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Original article

Cardiomyocyte-specific ablation of CD36 improves post-ischemic functional recovery

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ABSTRACT

Although pre-clinical evidence has suggested that partial inhibition of myocardial fatty acid oxidation (FAO) and subsequent switch to greater glucose oxidation for ATP production can prevent ischemia/reperfusion injury, controversy about this approach persists. For example, mice with germline deletion of the FA transporter CD36, exhibited either impaired or unchanged post-ischemic functional recovery despite a 40–60% reduction in FAO rates. Because there are limitations to cardiac studies utilizing whole body CD36 knockout (total*CD36*KO) mice, we have now generated an inducible and cardiomyocyte-specific CD36 KO (ic*CD36*KO) mouse to better address the role of cardiomyocyte CD36 and its regulation of FAO and post-ischemic functional recovery. Four to six weeks following CD36 ablation, hearts from ic*CD36*KO mice had significantly decreased FA uptake compared to controls, which was paralleled by significant reductions in intramyocardial triacylglycerol content. Analysis of cardiac energy metabolism using *ex vivo* working heart perfusions showed that reduced FAO rates were compensated by enhanced glucose oxidation in the hearts from ic*CD36*KO mice. In contrast to the total*CD36*KO mice, hearts from ic*CD36*KO mice exhibited significantly improved functional recovery following ischemia/reperfusion (18 min of global no-flow ischemia followed by 40 min of aerobic reperfusion). This improved recovery was associated with lower calculated proton production prior to and following ischemia compared to controls. Moreover, the amount of ATP generated relative to cardiac work was significantly lower in the hearts from ic*CD36*KO mice compared to controls, indicating significantly increased cardiac efficiency in the hearts from ic*CD36*KO mice. These data provide genetic evidence that reduced FAO as a result of diminished CD36-mediated FA uptake improves post-ischemic cardiac efficiency and functional recovery. As such, targeting cardiomyocyte FA uptake and FAO *via* inhibition of CD36 in the adult myocardium may provide therapeutic benefit during ischemia–reperfusion.

1. Introduction

Myocardial ischemia occurs as a consequence of insufficient blood flow and subsequent oxygen delivery to the myocardium, resulting in a variety of clinical conditions ranging from mild angina to myocardial infarction. Myocardial injury and contractile dysfunction are directly correlated with the length and severity of an ischemic event. Although many mechanisms contribute to ischemic injury (see [1] for review), there is clear evidence that contractile dysfunction during and after myocardial ischemia is mediated, at least in part, by the predominant type of energy substrate metabolized by the heart [2,3].

In the healthy heart, mitochondrial oxidation of long-chain fatty acids (FAs) provides the majority of ATP needed for proper cardiac function [4]. Notwithstanding this, pre-clinical evidence has suggested that partial inhibition of myocardial FA oxidation (FAO) and a subsequent switch to greater glucose oxidation for ATP production can prevent ischemia/reperfusion (I/R) injury (see [2,4] for reviews). Consistent with this finding, partial inhibitors of FAO in clinical use, such as trimetazidine [5,6] and ranolazine [7–10], have shown to improve and preserve cardiac function in patients suffering from ischemic heart disease and I/R injury [11,12]. However, more recent reports suggest that mechanistically both drugs may act *via* alternative pathways and thus exert their beneficial effects independent of partial inhibition of FAO [12–14]. There is also a growing body of evidence suggesting that partial inhibition of myocardial FAO may actually contribute to cardiac dysfunction [15,16] as a result of mismatch between FA uptake into the

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cardiomyocyte and subsequent utilization, leading to excessive and pathological lipid accumulation. It thus remains unclear as to whether partial inhibition of FAO is truly beneficial to the injured or diseased myocardium.

To address the potential limitations inherent in existing pharmacological therapies aimed at optimizing myocardial energetics, genetically modified mouse models designed to partially inhibit FA uptake and/or oxidation could be utilized. One such model is the whole body CD36 knockout (total*CD36*KO) mouse [17]. CD36 is a transmembrane sarcolemmal protein involved in facilitating approximately 50% of cardiomyocyte FA uptake and is consequently responsible for controlling 40–60% of FAO rates in the working mouse heart [18–20]. Although total*CD36*KO mice appeared to be suitable to address how partial inhibition of FA uptake and oxidation can influence ischemic injury, conflicting reports about the extent of myocardial I/R injury in these mice have emerged [21,22]. The precise reason(s) for the different outcomes is not known, but may include perfusion conditions, mouse genetic background, type of FA used, age of mice, *etc*. Additionally, germline deletion of CD36 modifies a variety of metabolic pathways in multiple tissues [23] and subsequently whole body metabolism, which may make it difficult to determine the effects of cardiomyocyte-specific CD36-mediated alterations in metabolism on I/R injury. Moreover, compensatory alterations resulting from chronic CD36 ablation in other metabolic processes within the cardiomyocyte may have occurred during development that could influence I/R injury.

To overcome the challenges inherent to the total*CD36*KO mouse, we have generated a cardiomyocyte-specific and tamoxifen-inducible CD36 KO (ic*CD36*KO) mouse. Using short-term inducible cardiomyocyte-specific CD36 ablation we tested the hypotheses that: 1) ic*CD36*KO mouse hearts have reduced FA uptake, utilization and storage and 2) this alteration in cardiac substrate utilization leads to improved post-ischemic functional recovery. These studies will allow us to determine if the concept of a combined strategy of limiting FA uptake and partially inhibiting FAO is a beneficial therapeutic approach to reducing ischemic injury [24,25].

2. Methods

2.1. Mice

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with the Canadian Council on Animal Care guidelines. The University of Alberta Health Sciences Animal Welfare Committee and the Institutional Animal Care and Use Committee of the Cleveland Clinic approved all animal procedures.

The targeting vector (Fig. 1A) was engineered to generate flox/flox CD36 mice for creation of the inducible CD36 cardiomyocyte-specific mutant. This vector contains 3 loxP and 2 FRT sites, which are sites for Cre and FLP recombinases, respectively. The vector targets mouse CD36 exons 2 and 3, and contains a Neomycin resistance gene cassette (NEO) for selection in embryonic stem (ES) cells. We previously successfully targeted exon 3, which contains the translation start site (black arrow), to create total*CD36*KO mice [17]. An important difference between our previous strategy [17] and this one is that the murine CD36 gene in this case was identified and cloned from a C57Bl/6 bacterial artificial chromosome (BAC) library and transfected into C57Bl/6 ES cells. The resulting mice are 100% C57Bl/6 and this avoids the potential contribution of strain differences to phenotype ("passenger effect"). The use of the Cre-Lox and FLP-FRT systems allowed for selection of ES cells in G418, identification of the homologously recombined allele, and then removal of the NEO cassette, using FLP recombinase (an extra loxP site was engineered around the NEO cassette in case FLP recombinase failed). This precludes any potential contribution of the NEO cassette to normal expression and regulation of the endogenous allele prior to cell/tissue specific removal of the exons of interest. After FLP mediated removal of NEO, loxP sites remain encompassing exons 2 and 3. When Cre recombinase is expressed, excision of these exons occurs.

After transfection by electroporation and selection using G418, ES cell genomic DNA was isolated from surviving colonies and Southern blot analysis using a probe that would differentiate the homologously recombined allele from the endogenous allele was performed and potential clones identified (data not shown). PCR and Southern blot confirmed that the integration was through homologous recombination and not random integration, by using probes/primers outside the targeting vector (data not shown). The individual positive clones were also sequenced for confirmation of integration of all 3 loxP sites. Five ES clones positive for homologous recombination by Southern blotting were identified; only one was confirmed by sequencing to have all 3 loxP sites. This clone, 186, was injected into 129svj blastocysts and resulted in chimeras with significant male skewing. (This is of note because the ES cells were male derived.) High level male chimeras were mated to C57Bl/6 mice, and black pups were born (offspring of the 129svj genome are agouti), indicating germ line transmission of our ES clone. These mice were brother–sister mated to generate mice for subsequent mating to Cre lines.

We devised a specific PCR strategy to differentiate flox/flox from flox/wt (Fig. 1B) and then mated against the tamoxifen-inducible myosin heavy polypeptide 6, cardiac muscle alpha Cre mouse (cardiomyocyte specific, Jackson Labs strain 005657, B6.FVB(129)-Tg(Myh6-cre/Esr1*)1Jml/J, 15× back crossed to C57Bl/6). Mice used for experimental purposes were housed on a 12-h light/12-h dark cycle with *ad libitum* access to chow diet (#5001, Lab Diet, St. Louis, MO; 13.5% kcal from fat) and water. Both male and female mice exhibited similar baseline metabolic alterations as a result of CD36 deletion and therefore both sexes were used for experimentation.

2.2. Tamoxifen administration and CD36 ablation

Tamoxifen (T5648, Sigma) dissolved in corn oil was administered orally to adult 12–16 week-old control and littermate ic*CD36*KO mice at a dose of 100 mg/kg/day for 5 consecutive days. All experiments were performed 4–6 weeks following final tamoxifen administration. This time point was chosen to avoid any confounding influence from possible transient cardiomyopathy due to tamoxifen-induced nuclear Cre translocation [26] in male and female ic*CD36*KO and littermate control mice.

2.3. Echocardiography

Mice were mildly anesthetized using 0.75% isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics) as described previously [26,27].

2.4. Heart perfusions

Hearts were perfused in the working heart mode at 11.5 mm Hg preload and 50 mm Hg afterload with Krebs–Henseleit buffer containing 1.2 mmol/L palmitate prebound to 3% delipidated bovine serum albumin (BSA), 5 mmol/L glucose, and 50 μU/mL insulin. Hearts were aerobically perfused for 30 min, or aerobically perfused for 30 min followed by 18 min of global no-flow ischemia and 40 min of reperfusion. At the end of aerobic perfusion or reperfusion, hearts were immediately frozen in liquid N_2 with a Wollenberger clamp and stored at −80 °C as described previously [26]. For metabolic measurements, palmitate and glucose were labeled using either a combination of [9,10-^{3}H] palmitate and [U-^{14}C]glucose (for determination of FAO and glucose oxidation) or [U-^{14}C]glucose and [5-^{3}H]glucose (for determination of