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Fatty acid oxidation in cardiac and skeletal muscle mitochondria is unaffected by deletion of CD36

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Abstract

Recent studies found that the plasma membrane fatty acid transport protein CD36 also resides in mitochondrial membranes in cardiac and skeletal muscle. Pharmacological studies suggest that CD36 may play an essential role in mitochondrial fatty acid oxidation. We isolated cardiac and skeletal muscle mitochondria from wild type and CD36 knock-out mice. There were no differences between wild type and CD36 knock-out mice in mitochondrial respiration with palmitoyl-CoA, palmitoyl-carnitine or glutamate as substrate. We investigated a potential alternative role for CD36 in mitochondria, i.e. the export of fatty acids generated in the matrix. Palmitate export was not different between wild type and CD36 knock-out mice. Taken together, CD36 does not appear to play an essential role in mitochondrial uptake of fatty acids or export of fatty acid anions.
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Humans and mice deficient in CD36 exhibit decreased cardiac long chain fatty acid (LCFA)[1] uptake [1,2], which has been attributed to decreased CD36 mediated LCFA transport across the sarcolemmal membrane [2]. LCFAs are key substrates for mitochondrial oxidative metabolism in heart and skeletal muscle and once activated are transported into the mitochondria via the carnitine-palmitoyl transferase (CPT) system where they undergo β-oxidation [3]. Recent reports noted that the plasma membrane fatty acid translocase CD36 is found in mitochondrial membranes, however the function of CD36 in mitochondria is unclear. Studies using a putative inhibitor of CD36, sulfo-*N*-succinimidyl oleate (SSO), observed decreased LCFA oxidation in skeletal muscle mitochondria, suggesting that CD36 is involved in LCFA transport into the mitochondrial matrix [4–6]. Although an attractive thesis, earlier studies showed SSO inhibits CPT-I activity [4], thus effects of SSO on fatty acid oxidation may not be specific for CD36. A potential alternative role for CD36 in mitochondria is as an exporter of fatty acids generated in the mitochondrial matrix. Recent studies on cardiac mitochondria noted the hydrolysis of LCFA-CoA to LCFA by mitochondrial thioesterase I (MTE-I) in the matrix, and these LCFAs are subsequently exported out of the mitochondria by an unidentified protein carrier [7,8]. It has been proposed that uncoupling protein 3 (UCP3) is responsible for LCFA export from the matrix [7], however we recently observed that both MTE-I and fatty acid export are

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[1] *Abbreviations used:* LCFA, long chain fatty acid; CPT, carnitine-palmitoyl transferase; SSO, sulfo-*N*-succinimidyl oleate; CS, citrate synthase.

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up-regulated ~5-fold by diabetes without a significant change in UCP3 protein levels [8]. A likely candidate for this process is CD36, however its role in mitochondrial LCFA export has not been assessed.

The present study tested the hypothesis that CD36 is essential for normal mitochondrial oxidation of LCFA in cardiac and skeletal muscle. Mitochondrial oxidation of palmitoyl-carnitine and palmitoyl-CoA, and palmitate export was assessed in isolated mitochondria from WT and CD36-KO mice [9].

Materials and methods

Animals

Experiments were performed on 8- to 16-week-old male WT and CD36-KO mice (6× backcrossed to C57Bl/6) [9]. Mice were housed in an AAALAC accredited specific pathogen free facility and all procedures were prior approved by the Institutional Animal Care and Use Committee.

Study design

We used mitochondria isolated from cardiac and skeletal muscle from WT and CD36-KO mice for measurements of respiration, protein expression of CD36, citrate synthase (CS) activity, and MTE-I activity. Cardiac mitochondria were also used for measurement of palmitate extrusion. Due to the small amount of mitochondria obtained from a single mouse, tissue was pooled from four animals for each data point. Mice were maintained on a 14/10 light/dark cycle, and were sacrificed by pentobarbital overdose (200 mg/kg) in the fed state during the beginning of the light phase. The heart was exposed by sternotomy and harvested, and hindlimb skeletal muscle was removed. Blood samples were also taken for plasma analysis.

Isolation of mitochondria

Cardiac muscle was minced in Buffer A (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM $MgSO_4$ $7H_2O$, and 1 mM ATP; pH 7.4 at 4 °C). Skeletal muscle was minced in Buffer A but without EGTA. Mitochondria were isolated from cardiac muscle as previously described [10]. Briefly, cardiac tissue suspended in Buffer A with 0.2% bovine serum albumin (BSA) was homogenized with a polytron tissue processor (Brinkman Instruments, Westbury, NY) for 2.5 s at a rheostat setting of 6.5, digested with trypsin (5 mg/g wet weight) for 10 min at 4 °C, further homogenized with a Potter–Elvejhem homogenizer at a setting of 3 and centrifuged at 600*g*. The supernatant was centrifuged at 3100*g* and the pellet was resuspended in KME (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA). Skeletal muscle mitochondria were released with a combination of trypsin (10 mg/g wet weight) and neutral protease (1 mg/g wet weight) for 10 min, and homogenization with the Potter–Elvejhem homogenizer. After removing the proteases, the mitochondria-rich supernatant was collected by centrifugation at 349*g*. Skeletal muscle mitochondria were then pelleted by centrifugation at 7059*g* and resuspended in KME.

Mitochondrial respiration measurements

Mitochondrial oxygen consumption was measured using an oxygen electrode at 30 °C in 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg/ml BSA. The following substrates were used to measure oxidative phosphorylation: 20 mM glutamate, 2 μM palmitate, 20 μM palmitoyl-CoA + carnitine + malate, and 40 μM palmitoyl-carnitine + malate, state III (ADP-stimulated) respiration, state IV (ADP-limited) respiration. Additional measurements of mitochondrial respiration were made with palmitoylcarnitine as the substrate in the presence and absence of 150 μM SSO (a gift from Prof. Jan Glatz, University of Maastricht, the Netherlands).

Mitochondrial palmitate export

Palmitate export was measured in isolated mitochondria from WT and CD36-KO mice, as previously described [7,8]. Isolated mitochondria were incubated with 20 μM [1-^{14}C]palmitoyl-carnitine at 37 °C for 6 min. The reaction was terminated as previously described [7], and the extramitochondrial fluid was separated by centrifugal filtration (0.45 μm). Lipids were extracted and fatty acid moieties separated by thin layer chromatography, and the palmitic acid spot visualized and counted [7,8].

Western blot analysis

Western blot analysis for CD36 was performed on frozen cardiac and skeletal muscle tissue and on isolated mitochondria by loading 100 μg protein for cardiac samples and 200 μg protein for skeletal muscle samples. Following electrophoresis in 10% Tris–HCl SDS–PAGE gels the separated proteins were transferred onto a PVDF membrane. Membranes were blocked with TBS–Tween containing 5% milk and then incubated with primary polyclonal rabbit antibodies to CD36 (1:5000) [11], followed by donkey anti-rabbit secondary antibody and development with enhanced chemiluminescence (Amersham). Bands were quantified using commercially available software. A macrophage lysate was used as a positive control for CD36 at 88 kDa.

Metabolites and enzyme activities

Plasma triglyceride and free fatty acid levels were determined using enzymatic spectrophotometric assays [12]. Citrate synthase (CS) activity was assessed spectrophotometrically on frozen cardiac and skeletal muscle tissue and mitochondria as previously described [13]. MTE-I activity was measured spectrophotometrically on mitochondria with palmitoyl-CoA by following the increase in absorbance at 412 nm in the presence of 5,5′-dithiobis 2-nitrobenzoic acid [14].

Statistical analysis

Differences between WT and CD36-KO mice within each tissue were determined using a 2-tailed *t*-test. Data are presented as means ± SEM, and $p < 0.05$ was considered significant.

Results

There were no differences in heart weight, body weight, or heart/body weight ratio between WT and CD36-KO mice (data not shown). There was a significant increase in plasma free fatty acid levels in CD36-KO mice in the fed state (0.11 ± 0.01 vs. 0.07 ± 0.01 mM; $p < 0.05$) and with overnight fasting (1.43 ± 0.06 vs. 1.21 ± 0.06 mM; $p < 0.05$). Plasma triglyceride levels were measured in the fed state, and were not different between groups (70.0 ± 3.1 vs. 74.7 ± 5.0 mg/dL).

Protein expression

Western blot analysis confirmed the presence of CD36 in heart and skeletal muscle mitochondria and whole tissue homogenates from WT but not CD36-KO mice (Fig. 1a). Interestingly, cardiac mitochondria had approximately 10-fold greater CD36 per mg mitochon-

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