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Mitochondrial Transporter ATP Binding Cassette Mitochondrial Erythroid Is a Novel Gene Required for Cardiac Recovery After Ischemia/Reperfusion

Marc Liesa, PhD*; Ivan Luptak, MD, PhD*; Fuzhong Qin, MD, PhD; Brigham B. Hyde, PhD; Ergun Sahin, MD, PhD; Deborah A. Siwik, PhD; Zhengkun Zhu, MSc; David R. Pimentel, MD; X. Julia Xu, PhD; Neil B. Ruderman, MD, PhD; Karl D. Huffman, BS; Susan R. Doctrow, PhD; Lauren Richey, DVM, PhD; Wilson S. Colucci, MD; Orian S. Shirihai, MD, PhD

Background—Oxidative stress and mitochondrial dysfunction are central mediators of cardiac dysfunction after ischemia/reperfusion. ATP binding cassette mitochondrial erythroid (ABC-me; ABCB10; mABC2) is a mitochondrial transporter highly induced during erythroid differentiation and predominantly expressed in bone marrow, liver, and heart. Until now, ABC-me function in heart was unknown. Several lines of evidence demonstrate that the yeast ortholog of ABC-me protects against increased oxidative stress. Therefore, ABC-me is a potential modulator of the outcome of ischemia/reperfusion in the heart.

Methods and Results—Mice harboring 1 functional allele of ABC-me (ABC-me^{+/−}) were generated by replacing ABC-me exons 2 and 3 with a neomycin resistance cassette. Cardiac function was assessed with Langendorff perfusion and echocardiography. Under basal conditions, ABC-me^{+/−} mice had normal heart structure, hemodynamic function, mitochondrial respiration, and oxidative status. However, after ischemia/reperfusion, the recovery of hemodynamic function was reduced by 50% in ABC-me^{+/−} hearts as a result of impairments in both systolic and diastolic function. This reduction was associated with impaired mitochondrial bioenergetic function and with oxidative damage to both mitochondrial lipids and sarcoplasmic reticulum calcium ATPase after reperfusion. Treatment of ABC-me^{+/−} hearts with the superoxide dismutase/catalase mimetic EUK-207 prevented oxidative damage to mitochondria and sarcoplasmic reticulum calcium ATPase and restored mitochondrial and cardiac function to wild-type levels after reperfusion.

Conclusions—Inactivation of 1 allele of ABC-me increases the susceptibility to oxidative stress induced by ischemia/reperfusion, leading to increased oxidative damage to mitochondria and sarcoplasmic reticulum calcium ATPase and to impaired functional recovery. Thus, ABC-me is a novel gene that determines the ability to tolerate cardiac ischemia/reperfusion. (Circulation. 2011;124:00-00.)

Key Words: ABCB10 • ischemia • mitochondria • oxidative stress • reperfusion

A cute coronary occlusion leading to cardiac ischemia/reperfusion is a major cause of mortality in Western societies. Multiple lines of evidence demonstrate that increased oxidative stress and mitochondrial dysfunction are key mediators of the cardiac dysfunction induced by ischemia/reperfusion.1–15 After ischemia/reperfusion, oxidative stress may impair mitochondrial electron transport,2,4,8–10 leading to decreased mitochondrial ATP synthesis and respiration and thereby contributing to diastolic stiffness and contractile dysfunction.8–10,16 Conversely, the sudden increase in mitochondrial respiration during early reperfusion and the impairment in electron transport chain activity may increase the production of reactive oxygen species and contribute to oxidative damage.4,5,8,12,17 Furthermore, the opening of the mitochondrial transition pore and mitochondrial fission promote cardiac myocyte death after ischemia/reperfusion.7,11,18

Clinical Perspective on p 000

The central role that oxidative stress plays in myocardial recovery from ischemia/reperfusion has been well illustrated. Targeting antioxidants to mitochondria or overexpression of global and mitochondrial antioxidant enzymes (such as catalase or superoxide dismutase sod1 and sod2) protects and improves the recovery from myocardial ischemia/reperfusion.
injury. In contrast, partial loss of function of antioxidant enzymes (such as mitochondrial sod2) worsens the recovery from ischemia/reperfusion. In the latter model, impaired recovery is independent of changes in cell viability, suggesting that oxidative stress leads to functional impairment of the contractile machinery and/or mitochondria (and the concomitant bioenergetic defect). In this regard, inactivation of sarcoplasmic reticulum calcium ATPase (SERCA) by sulfonic acid oxidation of the thiol on cysteine 674 is increased after ischemia/reperfusion, impairing calcium handling and contractility in cardiomyocytes. In addition, increased mitochondrial lipid oxidation has been shown to be one of the major alterations decreasing the electron transport chain activity after ischemia/reperfusion. Therefore, any novel gene that protects mitochondrial and/or contractile function from oxidative damage is a potential modulator of cardiac recovery after ischemia/reperfusion.

ATP binding cassette mitochondrial erythroid (ABC-me, ABCB10 or mABC2) was first discovered in the erythroid tissue, where it is induced during erythroid differentiation. ABC-me is a mitochondrial exporter of unknown substrate/s located in the inner mitochondrial membrane, with its nucleotide binding domain in the matrix. In erythroid cell lines, ABC-me has been shown to regulate hemoglobin synthesis and to stabilize mitoferrin 1, a mitochondrial iron importer. Although cardiac tissue is one of the main sites of ABC-me expression, its function in the heart has not been studied. Therefore, it is likely that, in such nonerythroid tissues, ABC-me plays a role not directly related to hemoglobinization.

Studies of ABC-me orthologs suggest that ABC-me may alter the capacity of cells to handle oxidative stress. In this study, we tested the hypothesis that ABC-me plays a role in the protection of mitochondrial and contractile functions from increased oxidative stress induced by ischemia/reperfusion in the heart. We found that hearts from mice harboring only 1 functional allele of ABC-me (ABC-me<sup>−/−</sup>) show impaired hemodynamic recovery and increased oxidative damage of both mitochondrial lipids and SERCA and exhibit severe mitochondrial dysfunction after ischemia/reperfusion. These defects were selectively restored in ABC-me<sup>+/−</sup> hearts by treatment with an SOD/catalase mimetic.

**Methods**

**Animals**

ABC-me<sup>−/−</sup> mice were generated by a gene-targeted knockout strategy through Lexicon Genetics (ABC-me exons 2 and 3 were replaced by a neomycin resistance cassette) on a C57BL6/129SvEvBrd mixed background and backcrossed up to 4 generations onto C57BL6 background (Figure I in the online-only Data Supplement). Three-month-old male or female wild-type and ABC-me<sup>−/−</sup> littermates were used (Inactivation of 1 allele of ABC-me did not alter the capacity of cells to handle oxidative stress). In this study, we tested the hypothesis that ABC-me plays a role in the protection of mitochondrial and contractile functions from increased oxidative stress induced by ischemia/reperfusion in the heart. We found that hearts from mice harboring only 1 functional allele of ABC-me (ABC-me<sup>−/−</sup>) show impaired hemodynamic recovery and increased oxidative damage of both mitochondrial lipids and SERCA and exhibit severe mitochondrial dysfunction after ischemia/reperfusion. These defects were selectively restored in ABC-me<sup>+/−</sup> hearts by treatment with an SOD/catalase mimetic.

**Mitochondria Isolation**

Hearts were incubated and minced in ice-cold ischemia relaxation buffer (for ~10 minutes; KCl 100 mmol/L, EGTA 5 mmol/L, HEPES 5 mmol/L adjusted with KOH to pH 7.4, to help releasing intermyofibrillar mitochondria), and they were homogenized in 2 mL HES buffer (HEPES 5 mmol/L, EDTA 1 mmol/L, sucrose 0.25 mol/L, pH 7.4 adjusted with KOH 1 mol/L) with a glass dounce homogenizer (20 strokes with loose pestle, 20 strokes tight pestle). The homogenate was centrifuged at 500 g for 10 minutes at 4°C (The pellet was discarded and supernatant recentrifuged at 500g). The supernatant was centrifuged at 9000 g for 15 minutes at 4°C, and the mitochondrial pellet was resuspended in 100 to 200 μL HES buffer with 0.2% BSA fatty acid free. Protein was quantified with BCA (Pierce), and the value of protein measured in HES-BSA 0.2% buffer alone was subtracted.

**Mitochondrial Oxygen Consumption Measurements**

Isolated mitochondria (20 to 40 μg in HES-BSA 0.2% buffer per well, n=3 to 4 replicates per mouse) were loaded in a V7 24-well Seahorse plate on ice and 440 μL ice-cold mitochondrial assay buffer (MAS; sucrose 70 mmol/L, mannitol 220 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 5 mmol/L, MgCl<sub>2</sub> 5 mmol/L, EDTA 2 mmol/L, EGTA 1 mmol/L, BSA fatty acid free 0.2%, pH 7.4 adjusted with KOH 1 mol/L) plus 50 μL MAS buffer with 10X substrates (complex II: succinate 50 μmol/L plus rotenone 20 μmol/L; complex I: pyruvate plus malate, 50 μmol/L each) were added on top. The 4 sequential injection ports of the Seahorse cartridge contained (in MAS solution and adjusted to pH 7.4) the following: port A, 50 μL of 10X substrate and ADP 2.5 mmol/L; port B, 55 μL oligomycin 20 μmol/L; port C, 60 μL 2,4-dinitrophenol 1 mmol/L; and port D, 65 μL antimycin A 40 μmol/L. Oxygen consumption rates were monitored in real time after the injection. State III was determined after port A injection; state IV, after port B; and uncoupled respiration rates, after port C. Antimycin A was used as a control because it blocks ETC oxygen consumption. See the online-only Data Supplement and www.shirihai-lab.org for more details.

**Data Supplement and www.shirihai-lab.org for more details.**

**Western Blot and Protein Carboxylation**

We performed SDS-PAGE and transfer as described previously. See the online-only Data Supplement for details.

**References**

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isolated mitochondria at 37°C. Between the first 5 to 30 minutes after addition of Mitosox on slope of Mitosox (Invitrogen) fluorescence increase was determined in a 96-well microplate reader in a 200-μL reaction volume. The ATP concentrations were measured spectrophotometrically in respiring mitochondrial fractions with the ATP Kit CLS II (Roche). See the online-only Data Supplement for additional information.

### Lipid Oxidation Measurements by Thiobarbituric Acid Reactive Substances
Isolated mitochondria (100 μg) were solubilized with SDS, and nanomoles of thiobarbituric acid reactive substances per mg protein were measured with the Cell Biolabs kit and following manufacturer’s indications on the basis of previously reported methodologies (eg, see Reference 10).

### Statistical Analysis
Nonparametric (unpaired, two-tailed) tests were used: Mann-Whitney U for n ≥ 4 and a test based on the Chebyshev inequality for n ≤ 3. Statistically significant differences and exact P values are stated in the figure legends.

### Results

#### ABC-me+/− Hearts Have Normal Hemodynamic and Mitochondrial Function Under Basal Conditions
ABC-me+/− mice were found to be embryonic lethal (data not shown). ABC-me−/− mice did not show any apparent phenotype under basal conditions, and their fertility was similar to that of wild-type mice. No differences in body weight, heart weight, or the ratio of heart weight to body weight were observed between wild-type and ABC-me+/− mice (the Table). Cardiac function was assessed by ex vivo Langendorff preparation, echocardiography, and tissue Doppler. Systolic and end-diastolic pressure-volume curves and developed pressure were similar over a range of LV volumes in wild-type and ABC-me+/− hearts (Figure 1A). Echocardiography showed that LV end-diastolic volume, end-systolic volume, wall thickness, mass index, and fractional shortening were similar in wild-type and ABC-me+/− hearts (the Table). Tissue Doppler also revealed similar diastolic function (Figure 1B and the Table).

Consistent with normal hemodynamic function, the rate of mitochondrial oxygen consumption measured in the presence of substrates that drive respiration through complex I or II was also normal in ABC-me+/− hearts (Figure 2A and 2B). This was also confirmed by the lack of differences in mitochondrial structure visualized by confocal microscopy (data not shown) or in maximal respiratory capacity and respiration linked to ATP synthesis measured with the Sea-

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**Table. Heart Weight, Echocardiographic Dimensions, and Doppler Analysis**

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**Figure 1.** ATP binding cassette mitochondrial erythroid+/− (ABC-me+/−) hearts have normal structure and function. A, Left ventricular pressure-volume relationships in isolated Langendorff-perfused wild-type (WT; black symbols) and ABC-me−/− hearts (open symbols) (n = 7). Upper traces represent systolic pressure and lower traces represent end-diastolic pressure. B, Representative tracings of mitral inflow and tissue Doppler analysis of WT and ABC-me+/− hearts. For values, see the Table.

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BW indicates body weight; HW, heart weight; IVSTh, interventricular septal thickness in diastole; PWTth, posterior wall thickness in diastole; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVMI, LV mass index; FS, fractional shortening; HR, heart rate; E, early diastolic velocity; A, late diastolic velocity; and m, mitral. Data are shown as mean±SEM. n = 3 to 6 per group.

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**Histology to Detect Sarcoplasmic Reticulum Calcium ATPase Oxidation**
This methodology was performed as described elsewhere.21

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After Ischemia/Reperfusion Recovery and Increased Oxidative Stress

used as a positive control because it increases superoxide production (solid bars, WT; open bars, ABC-me). In contrast, in ABC-me hearts there was a 55% recovery of developed pressure after 20 minutes of reperfusion (Figure 3A and 3D). In wild-type hearts, the recovery of developed pressure was absolute levels that exceeded that of untreated wild-type hearts (Figure 3). Of note, EUK-207 increased developed and systolic pressures in both ABC-me hearts and wild-type hearts to similar absolute values observed in wild-type hearts (Figure 3). Importantly, the degree of developed inhibition of oxidative metabolism in ABC-me hearts caused by ischemia/reperfusion (Figure 3B). Of note, EUK-207 increased developed and systolic pressures in both ABC-me hearts and wild-type hearts to similar absolute values observed in wild-type hearts (Figure 3). Importantly, the degree of developed

horse XF24 in intact cardiomyocytes isolated from adult ABC-me and wild-type mice (Figure II in the online-only Data Supplement). In addition, under basal conditions, there was no evidence of increased oxidative stress in ABC-me hearts, as reflected by protein carbonylation levels (Figure 3B). Of note, EUK-207 increased developed and systolic pressures in both ABC-me hearts and wild-type hearts to similar absolute values observed in wild-type hearts (Figure 3). Importantly, the degree of developed

**ABC-me hearts show impaired hemodynamic recovery and increased oxidative stress after ischemia/reperfusion**

To study the role of ABC-me in the recovery from ischemia/reperfusion, Langendorff preparations of wild-type and ABC-me hearts after Langendorff (n = 4). State III was induced by incubating mitochondria with 0.25 mmol/L ADP, state IV by 2 μmol/L oligomycin, and uncoupled respiration by 100 μmol/L of 2,4-dinitrophenol (DNP). Respiration was driven by (A) complex I (5 mmol/L pyruvate and 5 mmol/L malate) or (B) complex II (5 mmol/L succinate and 2 μmol/L rotenone). C, Representative Western blot analysis from total lysates of WT and ABC-me hearts (n = 7) to detect carbonylated proteins (Coomassie blue staining of the transferred gel was used as a loading control). D, Mitochondrial superoxide production rates were addressed by monitoring the linear increase of Mitosox fluorescence in a 96-well plate with 5 to 10 μg mitochondria from WT and ABC-me hearts. Antimycin A was used as a positive control because it increases superoxide production (solid bars, WT; open bars, ABC-me; n = 6). E, Representative Western blot analysis from total lysates of WT and ABC-me hearts (n = 7) to detect mitochondrial superoxide dismutase (sod2) and catalase. The Coomassie blue staining of the transferred gels and Porin were used as loading controls. Data are shown as mean ± SEM.

**A Superoxide Dismutase/Catalase Mimetic (EUK-207) Rescues Contractile and Mitochondrial Function After Ischemia/Reperfusion in ABC-me hearts**

To test to which extent increased oxidative stress mediates the reduced recovery from ischemia/reperfusion in ABC-me hearts, 20 minutes before the start of ischemia, we perfused hearts with EUK-207, a catalytic antioxidant with catalase and SOD activities.22 EUK-207 pretreatment completely prevented the impairment in recovery of hemodynamic function in ABC-me hearts caused by ischemia/reperfusion (Figure 3). EUK-207 normalized end-diastolic pressure in ABC-me hearts to the absolute values observed in wild-type hearts (Figure 3B). Of note, EUK-207 increased developed and systolic pressures in both ABC-me hearts and wild-type hearts to similar absolute levels that exceeded that of untreated wild-type hearts (Figure 3A, 3C, and 3D). Importantly, the degree of developed

**Figure 2.** ATP binding cassette mitochondrial erythroid 2/ (ABC-me) hearts have normal mitochondrial function and oxidative status under basal conditions. A and B, Oxygen consumption rates (OCR) of isolated mitochondria (20 μg) from wild-type (WT) and ABC-me hearts after Langendorff (n = 4). State III was induced by incubating mitochondria with 0.25 mmol/L ADP, state IV by 2 μmol/L oligomycin, and uncoupled respiration by 100 μmol/L of 2,4-dinitrophenol (DNP). Respiration was driven by (A) complex I (5 mmol/L pyruvate and 5 mmol/L malate) or (B) complex II (5 mmol/L succinate and 2 μmol/L rotenone). C, Representative Western blot analysis from total lysates of WT and ABC-me hearts (n = 7) to detect carbonylated proteins (Coomassie blue staining of the transferred gel was used as a loading control). D, Mitochondrial superoxide production rates were addressed by monitoring the linear increase of Mitosox fluorescence in a 96-well plate with 5 to 10 μg mitochondria from WT and ABC-me hearts. Antimycin A was used as a positive control because it increases superoxide production (solid bars, WT; open bars, ABC-me; n = 6). E, Representative Western blot analysis from total lysates of WT and ABC-me hearts (n = 7) to detect mitochondrial superoxide dismutase (sod2) and catalase. The Coomassie blue staining of the transferred gels and Porin were used as loading controls. Data are shown as mean ± SEM.
pressure recovery in ABC-me+/− hearts treated with EUK-207 was greater (from 27% to 90%; 3.3-fold increase) than in wild-type hearts (from 55% to 82%; 1.5-fold increase; Figure 3D), demonstrating the selectivity of the rescue by EUK-207 in ABC-me+/− hearts. To further address the mechanism leading to impaired ischemia/reperfusion recovery and to confirm the relevance of increased mitochondrial lipid oxidation in ABC-me+/− hearts, we measured mitochondrial respiration rates after 20 minutes of reperfusion. Respiration measurements were made in state III (respiration linked to maximal ATP synthesis rate), state IV (respiration not linked to ATP synthesis), and uncoupled respiration (by adding 2,4-dinitrophenol, which uncouples oxygen consumption from ATP synthesis). Ischemia/reperfusion selectively decreased mitochondrial respiration rates in all states and under 2,4-dinitrophenol in ABC-me+/− hearts compared with wild-type hearts, suggesting impairment of the electron transport chain (Figure 4A). This impairment also caused a marked decrease in mitochondrial ATP synthesis rates (Figure 4B). Of note, pretreatment with EUK-207 completely prevented the decrease in mitochondrial respiration caused by ischemia/reperfusion in ABC-me+/− hearts but had no effects on mitochondrial respiration in wild-type hearts after reperfusion (Figure 4A). This lack of EUK-207 effect in wild-type mitochondrial respiration demonstrated again the specificity of the mitochondrial defect in ABC-me+/− hearts after reperfusion and the selectivity of its correction by EUK-207 treatment. The correction of mitochondrial respiration by EUK-207 was accompanied by complete restoration of mitochondrial ATP synthesis rates in ABC-me+/− hearts (Figure 4B). This was associated with a marked increase in total ATP levels (Figure 4C). Further-

Figure 3. ATP binding cassette mitochondrial erythroid+/− (ABC-me+/−) hearts have increased oxidative damage and impaired recovery after ischemia/reperfusion (I-R), which is specifically rescued by the superoxide dismutase/catalase mimetic EUK-207 (EUK). Continuous recording of left ventricular pressure (baseline and during ischemia/reperfusion) was performed in isolated Langendorff-perfused hearts. A, Developed pressure (difference between systolic and diastolic pressures in mm Hg), B) end-diastolic pressure, and C) systolic pressure of wild-type (WT; solid symbols) and ABC-me+/− hearts (+/−; open symbols; n=7) pretreated (20 minutes before ischemia) with vehicle (continuous line) or 50 μmol/L EUK-207 (dashed line). D, Percentage of recovery of contractile function (ratio of developed [Dev] pressure at the end of reperfusion vs baseline) of WT and ABC-me+/− hearts (n=7) pretreated with vehicle or 50 μmol/L EUK-207. *P=0.02, WT vs ABC-me+/−; #P=0.002, vehicle ABC-me+/− vs EUK-207 ABC-me+/−; †P=0.03, WT vs WT EUK-207. E, Quantification of lipid oxidation by thiobarbituric acid reactive substances (TBARS) in isolated mitochondria from WT and ABC-me+/− hearts after ischemia/reperfusion or ABC-me+/− hearts pretreated with EUK-207 or with vehicle after ischemia/reperfusion (n=5). All data are shown as mean±SEM. *P=0.0079, WT vs ABC-me+/−; †P=0.03, vehicle ABC-me+/− vs EUK-207. F, Representative images of heart sections immunostained with an antibody detecting sulfonlated sarcoplasmic reticulum calcium ATPase on cysteine 674 from WT and ABC-me+/− hearts under basal conditions and after ischemia/reperfusion (IR). Scale bar, 25 μm.
more, EUK-207 treatment prevented the increase in both SERCA and mitochondrial lipid oxidation (Figure 3E and 3F). In addition, this increased oxidative damage caused some necrosis in ABC-me+/− hearts (detected as triphenyltetrazolium chloride–unstained areas within viable tissue), which was also prevented by EUK-207 (data not shown).

**Discussion**

We describe, for the first time, the phenotype of the ABC-me+/− mouse model and a role for the mitochondrial transporter ABC-me in the recovery of cardiac function after ischemia/reperfusion. This conclusion is supported by the impaired recovery of hemodynamic function after ischemia/reperfusion in mice having inactivation of 1 allele of ABC-me. Hemodynamic dysfunction was due to abnormalities in diastolic pressure and contraction, both of which are ATP dependent and thus reliant on normal mitochondrial oxidative phosphorylation.8,16 In this regard, we show an impaired oxidative status in mitochondria from ABC-me+/− hearts after reperfusion. This is demonstrated by the increase in mitochondrial respiration and ATP synthesis rates in ABC-me+/− hearts after reperfusion. These results are in line with several reports showing that lipid oxidation decreases mitochondrial respiration.8–10 Furthermore, we also observe an increase in SERCA oxidation in ABC-me+/− hearts, corroborating a lack of protection from oxidative stress induced by ischemia/reperfusion. In addition, this increased oxidative damage can explain the detection of some necrotic areas in ABC-me+/− hearts. Therefore, increased oxidative stress is sufficient to explain the hemodynamic dysfunction in ABC-me+/− hearts after ischemia/reperfusion.

To confirm that the alteration in the oxidative status was sufficient to cause the impaired recovery from ischemia/reperfusion in ABC-me+/− hearts, they were perfused with EUK-207, a catalytic antioxidant with catalase and SOD activities.28–30,36 EUK-207 treatment prevented the appearance of necrotic areas and the increase in oxidation of mitochondrial lipids and SERCA. Furthermore, the functional alterations in mitochondria and contractility of ABC-me+/− hearts were completely and selectively prevented by perfusion with EUK-207 shortly before the onset of ischemia. The
protective effect of this SOD/catalase mimetic suggests that inactivation of 1 allele of ABC-me decreases the ability of the cardiac myocyte to protect mitochondrial and contractile function from oxidative stress in the setting of ischemia/reperfusion. Consistent with this line of evidence, the ABC-me yeast ortholog (Mdl1p, 42% amino acid sequence similarity) protects against increased oxidative stress caused by iron accumulation in mitochondria triggered by ATM1 deletion.26 Also consistent with our observation, partial loss of sod2 (mitochondrial SOD), but not of sod1 (cytosolic), causes a worsening of the recovery from ischemia/reperfusion that is similar to that observed with ABC-me+/−/− hearts.3

We selected EUK-207 for these experiments because it exerts both SOD and catalase activities. Similar pretreatments with a predominant SOD mimic (eg, MnTBAP in cardiac ischemia/reperfusion) have been shown to increase the accumulation of hydrogen peroxide and to impair recovery after ischemia/reperfusion.6 On the other hand, when MnTBAP was administered with catalase, recovery after ischemia/reperfusion was improved compared with hearts treated with catalase alone or untreated hearts.6 Thus, it is likely that increasing both SOD and catalase activities, shortly before the onset of ischemia, is a better strategy to improve functional recovery from ischemia/reperfusion than short-term treatments predominantly increasing either SOD or catalase activities.

In contrast to the impaired response to ischemia/reperfusion, under basal conditions, ABC-me+/−/− hearts have normal mitochondrial respiration and hemodynamic function. Under basal conditions, there was also no evidence of increased oxidative stress as reflected by protein carbonylation, the upregulation of sod2 and catalase protein expression, the production of mitochondrial superoxide, or the detection of oxidized SERCA. This finding further suggests that the impaired response to ischemia/reperfusion is not due to an increase in basal oxidative stress. The lack of a basal cardiac phenotype is similar to observations in sod2+/− mice.3

A possible explanation for impaired mitochondrial oxidative status after ischemia/reperfusion is that partial loss of ABC-me affects heme synthesis and/or mitochondrial iron import in heart because ABC-me overexpression increases hemoglobinization and stabilizes the iron importer mitoferrin-1 in erythroid cell lines.23,25 Indeed, alterations in iron and/or in heme homeostasis are widely known to trigger mitochondrial dysfunction and/or oxidative stress. For example, the mouse heart model of Friedrich ataxia shows dramatic defects in mitochondrial iron homeostasis and is associated with a severe cardiac phenotype characterized by dilated cardiomyopathy at 4.5 to 9 weeks of age that is prevented by iron chelation.37 In this model, there is a marked decrease in the level of the complex II subunit Sdhα.37 However, in marked contrast, ABC-me+/−/− hearts have normal basal structure and function and do not develop dilated cardiomyopathy or changes in Sdhα or mitoferrin-1 levels (Figure IV in the online-only Data Supplement). Thus, it is unlikely that inactivation of 1 allele of ABC-me causes important defects in iron and/or heme homeostasis in heart under basal conditions.

Another potential explanation is that ABC-me transport activity is increased by ischemia/reperfusion and mediates protection from oxidative stress either by preventing the accumulation of molecules that would increase oxidative stress inside the mitochondria or by stimulating and/or enabling a cellular antioxidant response.

This study has potential clinical implications. First, these findings raise the possibility that genetic variations (mutations and/or polymorphisms) and/or drugs inactivating ABC-me may decrease tolerance for ischemia/reperfusion in humans. Second, this study demonstrates that pretreatment with antioxidants could be used to restore normal tolerance for ischemia/reperfusion in subjects showing genetic variations in ABC-me or to prevent the toxicity of drugs that could inactivate ABC-me.

Acknowledgments
We are thankful to Dani Dagan, PhD for comments on the manuscript, Richard A. Cohen, MD for SERCA antibodies, Janice M. Weinberg, ScD for advices on statistics, Wuhbet Abraham for technical assistance, and the rest of the members in Dr Shirihai’s laboratory. This work was supported by the Evans Center and the mitochondria-Affinity Research Collaborative (mARC).

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Disclosures
None.

References
SUPPLEMENTAL MATERIAL

Supplementary Methods.

Mitochondrial oxygen consumption measurements using XF24.

1.1 V7 plate loading:

The amount of mitochondria was titrated, as the absolute amount of protein loaded is highly dependent on the mitochondrial isolation protocol, the tissue, species and the loading methodology used. Titration is required in order to avoid O₂ depletion in the well. As a guideline, State II oxygen consumption rates (OCR) should be ~100-200 pmol/min/well and State III or uncoupled rates should not exceed 1600-1800 pmol/min/well.

Isolated mitochondria (20-40 μg in HES-BSA 0.2% buffer per well n=3-4 replicates per mouse) were loaded in the center of the well (using 5-10 μl, low volume that facilitates the contact and adhesion of mitochondria to the bottom of the well) of a V7 plate on ice and 440-445 μl of ice cold Mitochondrial Assay Solution (MAS: Sucrose 70 mM, Mannitol 220 mM, KH₂PO₄ 5 mM, MgCl₂ 5 mM, HEPES 2 mM, EGTA 1 mM, BSA fatty acid-free 0.2 %, pH 7.4 adjusted with KOH 1 M) + 50 μl of MAS buffer with 10x substrates (complex II driven respiration: succinate 50 mM + rotenone 20 μM; complex I: pyruvate + malate, 50 mM; or glutamate + malate, 50 mM each) were added on top (final concentration of substrates is 5 mM each and 2 μM for rotenone).

Alternatively, the mitochondrial suspension can be diluted in 1x MAS to 0.04-0.2 mg/ml and 50 μl loaded into the V7 plate wells (2-10 ug per well, depending on the species/tissue/substrates used). After loading, centrifugation of the V7 plate (20 minutes at 2000 x g at 4ºC) is performed to attach the mitochondria at the bottom of the plate.
After centrifugation, 450 µl of MAS + 1x substrates (ice cold, 10 mM substrate(s), 2 µM rotenone for complex II) are gently added to each well. This step was successfully developed by Dr. George W. Rogers, Dr. Alvaro E. Elorza and Dr. Anne N. Murphy.

The loaded V7 plate was incubated for 7-8 minutes at 37°C (no CO₂) before loading it into the XF24. Mitochondria attachment to the bottom of the plate can be observed using a microscope (20x) before and after the measurements. The initial consumption rate of oxygen measured before the first injection (port A) is state II (no ADP present, only respiration due to proton leak and contaminant ADP, also known as pseudo-state IV; see below).

Scheme plate loading (2 alternative methods):

1.2 Loading the cartridge: The dilutions of ADP and the different mitochondrial chemicals are freshly prepared the day of the experiment from concentrated stocks.
The four sequential injection ports of the Seahorse cartridge contained (in 1X MAS solution and adjusted to pH 7.4):

A (first port injected): 50 μl 10x substrate and ADP 2.5 mM;
B: 55 μl Oligomycin 20 μM (ATP synthase inhibitor);
C: 60 μl 2,4-dinitrophenol (DNP; uncoupler) 1 mM;
D: 65 μl Antimycin A 40 μM (complex III inhibitor).

Therefore the final concentrations are ADP 250 μM, Oligomycin 2 μM, DNP 100 μM and Antimycin 4 μM.

Oxygen consumption rates (pmols oxygen/min) were monitored in real time after the injection. State III was determined after port A injection, State IV after port B and uncoupled respiration rates after port C. Antimycin A was used as a control, as it blocks mitochondrial oxygen consumption linked to the electron transport chain. The ratio between state III and state IV is used as a control for the quality of the mitochondrial preparation and it is known as RCR (respiratory control ratio).

1.3 Measurement protocol (performed at 37 ºC)

Total XF Assay Time is ~ 65 min.: Load Cartridge (29 min calibration)
-Load V7 plate.

1) Equilibration (3 cycles of 2 min Mix, 2 min Wait).

2) State II (2 cycles of 25 sec Mix, 4 min Measure).

3) Injection port A.

4) State III (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).

5) Injection port B.
6) State IV (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).

7) Injection port C.

8) Uncoupled (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).

9) Injection port D.

10) Antimycin A (1 cycle of 25 sec Mix, 4 min Measure).

* Adjust the time to ensure optimal replenishment of oxygen in the sensor.

Below, you will find a table with all the commands of the measurement protocol summarized above:

<table>
<thead>
<tr>
<th>Command</th>
<th>Time</th>
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<tr>
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</tr>
<tr>
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<td>A</td>
</tr>
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</tr>
<tr>
<td>Mix</td>
<td>1 min</td>
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</tr>
<tr>
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<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Measure</td>
<td>3 min</td>
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</table>

† The mixing commands AFTER measurement commands are optional and facilitate the sensors returning to ambient
O2 concentration. These steps are useful if the basal respiration rate (OCR) is above 200 pmol/min.

1.4 Representative OCR tracings.

For state III and DNP (uncoupled respiration), the higher point to point value of OCR (oxygen consumption rates, pmols oxygen consumed per minute, calculated using the AKOS algorithm (which is a component of the XF Reader Software) for each sample was selected. For state IV and antimycin A, the lower point to point OCR values were selected. The values selected in this particular trace are highlighted with arrows. In this representative tracing, we show isolated mitochondria from wild type hearts after Langerdorff (20 μg protein/well, n=4 replicates ± SEM) using 5 mM Succinate and 2 μM Rotenone. State III/State IV, the RCR = 5.5, showing that it is a good quality mitochondrial preparation, together with the inhibition with Antimycin A. Increase of respiration with the uncoupler (port C) must be close to state III or higher (also known as state IIIu).
*The mixing time after injection determines the optimal replenishment of O₂ after injection (it should be close to ambient O₂, i.e. 90 mmHg minimum). In addition, O₂ should never reach 0 mmHg

**Echocardiography and tissue Doppler.** Left Ventricle (LV) dimensions and systolic function were measured in mice, not anesthetized, using an Acuson Sequoia C-256 echocardiograph machine equipped with a 15 MHz linear transducer (model 15L8). Briefly, the heart was imaged in the 2-D parasternal short-axis view, and M-mode echocardiogram of the mid-ventricle was recorded at the level of papillary muscles. Interventricular septum thickness (IVSTh), posterior wall thickness (PWTh), LV end-diastolic (LVEDD) and end-systolic (LVESD) dimensions were measured from the M-mode image. LV fractional shortening (FS) was calculated as \([\text{LVEDD} - \text{LVESD}] / \text{LVEDD} \times 100\). LV mass was calculated as \(1.05 \times [(\text{LVEDD} + \text{IVSTh} + \text{PWTh})^3 - \text{LVEDD}^3] / \text{body weight}\). LV diastolic function was assessed in anesthetized mice by Tissue Doppler echocardiography using a VisualSonics Vevo 770 high-resolution imaging system (Toronto, Canada) equipped with a 30-MHz RMV-707B transducer. Images were collected to measure myocardial peak early (E, \(E_m\)) and late (A, \(A_m\)) diastolic velocities, and calculate E/A and \(E_m/A_m\) ratios.
**Mitochondrial ATP synthesis rates.** ATP synthesis rates were measured in mitochondrial fractions using the ATP Bioluminescence Assay Kit CLS II (Roche). The increase in luminescence was measured using the Infinite Tecan M1000 Microplate reader in a mitochondrial suspension (5-10 μg in 150 μl) respiring at room temperature in the presence of 1 mM ADP and 5mM Pyruvate/Malate. The slope of luminescence increase with time was transformed to nmols ATP/min.mg protein by using a standard curve of luminescence vs. different ATP concentration values.

**Western blot.** The antibodies used (rabbit polyclonal) were diluted in 1% BSA in PBS-Tween (0.05%): Catalase (Abcam, dilution 1/1000), sod2 (Stressgen, dilution 1/2000), Porin (Abcam, dilution 1/1000, used as mitochondrial protein loading control). Ponceau staining of the membrane and/or Comassie-blue staining of the transferred membranes were also used as loading controls. ABC-me antibody was used as previously described\(^1,2\)

**Protein carbonylation/oxidation detection.** Total heart lysates were derivatized with 2,4-dinitrophenylhydrazine and detected by Western blot using an anti-2,4-dinitrophenol antibody. This procedure was realized using Oxyblot kit (Chemicon, Millipore) following manufacturer’s instructions.

**Real time PCR.** Hearts (washed from blood using ice cold PBS) were frozen in liquid nitrogen and grinded using a mortar/pestle (in dry ice, to avoid tissue thawing). 30 mg of grinded heart were used to extract total RNA using RNeasy Plus Mini Kit from Qiagen®
(following manufacturer’s instructions). After extraction, 280 ng of RNA ($R_{260\text{nm}/280\text{nm}} >$ 2) were retro-transcribed to cDNA using High Capacity RNA to cDNA from Applied Biosystems (20 µl reaction, following manufacturer’s instructions). 10 ng of cDNA were loaded per well in a 96-well plate (20 µl reaction) per triplicate. Taq Man ® Gene Expression Master Mix 2x, GAPDH VIC Probe or TaqMan® Gene Expression Assay 20x (Mm00497926_m1) for ABC-me (ABCB10) were used. RT-PCR was performed using the StepOne Plus RT-PCR system (Applied Biosystems). ABC-me expression levels were calculated by ΔΔCt method using GAPDH as a control.

**Seahorse XF24 respirometry in intact adult mouse cardiomyocytes.** Cardiomyocytes were isolated from wild type and ABC-me +/- male mice (3 month-old). They were plated in isolation media (MEM Cat No 11575: 5.56 mM Glucose and amino acids, 2mM Glutamine) and 125 µl (2x10⁴ cells) were loaded per laminin-coated well (V7 plate). 30-45 minutes after plating, the isolation media was replaced by 700 µl of fresh MEM media (warm at 37°C) and the plate was loaded into the Seahorse XF24. Twenty replicates were performed per heart and the average value was taken per each mouse/experiment (from 20 wells, one heart per V7 plate). The first OCR values (oxygen consumption rates) measured reflect basal respiration (cardiomyocytes in MEM), then oligomycin (final concentration 2µM) was injected (port A) in order to measure respiration not-linked to mitochondrial ATP synthesis. FCCP 1.5 µM (uncoupler, port B) was injected to measure the maximal respiratory capacity and Antimycin 10 µM to measure not-mitochondrial-oxidative phosphorylation dependent respiration (inhibitor of complex III, port C). All dilutions were performed in MEM.
Supplementary Figure 1

A. Schematic diagram of the ABCB10 (ABC-me) gene with restriction enzyme sites and primer locations. 5' external probe 23+20 and 3' external probe 21+22.

B. Gel analysis of ABC-me +/- and ABC-me -/- embryos compared to WT. Primers used: 339 bp, 286 bp. KO insert/gene (Neo3a/11) and WT (3/4).

C. Western blot analysis of embryonic lysates for ABC-me (65 kDa) and Actin (42 kDa).

D. Cardiac ABC-me levels with relative mRNA levels shown for Wild type and ABC-me +/- with an asterisk (*) indicating a significant difference.

Supplementary Figure 1
Supplementary Figure 2

A

![Graph A showing OCR (pmols O₂/min) for Basal, Not-linked to ATP synthesis, and Maximal respiration.]

B

![Graph B showing respiration linked to ATP synthesis (% decrease after oligomycin injection) for Wild type and ABC-me +/-.]
Supplementary Figure 3

**Input**
- **Wt**
  - Serca
  - Sdha

**Pull down**
- **Wt**
  - Serca
  - Sdha
- **ABC-me +/-**
  - Serca
  - Sdha

**Non-oxidized SERCA cysteines**

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<tr>
<td><strong>ABC-me +/-</strong></td>
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</table>
Supplementary Figure 4

WT   ABC-me+/-

Mfrn1

37 kDa

WT   ABC-me+/-

Sdha

50 kDa

WT   ABC-me+/-

Porin

37 kDa

Ponceau
Supplementary Figure 5: Individual observations from the figures in the manuscript.

Figure 2B

Succinate + Rotenone

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<table>
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<tr>
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Figure 3E

<table>
<thead>
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<table>
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<tr>
<td>ABC-me +/-</td>
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</tr>
<tr>
<td>EUK-207</td>
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<tr>
<td>ABC-me +/-</td>
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</tbody>
</table>
Supplementary Figure 6: Individual observations from the figures in the manuscript.

Figure 4A

Succinate + Rotenone

OCR (pmols O₂/min per 40 ug protein)

WT, Het, EUK, HEUK

State III, State IV, DNP

Figure 4B

nmols ATP / min. mg protein

WT, ABC-me +/-, Vehicle, EUK-207

ABC-me +/-
Supplementary Figure 7: Individual observations from the figures in the manuscript.

Figure 4C
Supplementary Figure Legends

**Supplementary Figure 1.** (A) Schematic representation of the ABCB10 (ABC-me) gene and the pKOS 61 vector showing the insertion sites, the annealing sites for PCR genotyping primers (3,4, Neo3a and 11), restriction sites and genotype strategies are denoted. (B) PCR of Genomic DNA of wild type (WT), ABC-me +/- and ABC-me -/- using wild type (WT) (3,4) and KO insert (Neo3a, 11) primers. (C) Representative Western blot of cell lysates from wild type (WT) and ABC-me -/- embryos. (D) Real time PCR measurements of total RNA retro-transcribed to cDNA from wild type and ABC-me +/- hearts (n=3 mice per group) using Taq Man® probes. For ABC-me (ABCB10), the set of primers used for RT-PCR annealed in exon 2 and 3 (region deleted by the KO insert; TaqMan® Gene Expression Assays, Mm00497926_m1). GAPDH was used as a control. Relative expression values to wild type hearts are shown ± SEM. Unpaired, two tailed, non-parametric test (based on Chebyshev’s inequality) was performed and * shows statistical significance p=0.00794.

**Supplementary Figure 2.** Respirometry of wild type and ABC-me +/- cardiomyocytes isolated from 3-month old male mice under basal conditions (n=3-4 per genotype). Wild type OCR (oxygen consumption rate) values are shown in black bars; ABC-me +/-, open bars. (A) Bars represent the average (n=3-4 mice per group) of OCR values ± SEM after subtraction of not-mitochondrial-oxidative phosphorylation OCR (respiration after Antimycin 10 μM injection) under 3 different states: 1) Basal respiration (in the presence of MEM media, 5.6 mM Glucose and 2 mM Glutamine). 2) Respiration not-linked to ATP synthesis (in the presence of 2 μM Oligomycin). 3) Maximal respiration (in the
presence of the uncoupler FCCP, 1.5 µM). (B) Quantification of the respiration linked to mitochondrial ATP synthesis. The percentage of decreased respiration induced by oligomycin injection (related to basal respiration) was calculated in every experiment/mouse (same experiments shown in panel A). Bars show the average of n=4 experiments and mice per genotype ± SEM. No statistically significant differences were detected.

**Supplementary Figure 3.** Representative image and quantifications of Western blot assays detecting pulled-down BIAM-labeled SERCA (SERCA with free cys-SH) and SERCA input of wild type and ABC-me +/- heart lysates (n=3 per group) after ischemia-reperfusion. The same membranes were also probed for Sdha, showing no significant differences in loading. BIAM labeling was performed as previously described. Unpaired, two-tailed, non-parametric test (based on Chebyshev’s inequality) was performed and * shows statistically significance p<0.00446.

**Supplementary Figure 4.** Representative images of Western blot analyses to detect Sdha (complex II electron transport chain, 70 kDa) and Mfrn1 (around 37 kDa) expression in total lysates and mitochondrial fractions respectively of 3 month-old wild-type and ABC-me +/- hearts (n=3-7 per genotype, 20 µg of protein were loaded). Ponceau staining was used as a loading control for Mfrn1 antibody and Porin for Sdha detection in total lysates.
Supplementary Figures 5-7: Individual observations from the figures in the manuscript. See Figure legends in the manuscript for specific details.

Acknowledgements.

Mfrn1 antibody was a gift from Barry Paw (Harvard Medical School).

Supplementary references

