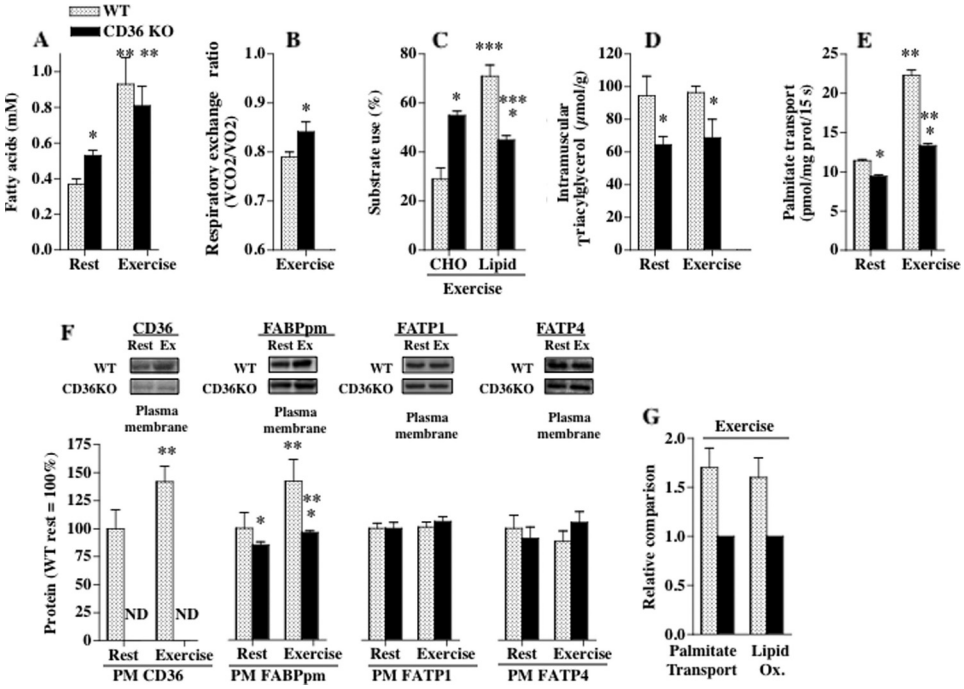

FIGURE 3. **Comparison of selected parameters at rest and in acutely exercised WT and CD36 KO mice.** *A*, circulating fatty acids. *B*, respiratory exchange ratio during exercise (17 m/min, 0% grade). *C*, relative (%) whole body carbohydrate and lipid utilization during exercise. *D*, intramuscular triacylglycerol. *E*, palmitate transport into giant vesicles. *F*, fatty acid transporters at the plasma membrane (PM). *G*, relative comparison of exercise-induced palmitate transport and whole body lipid oxidation (*ox.*) (CD36 KO is set to 1, WT expressed relative to CK36 KO). These various measurements are described under "Experimental Procedures." Mean ± S.E. $n = 5–7$ independent determinations for each parameter. For fatty acid transport determinations into giant sarcolemmal vesicles, lower hindlimb muscles from three animals were pooled to obtain sufficient tissue. This was repeated for each of five independently determined experiments. For Western blotting, equal quantities of protein were loaded and confirmed with Ponceau staining. Western blots of muscle plasma membranes at rest and at the end of exercise are shown for WT and CD36 KO muscles. Separation of the WT and CD36 KO blots indicates that these were run on different gels as the primary focus was to compare the effects of rest *versus* exercise on plasma membrane fatty acid transporters in each of the two groups (*F*). *ND,* not detected. Under resting conditions, the plasma membrane content of fatty acid transporters, when analyzed on the same gel, are comparable in WT (100%) and CD36 KO mice (FATP1, 98 ± 2%; FATP4, 100 ± 7%; FABPpm, 88 ± 8% ($n = 4$ for each determination)). Under conditions of acute exercise, total muscle content of CD36 is not altered (data not shown). The monocarboxylate transporter MCT1, which is present only at the plasma membrane (74), was not altered by exercise (data not shown). *, $p < 0.05$, CD36 KO *versus* WT. **, $p < 0.05$, exercise *versus* rest in each of WT and CD36 KO. ***, $p < 0.05$, substrate use: % CHO *versus* % lipid usage in each of WT and CD36 KO mice.

tion during exercise was 1.6-fold greater in WT mice compared with CD36 KO mice.

Fatty Acid Transport and Oxidation and Intramuscular Triacylglycerol during Exercise—In agreement with exercise studies in other models of altered lipid metabolism (55, 56), intramuscular triacylglycerol content was unchanged in both WT and CD36 KO animals during exercise (Fig. 3*D*), indicating that the exercising CD36 KO animals were not more reliant on these intramuscular lipids.

Although exercise increased fatty acid transport into muscle in WT and CD36 KO mice (Fig. 3*E*), the net increase (Δ = exercise-basal) in the exercise-induced rate of fatty acid transport was considerably greater in WT than in CD36 KO mice. Specifically, at the end of exercise, the net increase in the fatty acid transport rate in exercised WT mice was 1.7-fold greater than in the acutely exercised CD36 KO mice ($p < 0.05$, Fig. 3*E*). Similarly, when muscle contraction was performed under highly controlled conditions (electrically stimulated muscle contraction), we also found that fatty acid transport into WT muscle was 1.7-fold greater than in CD36 KO muscle (data not shown). These latter experiments confirm that the exercise-induced increase in the fatty acid transport rate was attributable to muscle contractile activity.

In WT mice, acute exercise did not alter either the total muscle content of CD36 nor other fatty acid transporters (data not shown). However, the exercise-induced up-regulation of fatty acid transport in WT mice was attributable to increases in plasma membrane CD36 (+42%) and FABPpm (+42%) (Fig. 3*F*). No exercise-induced changes were observed in plasma membrane FATP1 and -4 in WT mice (Fig. 3*F*).

The slight increase in muscle fatty acid transport in CD36 KO mice is possibly attributable to the small exercise-induced increase in plasma membrane FABPpm (10%, Fig. 3*F*). This transporter can by itself increase fatty acid transport (24, 57). Possible increases in plasma membrane FATP1 and FATP4, which might have compensated for the ablation of CD36 in the KO mice, were not observed (Fig. 3*F*).

Comparing Fatty Acid Transport and Fatty Acid Oxidation—Because intramuscular triacylglycerol appeared not to serve as a lipid source for muscle fatty acid oxidation during exercise in WT and CD36 KO mice (Fig. 3*D*), it appears that the relative differences in fatty acid transport during exercise (WT/CD36 KO ratio = 1.7:1) largely accounted for the relative differences in fatty acid oxidation determined from RER measurements during exercise (WT/CD36 KO ratio = 1.6:1) (Fig. 3*G*).

Muscle and Hepatic Glycogen Utilization during Exercise—Muscle and hepatic glycogen are well known to be key energy substrates for exercising muscle. Depletion of hepatic and muscle glycogen has been linked with the inability to sustain endurance exercise (17), although exercise-induced glycogen deple-

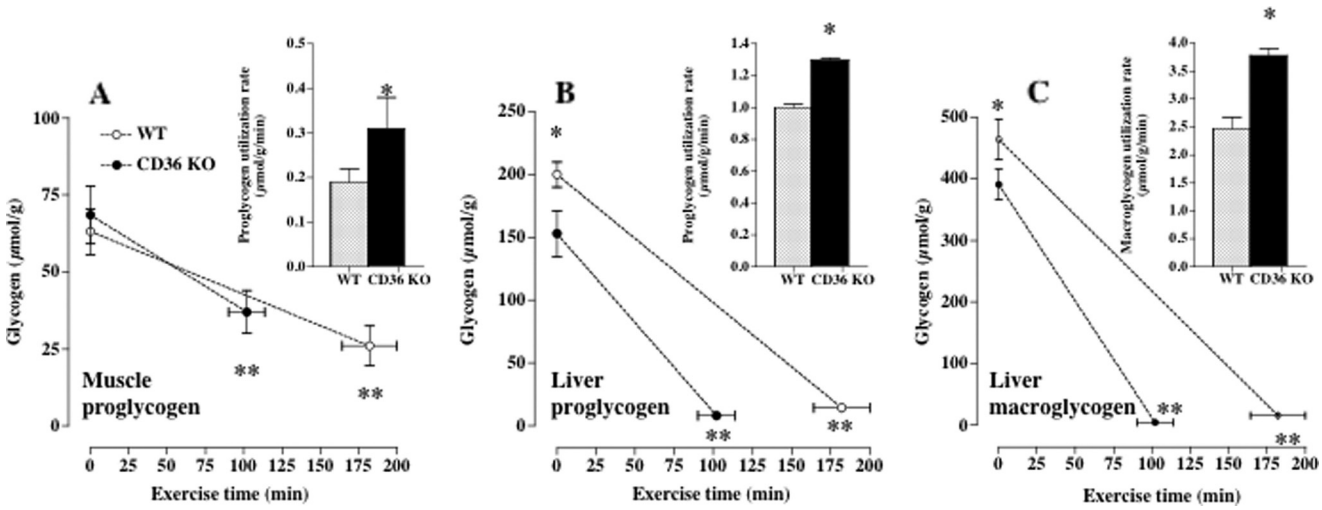

FIGURE 4. **Muscle proglycogen and hepatic pro- and macro-glycogen utilization during exercise to volitional cessation in WT and CD36 KO mice and rates of glycogen use during exercise (*insets*).** Measurements are described under "Experimental Procedures." Mean ± S.E. $n = 4–5$ independent determinations for each parameter. *, $p < 0.05$, CD36 KO *versus* WT. **, $p < 0.05$, exercise *versus* rest, in each of WT and CD36 KO.

tion is 5–10-fold greater in liver compared with skeletal muscle (Fig. 4) (17, 55).

In WT and CD36 KO mice, skeletal muscle proglycogen depots in muscle were reduced comparably in both WT (−37%) and CD36 KO mice (−32%) ($p < 0.05$, Fig. 4*A*), although muscle macroglycogen concentrations were not altered during exercise (data not shown). As expected (17), at the end of exercise the hepatic proglycogen (≥93% reduction, Fig. 4*B*) and macroglycogen (≥97% reduction, Fig. 4*C*) depots were almost fully depleted in both WT and CD36 KO mice. There were no differences between WT and CD36 KO mice in the hepatic glycogen concentrations at the end of exercise ($p > 0.05$, Fig. 4, *B* and *C*).

The absolute reductions in muscle and hepatic glycogen occurred more rapidly in CD36 KO mice (Fig. 4). Specifically, in the CD36 KO animals the utilization rates of muscle proglycogen (+55%, Fig. 4*A*, *inset*) and hepatic proglycogen (+27%, Fig. 4*B, inset*) and macroglycogen (+53%, Fig. 4*C, inset*) were considerably greater than in the WT mice. These data support the RER observed during exercise, which showed that the CD36 KO mice were almost twice as reliant on carbohydrate metabolism (55%) compared with WT mice (29%) (Fig. 3*C*).

Normal Training-induced Mitochondrial Biogenesis and Enzymatic Adaptation but Impaired Adaptation of Fatty Acid Oxidation in CD36 KO Mice

An increase in fatty acid oxidation has long been known to be a key metabolic adaptation to endurance exercise training. For many years, this has been attributed to an increase in muscle mitochondrial content and enzymes involved in fatty acid oxidation (*cf.* Refs. 3, 4). An underlying assumption for this explanation is that the blood-borne fatty acid provision for mitochondrial oxidation is more than adequate at rest and during a metabolic challenge such as exercise. However, the present acute exercise studies suggest that the regulation of mitochondrial fatty acid oxidation is highly dependent on CD36-mediated fatty acid transport into muscle. Therefore, we examined whether the normal training-induced adaptation in skeletal muscle fatty acid oxidation, such as occurs in WT animals, was impaired in CD36 KO mice. For these purposes, we exercise-trained both WT and CD36 KO mice.

Training-induced Mitochondrial Biogenesis, Selected Signaling and Metabolic Proteins, and Fatty Acid Transporters—The CD36 KO mice had an impaired exercise tolerance (Fig. 2*B*). Therefore, the exercise training program was carefully matched in the CD36 KO and WT mice for duration of running and the running speeds at each training session.

As expected, the exercise training program provoked changes in mitochondrial biogenesis and enzymes, consistent with changes observed with exercise training programs (58, 59). Specifically, in muscles of WT and CD36 KO mice, there were similar training-induced increases in widely used markers of mitochondrial biogenesis, including mtDNA (21–27%), citrate synthase (10–14%), and COXIV (22–30%) (Fig. 5). Exercise training did not alter signaling proteins involved in fatty acid oxidation (AMPK, pAMPK, ACC, and pACC) nor CPT-I expression in either WT or CD36 KO mice. However, β-HAD activity was increased comparably (~44%, $p > 0.05$, Fig. 5) in both WT and CD36 KO mice. At the level of fatty acid transporters, CD36 was increased (+44%, $p < 0.05$, Fig. 5) in muscles of WT mice, although in both WT and CD36 KO mice, only FABPpm was increased slightly (9–13%, $p < 0.05$), but FATP1 and -4 were not altered (Fig. 5).

Caffeine as a Stimulus to Assess Training-induced Changes in Fatty Acid Oxidation—To determine whether molecular changes induced by exercise training altered fatty acid oxidation, we examined palmitate oxidation in isolated skeletal muscles. This was done under basal conditions as well as with a metabolic challenge, as in isolated muscle metabolic rates are low, making experimental effects difficult to discern (60). Therefore, we used caffeine (3 mM), a well known metabolic stimulus, which in the low millimolar range (3 mM) increases calcium release from the sarcoplasmic reticulum while not inducing muscle contraction (61). Caffeine has recently been used to examine the regulation of muscle

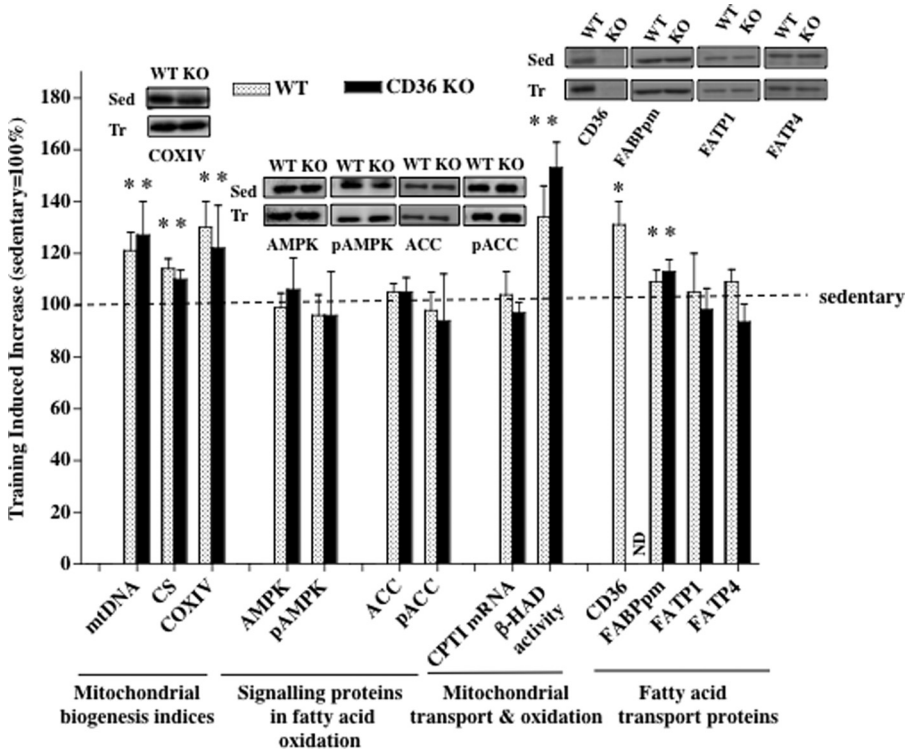

FIGURE 5. **Effects of 6 weeks of exercise training on indices of mitochondrial biogenesis, fatty acid oxidation signaling proteins, mitochondrial enzymes of fatty acid transport and oxidation, and fatty acid transport proteins in sedentary (*Sed*) and trained (*TR*) WT and CD36 KO mice.** Data are expressed relative (%) to mean of each parameter in respective control muscles of untrained WT and CD36 KO mice, as in sedentary animals none of the parameter differed (see Fig. 1) between WT and CD36 KO mice ($p > 0.05$). The various measurements are described under "Experimental Procedures." Mean ± S.E. *ND,* not detected. $n = 5$–7 independent determinations for each parameter. For Western blotting, equal quantities of protein were loaded and confirmed with Ponceau staining. Western blots for sedentary and trained muscles in WT and CD36 KO muscles were run on the same gels. Separation of blots for the sedentary conditions and training conditions was done for clarity of the presentation to accompany the *bar graphs*. *, $p < 0.05$, trained WT or CD36 KO muscle relative to respective sedentary muscle.

substrate utilization (45, 62, 63), including fatty acid oxidation (45, 64).

To determine the means by which caffeine alters skeletal muscle fatty acid oxidation, we examined the effects of caffeine on selected parameters in sedentary WT and CD36 KO muscles, including fatty acid transport, sarcolemmal fatty acid transporters, whole muscle fatty acid oxidation, and mitochondrial fatty acid-supported respiration. Muscle oxygen consumption rates in isolated muscles were similar in WT and CD36 KO mice under basal conditions and when muscles were stimulated with caffeine (WT muscle +22% and CD36 KO muscle +27%, data not shown).

In sedentary WT animals, caffeine increased the following: (i) whole muscle fatty acid oxidation (+60%); (ii) plasma membrane CD36 (+37%), as well as other fatty acid transporters (FABPpm +26%, FATP1 + 49%, and FATP4 + 38%, data not shown), and (iii) fatty acid transport (+70%) (Fig. 6*A*). In contrast, in sedentary CD36 KO muscles, the caffeine-induced stimulation of fatty acid oxidation and transport was markedly blunted (Fig. 6*A*), despite caffeine-induced increases in the plasma membrane content of other fatty acid transporters (FABPpm +26%, FATP1 + 70%, and FATP4 + 12%, data not shown).

In permeabilized sedentary muscles, we examined the effects of caffeine on mitochondrial rates of respiration (state III and IV) (Fig. 6*A*). In the presence of pyruvate, pyruvate + succinate, or palmitate, caffeine did not alter either mitochondrial state IV respiration (data not shown) or state III respiration (Fig. 6*A*) or the respiratory control ratios (6.00 ± 0.27, across all experiments) in either WT or CD36 KO muscles. Collectively, the observations in sedentary muscles (Fig. 6*A*) have shown that the caffeine-stimulated increase in whole muscle fatty acid oxidation (Fig. 6*A*) (45, 59) is primarily attributable to the increased rate of fatty acid transport into skeletal muscle (Fig. 6*A*).

Training-induced Changes in Muscle Fatty Acid Oxidation—The basal and caffeine-stimulated rates of fatty acid oxidation in WT and CD36 KO mice were examined in sedentary and trained animals (6 weeks). These experiments were performed in two types of muscles, namely in a slow twitch highly oxidative muscle (soleus) and in a fast twitch muscle (extensor digitorum longus) that harbors a mixture of highly glycolytic and highly oxidative muscle fibers.

As expected (60), under basal conditions training-induced changes in the rates of fatty acid oxidation in isolated muscles, measured 60 h after the last exercise bout, were not evident either in WT or CD36 KO muscles (Fig. 6*B*). In contrast, training-induced differences were revealed when muscles were challenged metabolically with caffeine (3 mM). This compound stimulated muscle fatty acid oxidation ($p < 0.05$, Fig. 6*C*), but to very different extents depending on genotype and training status. Specifically, in WT mice, exercise training markedly increased the net caffeine-stimulated fatty acid oxidation rate (net Δ = caffeine − basal) in the trained WT muscles (soleus, +90%; extensor digitorum longus, +40%) (Fig. 6*D*). In contrast,

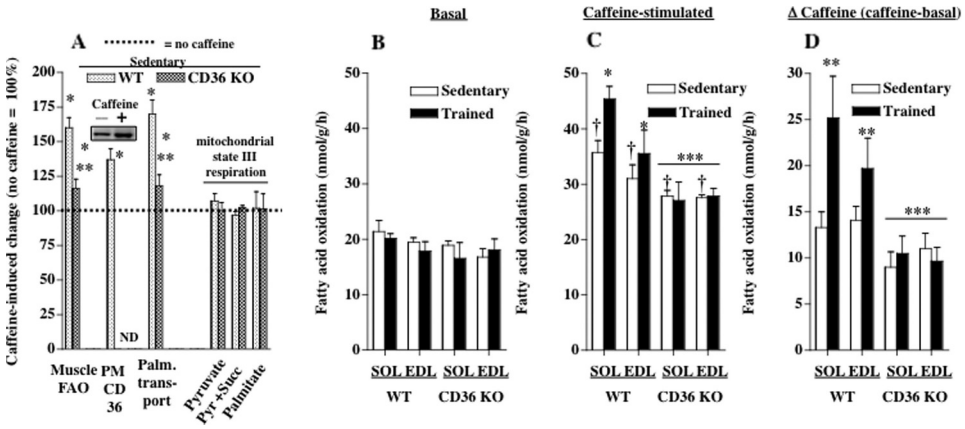

FIGURE 6. **Relative effects of a metabolic challenge (caffeine, 3 mM) on soleus muscle fatty acid oxidation, plasma membrane CD36, palmitate transport, and pyruvate-, pyruvate + succinate-, and fatty acid-stimulated state III rates of mitochondrial respiration in permeabilized soleus muscle fibers (*A*), and the effects of exercise training in WT and CD36 KO mice on basal (*B*), caffeine-stimulated (*C*), and net change (Δ = caffeine − basal) in fatty acid oxidation in intact soleus (*SOL*) and extensor digitorum longus (*EDL*) muscles (*D*).** These various measurements are described under "Experimental Procedures." Mean ± S.E. $n = 4–7$ independent determinations for each parameter. *A,* caffeine-stimulated data are shown relative (%) to treatments without caffeine (100%). The *dashed line* is the condition (set to 100%) in which no caffeine was provided. To obtain sufficient giant sarcolemmal vesicles for determinations of palmitate (*Palm.*) transport and plasma membrane (*PM*) CD36, lower hindlimb muscles from 3 to 4 animals were pooled for each of five independent experiments. This was repeated for each of five independently determined experiments. For Western blotting, equal quantities of protein were loaded and confirmed with Ponceau staining. Western blot of plasma membrane (*PM*) CD36 is only shown for WT, as CD36 is not present in CD36 KO mice. Blots for muscles treated with (+) or without (−) caffeine were run on the same gels. *Succ*, succinate; *Pyr*, pyruvate; *FAO*, fatty acid oxidation. *, $p < 0.05$, caffeine-treated muscle *versus* control (*no caffeine*). **, $p < 0.05$, caffeine-treated KO muscle *versus* caffeine-treated WT muscle. *B–D,* basal rates of muscle fatty acid oxidation are shown in *B*, and in *C* the caffeine-induced increase in fatty acid oxidation is shown. *D* represents the difference between data in *C* and *D* (*i.e.* Δ = caffeine-stimulated fatty acid oxidation − basal fatty acid oxidation). *C,* †, $p < 0.05$, caffeine-stimulated sedentary muscles in *C versus* respective basal sedentary muscles in *B*. *, $p < 0.05$, caffeine-stimulated trained muscle *versus* caffeine-stimulated sedentary muscle. *D,*** , $p < 0.05$, Δ caffeine-stimulated trained muscle *versus* Δ caffeine-stimulated sedentary muscle, where Δ is difference between basal and caffeine-stimulated conditions. *C* and *D,* ***, $p < 0.05$, CD36 KO *versus* WT (main effect, analysis of variance).

the net caffeine-stimulated rate of palmitate oxidation was not altered by exercise training in the CD36 KO mice (Fig. 6*D*), despite the comparable training-induced increases in mitochondrial biogenesis and β-HAD in muscles of WT and CD36-KO mice (Fig. 5).

CD36 Increases Fatty Acid Oxidation in Muscle without Altering Mitochondrial Content and Enzymes

In exercise-trained WT animals, muscle CD36, mitochondrial content and fatty acid oxidation enzymes, and fatty acid oxidation are all concomitantly increased. However, the training-induced increase in fatty acid oxidation rate in WT skeletal muscle appeared to be dependent on CD36. Therefore, we performed a gain of function study by transfecting CD36 into sedentary WT muscles. This enabled us to examine the independent role of CD36 on fatty acid oxidation in WT muscle, in the absence of concurrent changes in muscle mitochondrial content, and fatty acid oxidation enzymes.

Because CD36 was increased by 44% in trained WT muscle (Fig. 5), we overexpressed CD36 protein in sedentary WT muscle to the same level (+41%, Fig. 7*A*). The contralateral muscle in the same WT sedentary animals was transfected with empty vector, an experimental control that does not alter the expression of other proteins (data not shown and see Refs. 24, 57). In CD36-transfected sedentary WT muscles, no changes occurred in the other fatty acid transporters (FABPpm and FATP1 and -4), muscle mitochondrial content as measured by mtDNA and COXIV, or in selected enzymes (AMPK, ACC, β-HAD, and CPTI) involved in regulating fatty acid oxidation (Fig. 7*A*).

The basal rates of fatty acid oxidation in control and CD36-transfected muscles were not different ($p > 0.05$). Hence, we compared in sedentary WT muscles transfected with empty vector and with CD36 the effects of metabolic stimulation (caffeine, 3 mM) on the rates of fatty acid oxidation relative to these basal rates of fatty acid oxidation (Fig. 7*B*). Caffeine increased the rate of fatty acid oxidation (+49%) in control muscle (empty vector) ($p < 0.05$, Fig. 7*C*). In CD36-transfected muscle, the caffeine-stimulated rate of fatty acid oxidation was increased further (+ 84%, $p < 0.05$, Fig. 7*C*). These observations indicate that the level of muscle CD36 influences the rate of fatty acid oxidation.

Exercise Training and Genetic-induced Up-regulation of CD36 Increase Fatty Acid Oxidation Comparably

Because we controlled the CD36 overexpression in transfected muscle (+41%, Fig. 7) to the same levels that we observed with exercise training (+44%, Fig. 5), we were able to compare the effects of CD36 on fatty acid oxidation in the WT-trained muscles and in WT sedentary muscles transfected with CD36 (Fig. 8).

Interestingly, whether CD36 was increased with genetic overexpression or with exercise training, the net caffeine-stimulated increases in fatty acid oxidation (Δ = caffeine − basal) were comparable in the CD36-transfected sedentary WT soleus muscle (+84%) and in the exercise-trained WT soleus muscle (+90%) (Fig. 8). The up-regulation of fatty acid oxidation in WT-trained muscle was accompanied by concurrent changes in CD36, mitochondrial content, and enzymes involved in fatty acid oxidation (Fig. 8). In marked contrast, in the sedentary CD36-transfected WT muscles the increases in muscle fatty acid oxidation occurred independent of changes in mitochondrial content and enzymes involved in fatty acid oxidation (Fig. 8).

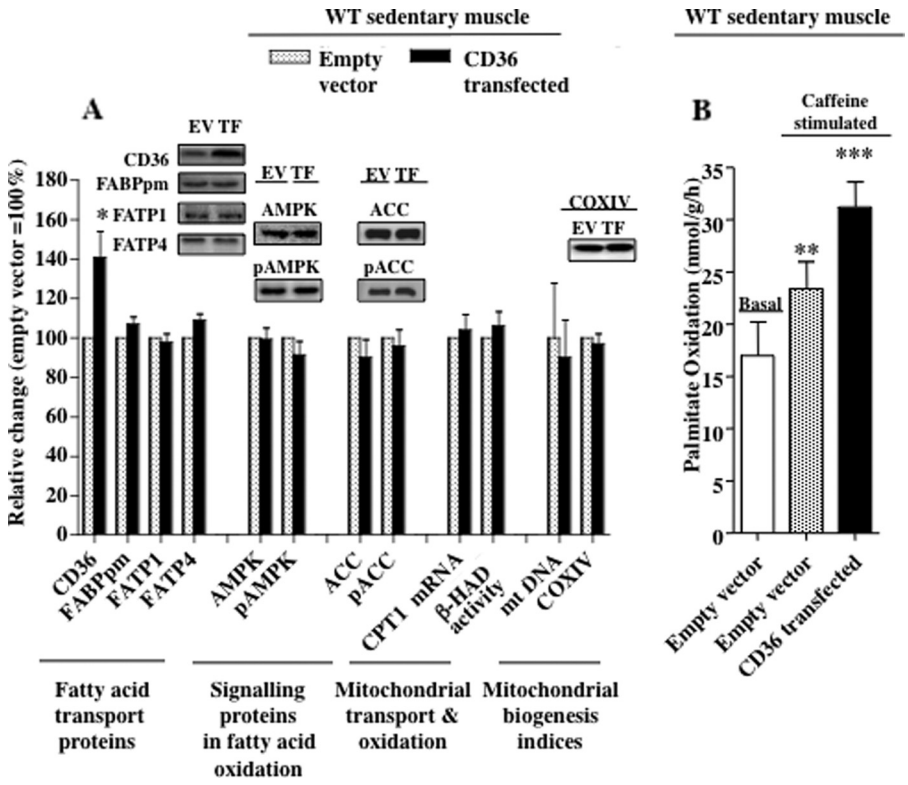

FIGURE 7. **Effects of CD36 overexpression in WT sedentary soleus muscle on fatty acid transporters, signaling proteins, fatty acid oxidation enzymes, and indices of mitochondrial biogenesis (*A*), and fatty acid oxidation under basal conditions and during a metabolic challenge (caffeine 3 mM) (*B*).** Mean ± S.E., $n = 5–7$ independent determinations for each parameter. The various measurements are described under "Experimental Procedures." *A,* CD36-transfected (*TF*) muscle data are expressed relative to the contralateral muscle transfected with empty vector (*EV*). For Western blotting, equal quantities of protein were loaded and confirmed with Ponceau staining. Muscles from the same animal transfected with empty vector and CD36 were run on the same gel. *, $p < 0.05$, CD36-transfected muscle (%) *versus* empty vector. *B,* rates of fatty acid oxidation are shown for basal conditions in muscles transfected with empty vector and in caffeine-stimulated muscles transfected either with empty vector or with CD36. **, $p < 0.05$, caffeine-stimulated EV-transfected muscle *versus* basal EV-transfected muscle. ***, $p < 0.05$, caffeine-stimulated CD36-transfected muscle *versus* caffeine-stimulated EV-transfected muscle.

DISCUSSION

Our results demonstrate that *in vivo* the plasma membrane fatty acid transporter CD36 is a key component of the molecular machinery required for regulating skeletal muscle fuel selection at rest and during an acute metabolic challenge (exercise), as well as influencing exercise endurance. In addition, we demonstrate that CD36-mediated fatty acid transport into muscle is essential for observing the well known exercise training-induced up-regulation of fatty acid oxidation, independent of changes in muscle mitochondrial enzymes and biogenesis. Collectively, our observations challenge long held understandings of the mechanism regulating skeletal muscle fatty acid oxidation during exercise and its adaptation to exercise training. Our work reveals a fundamental role for CD36 in these metabolic processes.

Metabolic Effects of CD36 Ablation under Basal Conditions—Consistent with previous reports (23, 51, 53), ablation of CD36 increased circulating fatty acids, impaired fatty acid transport into muscle, and reduced muscle fatty acid oxidation, intramuscular triacylglycerol synthesis rates, and lipid accumulation (triacylglycerol, diacylglycerol, and ceramide). These alterations were associated with better glucose tolerance, increased muscle insulin sensitivity (also shown previously (52)), and rates of glucose oxidation and reduced hepatic but not muscle glycogen. Thus, under basal conditions CD36 ablation shifts muscle fuel selection to an increased reliance on glucose utilization. This shift is not accompanied by concurrent changes in muscle mitochondrial content or selected enzymes involved in fatty acid oxidation.

Effects of CD36 Ablation during an Acute Metabolic Challenge (Exercise)—It is well known that altering the provision of circulating fatty acids to skeletal muscle alters their oxidation correspondingly, as increasing fatty acid delivery to muscle enhances the ability to sustain aerobic exercise, an effect that occurs independent of changes in muscle mitochondrial content and fatty acid-oxidizing enzymes (4, 17). To compare endurance exercise abilities in the WT and CD36 KO mice, we very carefully matched the exercise intensities ($\%VO_{2max}$) at which the animals ran on the treadmill and documented that the circulating fatty acid delivery to muscle was not compromised in exercising CD36 KO mice. Despite these important controls, in exercising CD36 KO mice the fatty acid transport into muscle (−41%) and whole body lipid utilization (−37%) were impaired comparably, as was aerobic exercise endurance (−44%). This impaired exercise capacity cannot be attributed to cardiac performance, as this is normal in CD36 KO mice (65). Collectively, our findings established a central role for CD36-mediated fatty acid transport in regulating skeletal muscle fatty acid oxidation during exercise and influencing aerobic exercise performance.

In exercising CD36 KO mice, the reduced fatty acid transport into muscle induced a profound shift in fuel selection. Specifi-

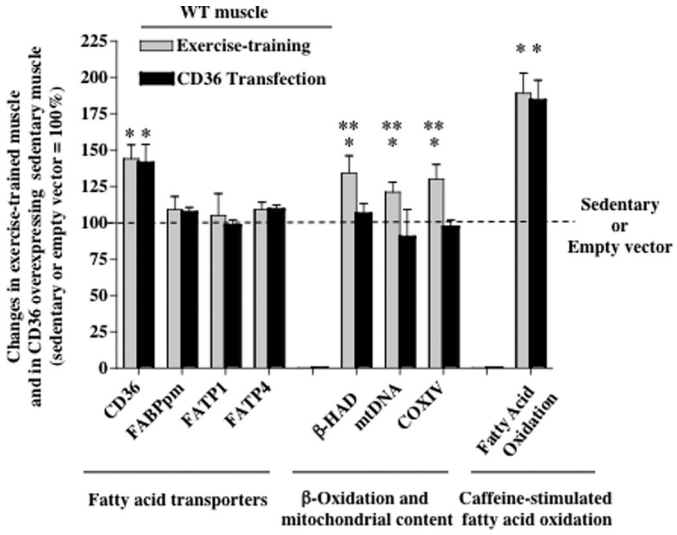

FIGURE 8. **Comparison of fatty acid transporters, β-oxidation enzymes, muscle mitochondrial content, and caffeine-stimulated muscle fatty acid oxidation in WT trained and in WT sedentary muscles transfected with CD36.** For exercise-trained muscles, data are expressed relative to untrained muscles, and for CD36-transfected muscles, data are expressed relative to muscles transfected with empty vector. The data are derived from Figs. 5 and 6*D* for trained WT soleus muscles, and from Fig. 7, *A* and *B,* for sedentary CD36-transfected WT soleus muscles. *Dashed line* refers to sedentary muscle or muscle transfected with empty vector. $n = 5–7$ independent determinations for each parameter. *, $p < 0.05$, trained or transfected muscles *versus* respective control. **, $p < 0.05$ exercise-trained *versus* CD36-transfected muscle.

cally, there was a markedly greater rate of glycogen utilization by muscle and a greatly enhanced depletion of hepatic glycogen. These observations parallel the increased rate of glycogen utilization during exercise observed in animal models in which fatty acid mobilization from adipose tissue and delivery to muscle are reduced (*i.e.* hormone-sensitive lipase (HSL) KO (54) and adipose triacylglycerol lipase (ATGL) KO mice (55, 56)) or when fatty acid delivery to muscle is altered experimentally (17). However, in contrast to these other experimental models, in exercising CD36 KO mice fatty acid delivery to muscle was not compromised but fatty acid transport into muscle was impaired. A compensatory response in the CD36 KO mice was the increased rate of muscle and hepatic glycogen utilization to provide fuel for the exercising muscles. This more rapid depletion of hepatic glycogen in the exercising CD36 KO mice likely accounted for limiting their exercise endurance ability. Depletion of hepatic glycogen has long been linked with an impaired exercise performance, and an accelerated depletion of this depot is typically observed whenever fatty acid utilization by exercising muscle is diminished (this study and Refs. 17, 54, 55).

Because the ratios of fatty acid oxidation (WT/CD36 KO ratio 1.6:1) and transport (WT/CD36 KO ratio 1.7:1) were comparable, it appears that fatty acid oxidation during exercise is highly dependent on fatty acid transport into muscle. Indeed, the impaired ability to adjust fatty acid utilization in CD36 KO mice during exercise has also been observed in these animals during a metabolic challenge with AICAR (23). In each instance, whether with exercise (this study) or with AICAR (23), the underlying cause of this impairment in CD36 KO mice is their inability to up-regulate fatty acid transport into muscle, a process that is dependent on the translocation of CD36 to the plasma membrane induced by metabolic stimuli (22, 27, 28), including acute exercise (this study).

In WT muscles other fatty acid transporters, in addition to CD36, are also induced to translocate to the plasma membrane, either by electrically induced muscle contraction (FABPpm, FATP1, and -4 (28)) or mild exercise (FABPpm only, this study). However, it appears that among fatty acid transporters CD36 is key, as its ablation (present study)[7] or specific blocking of sarcolemmal CD36 with reactive oleate or palmitate esters (27, 66) almost fully negate metabolic stimulus-induced up-regulation of fatty acid transport.

Clearly, fatty acid provision to muscle has a marked influence on muscle fuel selection during exercise. Until now, this has largely been viewed as being attributable to fatty acid delivery to muscle (blood flow × concentration) (67), an understanding developed prior to the recognition that fatty acid transport into muscle is a highly regulatable, protein-mediated process (*cf.* Ref.22). This study establishes that CD36 is a key molecular regulator required for the up-regulation of fatty acid oxidation in metabolically challenged (acute exercise) muscle *in vivo*.

Exercise Training-induced Adaptation of Fatty Acid Oxidation Is Critically Dependent on CD36—For the past 40 years or more, it has been widely recognized that aerobic exercise training increases skeletal muscle fatty acid oxidation. Classic studies by Holloszy and co-workers (for review, see Ref. 4) attributed this to an increase in mitochondrial density and to concomitant increases in enzymes regulating skeletal muscle fatty acid oxidation, although mitochondrial biogenesis appeared to be the key factor (68). In more recent years, PPARδ- and PGC-1α-induced up-regulation of skeletal muscle fatty acid oxidation has been attributed to the same mechanisms (69). However, the mechanisms involved in providing additional fatty acids for oxidation, when muscle mitochondria are increased, have not been addressed, although a number of observations have begun to suggest that CD36 has a central role in muscle fatty acid oxidation, as AICAR-stimulated fatty acid oxidation was impaired in CD36 KO mice (23), and the PGC-1α-mediated increase in fatty acid oxidation was accompanied by an increase in CD36 (32–34).

With exercise training, we observed the well known expected changes in muscle mitochondrial content and enzymes involved with fatty acid oxidation in both WT and CD36 KO mice. The conventional interpretation for WT mice would be that the training-induced increase in metabolically stimulated fatty acid oxidation was attributable to the training-induced mitochondrial biogenesis and enzymatic changes. In contrast, in exercise-trained CD36 KO mice, caffeine-stimulated fatty acid oxidation was not increased. Hence, our observations indicate that an increased rate of CD36-mediated fatty acid transport into muscle is critically important to support the well known exercise training-induced increase in muscle fatty acid oxidation.

Skeletal Muscle Fatty Acid Oxidation in Exercise-trained and CD36-overexpressing Muscle—The role of CD36 in the adaptation of fatty acid oxidation in muscle was further confirmed

[7] S. S. Jain and A. Bonen unpublished data.

when we overexpressed CD36 in sedentary muscle to the same extent as was induced by 6 weeks of exercise training. Importantly, only muscles of sedentary WT animals were transfected with CD36. This approach permitted us to compare directly the effects of CD36 overexpression in WT muscle on fatty acid oxidation in the absence of changes in mitochondrial content and fatty acid oxidation enzymes.

Just as in trained WT animals, in which CD36 was up-regulated (+44%) and in which caffeine-stimulated fatty acid oxidation was increased, overexpression of CD36 (+41%) in sedentary WT muscle also resulted in an increased rate of caffeine-stimulated fatty acid oxidation, despite the fact that mitochondrial content and fatty acid oxidation enzymes were not altered. This strongly suggests that training-induced increases in fatty acid oxidation are highly dependent on an increase in CD36-mediated fatty acid transport into muscles and can occur independent of changes in muscle mitochondrial content and enzymes involved in fatty acid oxidation, as has been widely believed for many years. Thus, our present results challenge the long held belief that mitochondrial biogenesis is necessarily required for the training-induced up-regulation of skeletal muscle fatty acid oxidation.

Possible Clinical Implications—In recent years genetic studies have identified common CD36 variants. As reviewed in detail elsewhere (70), these common CD36 genetic variants contribute to individual variability in lipid profiles and susceptibility to obesity-related complications but not with measures of obesity (70). Nevertheless, this study may have some clinical implications. Specifically, skeletal muscle genes that adapt to exercise training are frequently dysregulated in insulin resistance, along with reductions in muscle mitochondrial content (71–73). In human obesity and type 2 diabetes, an increase in sarcolemmal CD36 is highly correlated with intramuscular lipid accumulation (36). Although in this study CD36 is associated with an improved rate of fatty acid oxidation, it is important to note that this was observed when muscle mitochondrial content was unaltered or was up-regulated (training). In contrast, individuals with insulin resistance are often unfit and relatively inactive and can exhibit a reduced muscle mitochondrial content (71–73). Thus, the observed increase in sarcolemmal CD36 in insulin-resistant individuals (36), in whom muscle mitochondrial content is likely reduced, redirects the CD36-mediated increase in fatty acid transport to intramuscular lipid storage (36), because the reduced muscle mitochondrial content limits fatty acid oxidation. However, as shown in this study, exercise training increases both CD36 and muscle mitochondrial content. These combined training-mediated effects avoid the deleterious effects of excess fatty acids being trafficked into lipid depots,[8] as occurs when sarcolemmal CD36 is increased in the face of mitochondrial insufficiency in insulin-resistant human muscle.

Summary—We have shown that CD36-mediated fatty acid transport into skeletal muscle influences muscle fuel selection and exercise performance. In addition, we demonstrate that CD36-mediated fatty acid transport into muscle is essential for observing the well known exercise training-induced up-regulation of fatty acid oxidation. Our work provides the novel view that *in vivo* both the regulation of fatty acid oxidation at rest and during exercise and the adaptation of fatty acid oxidation by exercise training are highly CD36-dependent. These observations markedly alter the long held belief that the skeletal muscle fatty acid oxidation and its training-induced adaptation are regulated solely by mitochondrial density and selected enzymes involved in fatty acid oxidation.

[8] A. Bonen and G. P. Holloway, unpublished observations.

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