Published in final edited form as:

Arch Biochem Biophys. 2007 November 15; 467(2): 234–238. doi:10.1016/j.abb.2007.08.020.

Fatty Acid Oxidation in Cardiac and Skeletal Muscle Mitochondria is Unaffected by Deletion of CD36

Kristen L. King¹, William C. Stanley^{1,2,3}, Mariana Rosca⁴, Janos Kerner², Charles L. Hoppel⁴, and Maria Febbraio⁵

¹Department of Physiology and Biophysics, Case Western Reserve University; Cleveland, USA

²Department of Nutrition, Case Western Reserve University; Cleveland, USA

³Department of Medicine, Division of Cardiology, University of Maryland, Baltimore, USA

⁴Department of Pharmacology, School of Medicine, Case Western Reserve University; Cleveland, USA

⁵Department of Cell Biology, Cleveland Clinic, Lerner Institute; Cleveland, USA

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, ie. 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.

Keywords

fatty acids; lipotoxicity; myocardium

Introduction

Humans and mice deficient in CD36 exhibit decreased cardiac long chain fatty acid (LCFA) 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

Address for Correspondence: William C. Stanley, Ph.D., Division of Cardiology, Department of Medicine, University of Maryland-Baltimore, 20 Penn St, HSF2, Room S022, Baltimore, MD, 21201, USA, 410-706-3585 (Phone), 410-706-3583 (Fax), e-mail: E-mail: wstanley@medicine.umaryland.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 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 8-16-week old male WT and CD36-KO mice (6x 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 seconds at a rheostat setting of 6.5, digested with trypsin (5 mg/g wet weight) for 10 minutes at 4°C, further homogenized with a Potter-Elvehjem homogenizer at a setting of 3 and centrifuged at $600 \times g$. The supernatant was centrifuged at $3100 \times g$ 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 minutes, and homogenization with the Potter-Elvehjem homogenizer. After removing the proteases, the mitochondria-rich supernatant was collected by centrifugation at $349 \times g$. Skeletal muscle mitochondria were then pelleted by centrifugation at $7059 \times g$ 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 KH₂PO₄, and 1 mg/ml BSA. The following substrates were used to measure oxidative phosphorylation: 20mM glutamate, 2μM palmitate, 20μM palmitoyl-CoA + carnitine + malate, and 40μM 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 [1-¹⁴C]-palmitoyl-carnitine at 37°C for 6 minutes. The reaction was terminated as previously described [7], and the extramitochondrial fluid was separated by centrifugal filtration (0.45μm). 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 kd.

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 mean ± 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 (Figure 1A). Interestingly, cardiac mitochondria had approximately 10-fold greater CD36 per mg mitochondrial protein than skeletal muscle (Figure 1). No Na⁺K⁺ ATPase was found in mitochondrial fractions demonstrating that the presence of CD36 in mitochondria was not due to contamination by plasma membrane (data not shown).

Enzyme Activities

Cardiac and skeletal muscle tissue CS activity, indicative of mitochondrial content, was unaffected by absence of CD36 (Table 1). Similarly, there was no difference in mitochondrial recovery between WT and CD36-KO. CS and MTE-1 activities in isolated mitochondria from cardiac and skeletal muscle did not differ between CD36-KO and WT mice (Table 1).

Mitochondrial Respiration

Glutamate served as a control substrate because its oxidation by mitochondria is independent of LCFA transport, and, as expected, there were no differences in state III and state IV respiration, RCR or the ADP/O ratio in either cardiac or skeletal muscle mitochondria (Table 2) isolated from WT and CD36-KO mice. To assess if CD36 was involved either directly or indirectly (by interaction with known enzymes of the uptake pathway) we measured the oxygen consumption with substrates which enter fatty acid oxidation at different steps of the uptake pathway. As shown in Table 2, neither the CPT-1 dependent oxidation of palmitoyl-CoA nor the CPT-I independent oxidation of palmitoylcarnitine was affected by the deletion of CD36. Furthermore, the oxidation of palmitate, which requires CPT-I and long chain acyl CoA synthetase, was not affected (98 ± 18 vs. 145 ± 21 nA oxygen $\times$ min⁻¹ $\times$ mitochondrial protein⁻¹, $p > 0.05$, WT vs. CD36-KO, respectively). Palmitoylcarnitine respiration, measured in the presence and absence of 150 μ M SSO, an inhibitor of sarcolemmal fatty acid transport [15] and the activity of carnitinepalmitoyltransferase-I (CPT-I)[4], showed an approximately equal 34-42% decrease using mitochondria isolated from WT and CD36-KO mice (Figure 2).

Palmitate Extrusion from Mitochondria

Export of palmitate from cardiac mitochondria was not significantly different between WT and CD36-KO (Figure 3).

Discussion

The results of this study affirm the presence of CD36 in mitochondria, and demonstrate an approximate 10-fold greater abundance in heart compared with skeletal muscle mitochondria. However, there were no differences between WT and CD36-KO mice in the rate of mitochondrial respiration with palmitate, palmitoyl-CoA, or palmitoylcarnitine as the substrate. Thus these results do not support an essential role for CD36 in mitochondrial fatty acid oxidation in muscle. We hypothesized a potential alternative role for CD36 in mitochondria, that is to export LCFA out of the matrix, which would maintain CD36 polarity. Once again, however, we did not observe any differences between WT and CD36-KO mice in palmitate export from cardiac mitochondria. Taken together, CD36 does not appear to play an essential role in mitochondrial fatty acid uptake.

Evidence to suggest that CD36 might be required for the transport of fatty acyl units into the mitochondrial matrix comes from inhibition studies with SSO, and from the localization of CD36 to mitochondrial membranes[4-6;16]. Initial studies in rat skeletal muscle showed a decrease in palmitate oxidation upon addition of SSO, and proposed a role for CD36 as an

LCFA acceptor, receiving LCFAs from cytoplasmic fatty acid binding proteins and transferring them to the long chain acyl CoA synthetase on the mitochondrial outer membrane[4]. Subsequent studies in rat and human skeletal muscle found a decrease in mitochondrial palmitate and palmitoyl-carnitine oxidation with the addition of SSO[6]. Little information is available regarding the specificity of SSO for CD36, however it has been shown to be an effective inhibitor of skeletal muscle CPT-I[4], suggesting that the reduction in fatty acid oxidation by SSO may be independent of CD36 inhibition. The results of the present investigation show no change in mitochondrial respiration with palmitate, palmitoyl-CoA, or palmitoyl-carnitine in CD36-KO animals (Table 2), clearly demonstrating that CD36 is not *required* for normal mitochondrial utilization of long chain fatty acids.

A large body of data supports a role for CD36 in sarcolemmal LCFA transport, including the observations that humans and mice deficient in CD36 exhibit decreased LCFA uptake and subsequently decreased LCFA oxidation[1;2]. Since there was no difference in mitochondrial fatty acid oxidation between WT and CD36-KO mice in the present study, the data suggest that CD36 is involved in the regulation of muscle LCFA at the level of the plasma membrane. We hypothesized that an alternative role for CD36 in mitochondria may be to function as a fatty acid anion exporter from the mitochondrial matrix. We did not observe any difference between WT and CD36-KO in the rate of palmitate export, thus arguing that CD36 is not essential in this function either. At present, the function of CD36 in mitochondria remains to be elucidated. A novel finding of the present study is the 10-fold greater CD36 content in cardiac mitochondria compared to skeletal muscle, which suggests that whatever role CD36 plays in mitochondria, the capacity is ~10-fold greater in cardiac than in skeletal muscle.

While we believe our hypotheses were well reasoned and pertinent, they clearly were not supported by our data, thus the role of CD36 in mitochondria remains elusive. It is important to note that the CD36-KO mouse employed in these studies has a life-long deletion and thus we cannot exclude the possibility that compensatory mechanisms prevented changes in mitochondrial fatty acid metabolism in the knockout strain. To more thoroughly evaluate the role of CD36 in cardiac mitochondria a conditional cardiac-specific CD36-KO mouse should be used. To our knowledge, such a mouse is not currently available.

In conclusion, we provide direct evidence that although CD36 is abundant in cardiac mitochondria, it does not play an essential role in the uptake and oxidation of long chain fatty acids, nor the export of long chain fatty acids from the matrix.

Acknowledgments

This work was supported by NIH Grants HL074237 (CLH, JK, WCS) and HL70083 (MF). We thank George Nimako for excellent technical assistance with regard to CD36 Western Blots.

Reference List

1. Nozaki S, Tanaka T, Yamashita S, Sohmiya K, Yoshizumi T, Okamoto F, Kitauro Y, Kotake C, Nishida H, Nakata A, Nakagawa T, Matsumoto K, Kameda-Takemura K, Tadokoro S, Kurata Y, Tomiyama Y, Kawamura K, Matsuzawa Y. CD36 mediates long-chain fatty acid transport in human myocardium: complete myocardial accumulation defect of radiolabeled long-chain fatty acid analog in subjects with CD36 deficiency. *Mol Cell Biochem* 1999;192:129–135. [PubMed: 10331667]
2. Kuang M, Febbraio M, Wagg C, Lopaschuk GD, Dyck JR. Fatty acid translocase/CD36 deficiency does not energetically or functionally compromise hearts before or after ischemia. *Circulation* 2004;109:1550–1557. [PubMed: 15023869]
3. Kerner J, Hoppel C. Fatty acid import into mitochondria. *Biochim Biophys Acta* 2000;1486:1–17. [PubMed: 10856709]

4. Campbell SE, Tandon NN, Woldegiorgis G, Luiken JJ, Glatz JF, Bonen A. A novel function for fatty acid translocase (FAT)/CD36: involvement in long chain fatty acid transfer into the mitochondria. *J Biol Chem* 2004;279:36235–36241. [PubMed: 15161924]
5. Holloway GP, Bezaire V, Heigenhauser GJ, Tandon NN, Glatz JF, Luiken JJ, Bonen A, Spriet LL. Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. *J Physiol* 2006;571:201–210. [PubMed: 16357012]
6. Bezaire V, Bruce CR, Heigenhauser GJ, Tandon NN, Glatz JF, Luiken JJ, Bonen A, Spriet LL. Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. *Am J Physiol Endocrinol Metab* 2006;290:E509–E515. [PubMed: 16219667]
7. Gerber LK, Aronow BJ, Matlib MA. Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts. *Am J Physiol Cell Physiol* 2006;291:C1198–C1207. [PubMed: 16855217]
8. King KL, Young ME, Kerner J, Huang H, O'shea KM, Alexson SE, Hoppel CL, Stanley WC. Diabetes and activation of peroxisome proliferator activated receptor alpha increase mitochondrial thioesterase I protein expression and activity in the heart. *J Lipid Res*. 2007
9. Febbraio M, Abumrad NA, Hajjar DP, Sharma K, Cheng W, Pearce SF, Silverstein RL. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J Biol Chem* 1999;274:19055–19062. [PubMed: 10383407]
10. Moghaddas S, Stoll MS, Minkler PE, Salomon RG, Hoppel CL, Lesnefsky EJ. Preservation of cardiolipin content during aging in rat heart interfibrillar mitochondria. *J Gerontol A Biol Sci Med Sci* 2002;57:B22–B28. [PubMed: 11773203]
11. Bodart V, Febbraio M, Demers A, McNicoll N, Pohankova P, Perreault A, Sejlitz T, Escher E, Silverstein RL, Lamontagne D, Ong H. CD36 mediates the cardiovascular action of growth hormone-releasing peptides in the heart. *Circ Res* 2002;90:844–849. [PubMed: 11988484]
12. Okere IC, Chandler MP, McElfresh TA, Rennison JH, Sharov V, Sabbah HN, Tserng KY, Hoit BD, Ernberger P, Young ME, Stanley WC. Differential effects of saturated and unsaturated fatty acid diets on cardiomyocyte apoptosis, adipose distribution, and serum leptin. *Am J Physiol Heart Circ Physiol* 2006;291:H38–H44. [PubMed: 16443671]
13. Panchal AR, Stanley WC, Kerner J, Sabbah HN. Beta-receptor blockade decreases carnitine palmitoyl transferase I activity in dogs with heart failure. *J Card Fail* 1998;121–126. [PubMed: 9730105]
14. Alexson SE, Nedergaard J. A novel type of short- and medium-chain acyl-CoA hydrolases in brown adipose tissue mitochondrial. *J Biol Chem* 1988;263:13564–13571. [PubMed: 2901416]
15. Luiken JJ, Willems J, van der Vusse GJ, Glatz JF. Electrostimulation enhances FAT/CD36-mediated long-chain fatty acid uptake by isolated rat cardiac myocytes. *Am J Physiol Endocrinol Metab* 2001;281:E704–E712. [PubMed: 11551846]
16. Distler AM, Kerner J, Peterman SM, Hoppel CL. A targeted proteomic approach for the analysis of rat liver mitochondrial outer membrane proteins with extensive sequence coverage. *Anal Biochem* 2006;356:18–29. [PubMed: 16876102]

Figure 1.

CD36 protein expression in isolated mitochondria and whole cardiac and skeletal muscle homogenates from WT and CD36-KO mice. **Top panel:** Representative Western Blot demonstrating the presence of CD36 in mitochondria from heart and skeletal muscle of WT mice and its absence in CD36-KO mice. **Bottom panel:** Comparison of CD36 expression in cardiac and skeletal mitochondria and whole tissue homogenates from WT and CD36-KO mice. Data are expressed in arbitrary units and normalized to the mean of the WT group. *p<0.05 compared to cardiac WT.

Figure 2. Mitochondrial State III respiration measured in the presence and absence of 150 μ M SSO with palmitoylcarnitine as a substrate ($n = 3$ batches (pooled from 4 mice each) for both WT and CD36-KO). (* $p < 0.05$)

Figure 3.

The rate of palmitate extrusion from cardiac mitochondria in WT and CD36-KO animals. Results expressed as pmol mg mitochondria protein⁻¹ min⁻¹ (n = 7 batches (pooled from 4 mice each) for WT and n = 8 batches for CD36-KO).

Table 1

Enzyme activities in heart and skeletal muscle tissue, and isolated mitochondria.

	<u>Heart</u>		<u>Skeletal Muscle</u>	
	<u>WT</u>	<u>CD36-KO</u>	<u>WT</u>	<u>CD36-KO</u>
Tissue Citrate Synthase ($\mu\text{mol min}^{-1} \text{ gww}^{-1}$)	80.5 $\pm$ 12.6	89.3 $\pm$ 17.4	28.5 $\pm$ 3.0	29.1 $\pm$ 3.6
Mitochondrial Citrate Synthase ($\text{mmol min}^{-1} \text{ mg mito prot}^{-1}$)	2.48 $\pm$ 0.31	2.34 $\pm$ 0.41	2.23 $\pm$ 0.35	2.08 $\pm$ 0.34
Mitochondrial MTE-1 ($\mu\text{mol min}^{-1} \text{ mg mito prot}^{-1}$)	8.7 $\pm$ 1.1	7.1 $\pm$ 1.1	14.0 $\pm$ 1.8	10.7 $\pm$ 0.8

Means $\pm$ SEM (n = 5 batches (pooled from 4 mice each) for WT and CD36-KO tissue citrate synthase, and for mitochondrial measurement n = 5 batches for WT and n = 4 for CD36-KO).

Table 2

Respiration of isolated cardiac and skeletal mitochondria from WT and CD36-KO mice

	<u>Heart</u>		<u>Skeletal</u>	
	<u>WT</u>	<u>CD36-KO</u>	<u>WT</u>	<u>CD36-KO</u>
Palmitoyl-CoA				
State III	363 ± 28	364 ± 25	278 ± 40	210 ± 14
State IV	85 ± 6	82 ± 6	117 ± 15	97 ± 18
RCR	4.32 ± 0.18	4.48 ± 0.27	2.39 ± 0.15	2.46 ± 0.49
ADP/O	2.38 ± 0.11	2.57 ± 0.10	2.23 ± 0.19	2.35 ± 0.16
Palmitoylcarnitine				
State III	399±29	373±33	288±46	196±24
State IV	107±7	96±7	138±22	115±22
RCR	3.72±0.09	3.90±0.15	2.11±0.21	1.96±0.43
ADP/O	2.30±0.14	2.57±0.08	2.08±0.16	2.32±0.21
Glutamate				
State III	223±28	250±26	650±84	455±46
State IV	47±7	54±4	122±20	99±18
RCR	4.68±0.49	4.87±0.61	5.52±0.53	5.36±1.35
ADP/O	2.58±0.10	2.67±0.07	2.36±0.15	2.41±0.13

Means ± SEM (n = 5 batches (pooled from 4 mice each) for skeletal WT and CD36-KO groups, and n = 9 batches for cardiac WT and CD36-KO groups).

Respiratory rates are expressed as nA oxygen × min⁻¹ × mitochondrial protein⁻¹. RCR, respiratory control ratio; ADP/O, ADP-to-oxygen ratio.