ORIGINAL RESEARCH COMMUNICATION

Elucidating Mitochondrial Electron Transport Chain Supercomplexes in the Heart During Ischemia–Reperfusion

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Abstract

Aims: Mitochondrial supercomplexes (SCs) are the large supramolecular assembly of individual electron transport chain (ETC) complexes that apparently provide highly efficient ATP synthesis and reduce electron leakage and reactive oxygen species (ROS) production. Oxidative stress during cardiac ischemia–reperfusion (IR) can result in degradation of SCs through oxidation of cardiolipin (CL). Also, IR induces calcium overload and enhances reactive oxygen species (mitROS) in mitochondria that result in the opening of the nonselective permeability transition pores (PTP). The opening of the PTP further compromises cellular energetics and increases mitROS ultimately leading to cell death. Here, we examined the role of PTP-induced mitROS in disintegration of SCs during cardiac IR. The relationship between mitochondrial PTP, ROS, and SCs was investigated using Langendorff-perfused rat hearts subjected to global ischemia (25 min) followed by short-time (5 min) or long-time (60 min) reperfusion in the presence or absence of the PTP inhibitor, sanglifehrin A (SfA), and the mitochondrial targeted ROS and electron scavenger, XJB-5-131. Also, the effects of CL deficiency on SC degradation, PTP, and mitROS were investigated in tafazzin knockdown (TazKD) mice.

Results: Cardiac IR induced PTP opening and mitROS generation, inhibited by SfA. Percent distributions of SCs were significantly affected by IR, and the effects were dependent on the reperfusion time and reversed by SfA and XJB-5-131. TazKD mice demonstrated a 40% lower SC I+III+IV with reduced basal mitochondrial PTP, ROS, and ETC complex activity.

Innovation and Conclusion: Sustained reperfusion after cardiac ischemia induces disintegration of mitochondrial SCs, and PTP-induced ROS presumably play a causal role in SC disassembly. *Antioxid. Redox Signal.* 27, 57–69.

Keywords: mitochondria, cardiac ischemia–reperfusion, ETC supercomplexes, permeability transition pore, cardiolipin, cyclophilin D

Introduction

CORONARY HEART DISEASE is characterized by severe impairment of coronary blood supply, which produces myocardial ischemic injury and is accompanied by a wide range of clinical symptoms. Indeed, timely reperfusion or restoration of coronary perfusion is the only effective therapeutic intervention for protecting the heart against ischemic injury or myocardial infarction (33,44). However, although reperfusion salvages viable myocardium, it can also damage

the myocardium up to 50% more than ischemia alone, known as “reperfusion injury” that increases arrhythmia and infarction size, while diminishing myocardial contraction (44,66).

During ischemia, the loss of oxygen compromises mitochondrial ATP production and induces a rise in intracellular $[Ca^{2+}]$. Reperfusion further increases intracellular $[Ca^{2+}]$, producing a calcium overload in the cytoplasm and mitochondria, and enhancing the generation of reactive oxygen species (ROS) by damaged mitochondrial electron transport chain (ETC) complexes. These factors, along with high $[Pi]$,

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Innovation

Few studies, if any, have investigated a causal role of permeability transition pores (PTP) in supercomplex (SC) degradation in cardiac ischemia–reperfusion. This study, for the first time, demonstrates that SC disassembly occurs after a long-time (60 min) reperfusion that is prevented by sanglifehrin A (PTP inhibitor) and XJB (a mitochondria-targeted antioxidant), suggesting a causal role of PTP opening in SC degradation. Furthermore, long-time reperfusion induces remarkably higher PTP-dependent mitROS generation compared to short-time (5 min) reperfusion. Notably, PTP opening induced by short-time reperfusion has a greater causal role in mitochondrial swelling than long-time reperfusion. Finally, tafazzin knockdown hearts demonstrate enhanced degradation of the SCs and high basal mitochondrial swelling.

result in changes in mitochondrial membrane permeability defined as the mitochondrial permeability transition concurrently with opening of the nonselective, high-conductance permeability transition pores (PTP) in the inner mitochondrial membrane (IMM) (8,24). PTP opening further increases mitochondrial Ca^{2+} and ROS (mitROS) and stimulates protein and lipid oxidation in mitochondria. Oxidation of cardiolipin (CL), a hallmark phospholipid of the IMM, diminishes ETC activity, possibly by degrading ETC supercomplexes (SCs), which ultimately compromises cellular energetics leading to mitochondria-mediated cell death (11,45).

Stress associated with mitochondrial swelling has a strong impact on the proteome biology of cardiac mitochondria (67). Growing evidence indicates that ETC complexes are assembled into large supramolecular complexes, or SCs, under physiological conditions (14). Blue native gel electrophoresis (BNGE) revealed two types of SCs in mammalian mitochondria: $\text{I}_1+\text{III}_2+\text{IV}_1$ and III_2+IV_1 (2,56). The main SC, or respirasome $\text{I}_1+\text{III}_2+\text{IV}_1$, has been found in the mitochondria of rodent (62), dog (54), and bovine (56) hearts. According to the solid-state model, the assembly of SCs can provide high-efficiency electron flux throughout the ETC, increase ATP synthesis, and reduce electron leakage and mitROS production due to short diffusion distances between complexes (2,9,13,35). However, it should be noted that functional and catalytic advantages of SCs are disputed, and it is still not clear whether channeling in SCs is kinetically important to provide highly efficient ATP synthesis (10).

Normal assembly and stability of SCs require CL, a phospholipid that contributes to the function of many proteins and is actively involved in ETC integrity and activity. Electrophoretic (51,68), kinetic (69), and structural (4) studies provide strong evidence that CL is a key to the structural organization, stability, and function of SCs. Indeed, mitochondria lacking CL demonstrate degradation of SCs (51,68). Furthermore, mitROS-induced oxidation and depletion of CL in bovine heart mitochondria were associated with diminished activity of the ETC complexes I, III, and IV (46,47). Studies with artificial membranes indicate that CL peroxidation not only affects the organization and stability of individual ETC complexes but also of entire SCs (21). Likewise, mutation or lack of tafazzin, a

mitochondrial transacylase involved in the synthesis of CL, induces inactivation and destabilization of SCs in patients with Barth syndrome (43) and in tafazzin knockdown (TazKD) mice (26).

Few studies, if any, have investigated a link between SC degradation and the PTP in cardiac diseases. Heart failure induced by coronary microembolization decreased the amount of SC $\text{I}_1+\text{III}_2+\text{IV}_1$ in dogs (54). Ischemic preconditioning had no effect on the level of mitochondrial SCs during global ischemia–reperfusion (IR) in Langendorff-perfused mice hearts (64); however, isoflurane stabilized SCs consisting of complexes III and IV during *in vivo* cardiac IR (40). Elevated mitROS generation during cardiac IR may significantly destabilize mitochondrial SCs, possibly through CL oxidation, although there are no data confirming this. On the contrary, synthetic oxidized CL (48) and oxidation of endogenous CL (49) sensitized mitochondria to PTP opening only after addition of Ca^{2+} . Although many studies indicate the role of mitochondrial Ca^{2+} overload and CL oxidation in cardiac IR (37,50), there are no data on the cause-and-effect relationship between CL, SC disintegration, and the PTP.

In this study, we investigated the possible role of PTP opening in the disintegration of SCs, during cardiac IR in rats. We found that SC levels depended on the duration of reperfusion (5 and 60 min) following sustained global ischemia. Degradation of SCs occurs at late reperfusion and can be alleviated by inhibition of the PTP opening and ROS production. In addition, the loss of SC integrity is associated with decreased ETC activity, less ROS production, and mitochondrial swelling in the hearts of TazKD mice.

Results

Postischemic recovery of the heart

The experimental design of the studies is shown in Figure 1. We examined the effects of the PTP inhibitor, sanglifehrin A (SfA), on postischemic recovery of the hearts subjected to IR. SfA improved the recovery of left ventricular developed pressure (LVDP) from 23% in untreated hearts to 42% ($p < 0.05$, IR60 vs. IR60+SfA) at 60 min of reperfusion (Fig. 2A). In addition, we perfused the hearts with XJB-5-131 (XJB), a recently developed antioxidant (61) that targets mitochondria and provides mitROS and electron scavenging capacity by virtue of its conjugation to a 4-amino-2,2,6,6-tetramethylpiperidinoxy (4-Amino-TEMPO) moiety (20). We have previously shown that XJB protected against global cerebral IR in adult rats (32), and cardiac IR in aged rats (19).

XJB increased the recovery of LVDP from 23% (IR group) to 49% ($p < 0.05$, IR60 vs. IR60+XJB) at 60 min of reperfusion (Fig. 2A). Likewise, cardiac work expressed as the rate pressure product (RPP) was 79% and 47% ($p < 0.05$ for both) higher for SfA- and XJB-treated groups, compared to untreated hearts at the end of reperfusion (Fig. 2B). Hearts subjected to IR showed increased lactate dehydrogenase (LDH) activity in the coronary perfusate, indicating increased cell death. Administration of SfA and XJB also decreased the LDH activity at reperfusion (Fig. 2C).

mitROS production and PTP

Basal (no Ca^{2+} added) and Ca^{2+} -induced mitochondrial swelling was determined as a marker of PTP opening (Fig. 3A).

FIG. 1. Experimental design and groups. Groups: (i) P60, nonischemic hearts perfused for 60 min ($n=9$); (ii) I25, hearts subjected to 25-min global ischemia ($n=10$); (iii) I25+SfA, hearts subjected to 25-min global ischemia in the presence of $0.5 \mu\text{M}$ SfA ($n=7$); (iv) IR5, hearts subjected to 25-min global ischemia followed by 5-min reperfusion ($n=8$); (v) IR5+SfA, hearts subjected to 25-min global ischemia followed by 5-min reperfusion in the presence of $0.5 \mu\text{M}$ SfA ($n=7$); (vi) IR60, hearts subjected to 25-min global ischemia followed by 60-min reperfusion ($n=9$); (vii) IR60+SfA, hearts subjected to 25-min global ischemia followed by 60-min reperfusion in the presence of $0.5 \mu\text{M}$ SfA ($n=8$); and (viii) IR60+XJB, hearts subjected to 25-min global ischemia followed by 60-min reperfusion in the presence of $0.2 \mu\text{M}$ XJB. IR, ischemia-reperfusion; SfA, sanglifehrin A.

FIG. 2. Postischemic recovery of Langendorff-perfused hearts at the end of reperfusion (60 min). (A) LVDP. Data are presented as a percentile of preischemic values. (B) RPP. Data are given in mmHg·beats/min. (C) LDH activity in the coronary effluent of the hearts subjected to 25-min ischemia followed by 60-min reperfusion in the presence and absence of SfA or XJB. LDH activity is shown as IU per gram heart. * $p < 0.05$ IR60 versus IR60+SfA and IR60+XJB. * $p < 0.05$ versus IR60+SfA and IR60+XJB. LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; RPP, rate pressure product.

Mitochondria isolated from the I25 and IR5 groups showed 29% ($p < 0.05$) less absorbance, indicating a greater degree of swelling than the control group during ischemia and at the onset (5 min) of reperfusion. The basal swelling was attenuated by 49% ($p < 0.05$) in the hearts treated with SfA at 5 min, and by 38% ($p < 0.05$) at 60 min (Fig. 3B). Calcium-induced swelling in the IR5 group was higher than in the IR60 group, and this was relieved by 55% ($p < 0.05$ IR5 vs. IR5+SfA) in the presence of SfA. Interestingly, SfA failed to prevent Ca^{2+} -induced swelling of mitochondria isolated from heart after 60 min of reperfusion (Fig. 3C). These findings suggest that SfA-sensitive mitochondrial swelling opening occurs at early reperfusion, followed by PTP-independent swelling with progression of reperfusion.

To determine the role of mitochondrial swelling in mitROS production, we treated isolated mitochondria with gradually increasing concentrations of Ca^{2+} , which increased mitROS generation in a dose-dependent manner (Fig. 3D and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). Analysis of mitROS production in isolated mitochondria revealed remarkable increases of mitROS after both 5 and 60 min of reperfusion that were 2.1 ($p < 0.05$) and 5.7 times ($p < 0.001$) higher, respectively, than in control (P60) hearts.

Notably, sustained reperfusion (IR60) dramatically increased mitROS generation compared with short-time reperfusion (IR5) (Fig. 3E). During both periods of reperfusion, mitROS levels were significantly abrogated in the hearts treated with SfA, suggesting that mitROS production was mostly PTP dependent. SfA had no antioxidant effect on isolated mitochondria *in vitro* in the presence of Ca^{2+} (Supplementary Fig. S1). Likewise, mitROS production was significantly reduced in the presence of XJB at 60 min of reperfusion (Fig. 3E).

Analysis of the activity of individual ETC complexes revealed no remarkable changes after ischemia and reperfusion (5 and 60 min), except in the activity of complex III that was increased 23% ($p < 0.05$) at 5 min and decreased 19% ($p < 0.05$) at 60 min of reperfusion, compared with the control group (Supplementary Fig. S2A–D). Respiration rates (state 3) of mitochondria were lower in all experimental groups than the control group. Ischemia markedly reduced

FIG. 3. PTP opening and ROS production in mitochondria of rat hearts subjected to ischemia followed by 5-min (IR5) or 60-min (IR60) reperfusion in the presence or absence of SfA or XJB. (A) Representative traces of the mitochondrial swelling as the indicator of PTP opening. Swelling of mitochondria in the presence or absence of calcium was determined by monitoring the decrease in light scattering at 525 nm in the presence (gray line) and absence (black line) of 0.5 μ M SfA. Each arrow shows the addition of 100 μ M CaCl_2 . (B) Basal (no exogenous Ca^{2+} added) swelling of mitochondria. (C) Rates of swelling induced by 200 μ M CaCl_2 . (D) Rates of ROS production in the presence (black line, closed circles) and absence (gray line, open circles) of calcium in mitochondria isolated from control (nonischemic) hearts. The values are expressed in pmol of H_2O_2 produced per minute per mg protein. (E) Rates of ROS production in mitochondria isolated from the hearts in experimental groups. * $p < 0.05$. PTP, permeability transition pore; ROS, reactive oxygen species.

the respiration rate by 63% ($p < 0.05$) and there were no significant changes at reperfusion in the presence or absence of SfA and XJB (Supplementary Fig. S2E, F). Interestingly, the activity of complex I was not affected in early reperfusion although the respiration rate at complex I was significantly diminished. Our data are consistent with previous studies when the complex I-linked respiration was inhibited after IR with no changes of its enzymatic activity (59).

Degradation of ETC SCs and CL during IR injury

To analyze mitochondrial ETC SCs, isolated mitochondria were treated with the solubilization buffer containing digitonin or dodecyl maltoside (DDM). As reported earlier, only digitonin was able to maintain the structural integrity of SCs in the native electrophoresis condition, whereas DDM strongly induced disintegration of SCs (Fig. 4A). Second-dimension SDS-PAGE with subsequent immunoblotting was used to identify each individual ETC complex as individual bands (Fig. 4B). The results of two-dimensional (2D) BNGE revealed that 58% of complex I was involved in the structure of SCs. Interestingly, the highest molecular weight band, corresponding to the SC I+III+IV, showed a broad range of molecular weight of the SCs (Fig. 4B, C, peak 7).

The patterns of the bands were reproducible between different sets of experiments, so we were able to directly identify each SC band on the blue native gel (Fig. 4D). The SC I+III+IV band may contain the individual ETC complexes I, III, and IV in

different combinations, including SC I₁+III₂+IV₁. Additional blue native (BN) gel images and 2D Western immunoblots are shown in Supplementary Figures S3 and S4.

Analysis demonstrated that SCs, including complex I (Fig. 4D, marked as "S"), were decreased after 60 min of reperfusion and recovered by SfA or XJB treatment (Fig. 4E). Interestingly, cardiac mitochondria isolated from CypD (*Ppif*) knockout (KO) mice exhibited no changes in SC I+III+IV and ETC individual complex activity between wild-type (WT) and KO mice. Total mitochondrial protein level and citrate synthase activity were 17% and 12% ($p < 0.005$ for both) less, respectively, in CypD KO hearts compared to WT animals (Supplementary Fig. S5).

Analysis of oxidized and intact CL levels in mitochondria demonstrated that ischemia has no effect on CL. However, hearts after reperfusion for 60 min had an apparent trend toward high levels of oxidized CL and low intact CL. The ratio of oxidized CL to intact CL at 60 min of reperfusion (IR60) was 30% ($p < 0.11$) and 43% ($p < 0.05$) higher than control (P60) and ischemia (I25), respectively. It should be noted that the reason oxidized CL levels were high in all groups, including control hearts, is to prevent spontaneous lipid oxidation; the antioxidant butylated hydroxytoluene was added to a final pellet of mitochondria at the end of isolation (but not the beginning of isolation). Treatment with SfA or XJB reduced the oxidized CL to intact CL ratio (Fig. 4F). Likewise, mitochondrial protein carbonylation and lipid peroxidation were increased by the end of the 60-min reperfusion (Supplementary Fig. S6).

FIG. 4. Analysis of ETC SCs in cardiac mitochondria. (A) Representative BN gel image of mitochondria solubilized by DG or DDM demonstrating that DG but not DDM is able to maintain SCs in the native electrophoresis condition. (B) Representative immunoblot of SCs solubilized with DG and separated by BNGE. (C) Densitometry analysis of complex I spots from 2D BNGE-immunoblot. (D) Representative BN gel image with individual bands of SCs identified. S, respiratory SCs containing complexes I, III, and IV; V_m and V_d, monomeric and dimeric complex V; I, Complex I; III, Complex III. (E) Levels of ETC complex I containing SCs (S) normalized by total density (Total) of each lane. * $p < 0.05$ versus the other groups. (F) The ratio of oxidized CL to intact (nonoxidized) CL in cardiac mitochondria. * $p < 0.05$ versus I25. BN, blue native; BNGE, blue native gel electrophoresis; DDM, dodecyl maltoside; DG, digitonin; ETC, electron transport chain; SC, supercomplex.

PTP opening and mitROS generation in cardiac mitochondria of TazKD mice

Mitochondria isolated from TazKD mice demonstrated 27% ($p < 0.005$) more basal swelling (no Ca^{2+} added) than mitochondria from WT hearts (Fig. 5A). Furthermore, mitochondria of TazKD hearts responded 49% ($p < 0.001$) less to Ca^{2+} compared to WT hearts (Fig. 5B). Notably, SfA significantly reduced mitochondrial swelling in both WT and TazKD hearts, indicating the contribution of PTP opening to the swelling. Genetic ablation of tafazzin may induce basal mitochondrial swelling, independent of CL-related alterations. Therefore, we analyzed the protein level of CyP-D, a key PTP regulator in cardiac mitochondria of TazKD and WT mice.

Surprisingly, the CyP-D level in TazKD mice was 13% ($p < 0.05$) higher than in WT animals (Fig. 5C). Also,

although we revealed no differences in basal levels of mitROS between WT and TazKD mice (Fig. 5D), TazKD mice demonstrated 38% ($p < 0.05$) less Ca^{2+} -induced mitROS generation than WT mice (Fig. 5E). Thus, these findings demonstrate high basal but low Ca^{2+} -induced mitochondrial swelling in TazKD hearts, associated with increased expression of CyP-D.

Tafazzin deficiency results in the disassembly of SCs and reduces activity of individual ETC complexes

We analyzed mitochondria from WT and TazKD mice to evaluate the effects of CL deficiency on the assembly of ETC SCs. As shown in Figure 6A and B, mitochondria of TazKD mice demonstrated no remarkable changes in complexes I

FIG. 5. PTP opening and ROS production in cardiac mitochondria isolated from WT and TazKD mice. (A) Basal (no Ca²⁺ added) swelling of mitochondria measured as the decrease in light scattering at 525 nm. Results are shown as A₅₂₅. (B) Rates of swelling induced by 200 μ M CaCl₂. (C) Protein levels of CyP-D. Data were normalized to cytochrome c oxidase IV (COXIV), a mitochondrial housekeeping protein, and expressed as a percent of the WT group. (D) Rates of ROS production in mitochondria in the absence of calcium. (E) Rates of Ca²⁺-induced ROS production in mitochondria in the presence and absence of SfA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. TazKD, tafazzin knockdown; WT, wild type.

and IV, or SC II+III. However, the levels of SC I+III+IV, also known as the respirasome, in TazKD mitochondria were 40% ($p < 0.001$) lower than in WT mice. In contrast, the SC III+IV was 13% higher ($p < 0.01$), compared to mitochondria isolated from WT mice.

Cardiac mitochondria isolated from TazKD mice showed decreased activities of the individual ETC complexes I, II, and III. Interestingly, the activity of complex IV was higher (22%, $p < 0.05$) in the hearts of TazKD mice compared to WT animals (Fig. 6C–F).

Discussion

This study demonstrated the following. (i) Both short-time reperfusion and long-time reperfusion (5 and 60 min, respectively) induce the same degree of mitochondrial swelling

in the heart. (ii) PTP opening has a greater effect on mitochondrial swelling in short-time reperfusion than in long-time reperfusion, since SfA effectively inhibits the swelling after short-time reperfusion. (iii) Both short-time reperfusion and long-time reperfusion stimulate PTP-dependent mitROS generation; the level of mitROS after long-time reperfusion is significantly higher than that after short-time reperfusion. (iv) Disintegration of SCs occurs only after sustained (60 min) reperfusion, in an SfA-dependent manner. (v) A deficiency of CL synthesis in TazKD mice enhances the degradation of the SC I+III+IV and reduces the activity of complex III. (vi) TazKD mice demonstrate high basal mitochondrial swelling and CyP-D protein expression.

Mitochondrial ETC complexes are believed to dynamically arrange in SCs or respirasomes, although their functional and catalytic advantages remain elusive. Mitochondrial SC association limited production of ROS from complex I (42), and dynamic SC assembly has been shown to determine electron flux from different substrates through the respiratory chain (35) although a robust flux control analysis provided no evidence for substrate channeling between the enzymes in SCs (10).

Although many studies elucidate the structural identity of ETC SCs (34), few, if any, examine the role of SCs in cardiac diseases. Heart failure induced by coronary microembolization for 13 weeks decreased the levels of SC I₁+III₂+IV₁ in canine hearts (54), suggesting that sustained oxidative stress contributes to the disintegration of SCs. In addition, alterations in respiration rates of individual ETC complexes appeared concurrently with SC degradation, indicating that SCs, and not individual ETC complexes, lead to heart failure-induced mitochondrial dysfunction (54). Consistent with these findings, short-term reperfusion had no effect on SC levels; rather, it surprisingly increased the activity of complexes II and IV. Degradation of the SC I+III+IV appeared when the reperfusion period was extended to 60 min, and was associated with reduced activity of complex III.

Degradation of the SC I+III+IV was prevented in hearts treated with SfA, a PTP inhibitor, suggesting the PTP may cause disintegration of SCs. In addition to mitochondrial swelling, PTP-induced ROS presumably contribute to SC disassembly. Previous studies using the [³H]-deoxyglucose method in intact Langendorff-perfused rat hearts demonstrated that the PTP remains closed during cardiac ischemia, but opens upon reperfusion (23,24). However, despite the absence of pore opening, PTP-independent swelling of mitochondria occurs during ischemia (38). A lack of SC degradation at the onset of reperfusion (Fig. 4E) suggests that swelling of the matrix is not sufficient to alter the integrity of SCs. We propose that PTP-induced mitROS play a critical role in the disintegration of SCs via the oxidation of CL. As in previous studies (7), we demonstrated that PTP opening stimulates mitROS production upon reperfusion and is greatly abrogated by SfA treatment.

Notably, despite the same degree of initial pore opening (no exogenous Ca²⁺) in both periods of reperfusion, long-term reperfusion significantly (2.7-fold) increased mitROS production compared to short-term reperfusion. Indeed, *in vitro* studies with isolated mitochondria demonstrated that tert-butyl hydroperoxide (t-BuOOH), a lipid-soluble peroxide that promotes lipid peroxidation, induced CL peroxidation in isolated cardiac mitochondria only after the addition of exogenous Ca²⁺ (49). These data suggest that mitochondrial

FIG. 6. Analysis of ETC SCs and activity of individual ETC complexes in cardiac mitochondria isolated from WT and TazKD mice. (A) Representative image of Coomassie-stained BN-PAGE of mitochondria. **(B)** Protein levels of SCs and individual ETC complexes. Data were normalized to a total level of ETC SCs and complexes, and expressed as a fold change compared to the WT group. **(C–F)** The activity of the ETC complexes I–IV expressed in $\text{nmols min}^{-1} \cdot \text{mg}^{-1}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

swelling sensitizes CL to the mitROS attack, leading to degradation of SCs. Simultaneous increases in mitROS and PTP opening appear to be the key players in the promotion of SC disassembly through oxidation of CL.

Cardiac phenotype of TazKD mice mimics biochemical, histological, and clinical findings in humans with Barth syndrome. Tafazzin gene inactivation in mice results in defective CL metabolism associated with a drastic reduction of C18-CL content, and the accumulation of monolysocardiolipins in cardiac mitochondria, increased mitochondrial abnormalities, depressed systolic function, and dilated cardiomyopathy (1,53,57). In addition, fetal cardiomyopathy and embryonic death also have been reported (52).

In our studies, TazKD mice demonstrated a high degree of basal swelling in cardiac mitochondria and were less responsive to exogenous Ca^{2+} compared to WT mice. Similar to WT hearts, Ca^{2+} -induced mitochondrial swelling was abrogated by SfA in TazKD hearts, further supporting the proposed role of PTP. Nonetheless, TazKD mice have the following limitations for

elucidating the cross talk between PTP opening and SC degradation. First, the deficiency in CL remodeling induced by tafazzin knock down is different from CL oxidation induced by oxidative stress. We analyzed mitochondria of TazKD mice to establish if SC disassembling can be detected by BN-PAGE, and whether CL depletion induces any adaptive changes in mitochondria. Second, we have previously shown that the expression of mitochondrial proteins is remarkably affected in TazKD hearts (26), which may compromise interpretation of the results. Notably, the protein level of CyP-D, a main regulator of the PTP, changes in TazKD mice with age; it was 65% less in 3-month-old mice (26) and 13% higher in 8-month-old mice (Fig. 5C). Increased expression of CyP-D in TazKD hearts could explain, at least in part, high basal swelling due to increased PTP opening in these hearts. Hence, PTP-induced mitochondrial swelling, together with deficiency of CL synthesis, could enhance SC disassembly in TazKD mice. Recent studies demonstrated that TazKD does not enhance the vulnerability of the heart to IR injury and has no effect on mitochondrial

respiration (58). In contrast to these studies, where TazKD was initiated at an adult age, TazKD in our studies was induced at the early embryo stage (1,53). In addition, no changes in vulnerability can be explained with high basal swelling of mitochondria in TazKD hearts (Fig. 5A) that can protect the mitochondria against IR injury. Interestingly, the extent (40%) of CL reduction found in TazKD hearts (58) was the same for SC degradation found in our studies (Fig. 6B).

The IMM has emerged as an increasingly attractive target for pharmacological and conditional (e.g., ischemic pre/post-conditioning) therapies, with the goal of promoting cell survival during cardiac IR [reviewed in Refs. (11,44)]. Although many mitochondria-targeted compounds have been found to limit infarct size in animal models of cardiac IR injury, unfortunately, none has been approved for the treatment of myocardial infarction in humans. Among the many potential mitochondria-targeted therapies, those that inhibit the PTP seem to show the most promise. In particular, the PTP inhibitors, cyclosporine A (CsA) and SfA, have been shown to exert cardioprotective effects against IR in rodents (5,15,39). SfA is a CsA analogue that also potently inhibits the PTP by blocking CyP-D, and, unlike CsA, it does not inhibit calcineurin activity (15).

Intriguingly, CsA and SfA provide protective effects in a narrow concentration range and their effects strongly depend on severity of ischemia and timing of administration of the drugs. The drugs apparently exert protective effects when the heart is subjected to severe IR injury to protect the cells against PTP-induced necrosis. In isolated Langendorff-perfused hearts, severe damage occurs within the first few minutes of reperfusion when LDH release and PTP opening reach their maximum values (23) and this was found to be a critical time window as a cardioprotective strategy through inhibition of the PTP opening. Ca^{2+} -induced mitochondrial swelling was higher at 5 min compared to 60 min of reperfusion, and hence, inhibitory effects of SfA were higher at 5 min of reperfusion (Fig. 3B). Also, we do not exclude the role of PTP-independent swelling of mitochondria at reperfusion since SfA was able to reduce PTP opening up to a certain level for both 5 and 60 min of reperfusion (Fig. 3B).

Dependence of protective effects of CyP-D ablation on duration of ischemia has been shown in previous studies (55). Reperfusion injury at early phase (5 min) caused a moderate accumulation of ROS due to undamaged ETC activity; however, at late phase (60 min), it significantly increased ROS production presumably due to diminished scavenging capacity of mitochondria and dysfunction of ETC complexes, particularly complex III (Supplementary Fig. S2C and Fig. 3E). Accordingly, treatment with SfA or XJB reduced ROS production and preserved the complex III activity at 60 min of reperfusion. These data are in line with a recent study when targeting CyP-D decreased mitochondrial superoxide generation, improved vascular relaxation, and reduced hypertension in CyP-D KO mice (28).

Recent Cyclosporine and Prognosis in Acute Myocardial Infarction Patients (CIRCUS) clinical trial reported that CsA failed to protect reperfusion injury-related outcomes in patients receiving percutaneous coronary intervention for acute anterior ST-segment elevation myocardial infarction (16). Among many possible explanations for the lack of beneficial effects of CsA, the inappropriate time of administration of the drug was the most important, practical issue. CsA and SfA exert cardioprotective effects within a narrow concentration

range, early in reperfusion (24,27). In our studies, SfA was present before ischemia and throughout reperfusion, during which it improved heart function and prevented mitROS generation. Although SfA inhibited basal swelling of mitochondria at both early (5 min) and late (60 min) reperfusion, it abrogated Ca^{2+} -induced swelling at only early reperfusion with no effect at late reperfusion (Fig. 3B, C). Likewise, protective effects of CsA against brain ischemia were only observed when the drug was infused immediately upon reperfusion (18).

These data suggest that the PTP contributes less to swelling during late reperfusion, when other PTP-independent mechanisms can enhance the matrix swelling (36). We are tempted to speculate that (i) Ca^{2+} overload induced by sustained reperfusion exceeds the ability of SfA to inhibit CyP-D, but prevents mitROS production, and (ii) PTP-independent swelling at late reperfusion is possibly related to the structural destabilization of the respiratory SC I+III+IV. The latter is supported by the fact that the mitochondria isolated from TazKD mice exhibited more basal swelling, with no changes in mitROS generation (Fig. 5A, D). In addition, transient inhibition of complex I at the onset of reperfusion showed reduced cardiac injury (12,65). Currently, the lack of understanding of the mechanisms underlying the cross talk between PTP, CL oxidation, and SC degradation limits precise therapeutic targeting of mitochondria.

PTP opening stimulates mitROS generation and leads to dissociation of ETC SCs during sustained reperfusion followed by global ischemia. Inhibition of the PTP by SfA improves postischemic recovery of the heart, and prevents PTP-induced mitROS generation and SC degradation. Cardiac mitochondria of TazKD mice have high basal swelling, associated with increased expression of CyP-D. Deficiency in CL synthesis in TazKD mice leads to SC disintegration and modulates the enzymatic activity of individual ETC complexes.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 225–275 g were purchased from Charles River (Wilmington, MA). Eight-month-old WT and TazKD mice, mixed males and females, were used. Doxycycline-inducible shRNA-mediated TazKD mice have been described previously (1,53,57). All mice were in C57BL/6J background. TazKD was induced before conception by feeding females doxycycline-containing rodent chow (625 mg/kg) 3 days before mating. During the mating period, doxycycline was temporarily discontinued to avoid reported potential problems with male fertility. After detecting copulatory plugs, females were separated from males and placed back on the doxycycline-containing diet. This approach allows 85%–95% silencing of the tafazzin gene in heart and skeletal muscle (1). One- to 3-month-old WT and CyP-D KO mice, mixed males and females, were also used. All experiments were performed according to protocols approved by the University Animal Care and Use Committee and conformed to the National Research Council Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (2011, Eighth Edition).

Langendorff heart perfusion and animal groups

Rats were deeply anesthetized with anesthetic cocktail (179.2 mg/kg body weight, IP) containing 4.2 mg/kg xylazine,

87.5 mg/kg ketamine, and 87.5 mg/kg acepromazine to avoid any discomfort, distress, and pain in accordance to the *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition*. Depth of anesthesia was assessed by lack of response to the withdrawal reflex from a toe pinch, 15 min after the administration of anesthesia.

The hearts were rapidly removed and retrogradely perfused via a Langendorff perfusion system at constant flow as previously described (30). A water-filled latex balloon was inserted into the left ventricle for continuous monitoring of HR and left ventricular systolic (LVSP) and end diastolic (LVEDP) pressures. Measurements were recorded using Labscribe2 (iWorx 308T, Dover, NH). LVDP was calculated as the difference between LVSP and LVEDP (LVDP = LVSP – LVEDP). The RPP, an important marker of cardiac work, was calculated as the product of LVDP and HR (RPP = LVDP × HR) and given in mmHg · beats/min.

Protocols of perfusion are illustrated schematically in Figure 1. Animals were randomly assigned to the following treatment groups: (i) P60, nonischemic hearts perfused for 60 min ($n=9$); (ii) I25, hearts subjected to 25-min global ischemia ($n=10$); (iii) I25+SfA, hearts subjected to 25-min global ischemia in the presence of 0.5 μ M SfA ($n=7$); (iv) IR5, hearts subjected to 25-min global ischemia followed by 5-min reperfusion ($n=8$); (v) IR5+SfA, hearts subjected to 25-min global ischemia followed by 5-min reperfusion in the presence of 0.5 μ M SfA ($n=7$); (vi) IR60, hearts subjected to 25-min global ischemia followed by 60-min reperfusion ($n=9$); (vii) IR60+SfA, hearts subjected to 25-min global ischemia followed by 60-min reperfusion ($n=8$) in the presence of 0.5 μ M SfA; (viii) IR60+XJB, hearts subjected to 25-min global ischemia followed by 60-min reperfusion ($n=6$) in the presence of 0.2 μ M XJB.

Global normothermic ischemia was induced by switching off the pump after a total preischemic period of 25 min, with the heart immersed in buffer maintained at 37°C. In all experiments, an ischemic period of 25 min was followed by 5 or 60 min of reperfusion with flow at preischemic levels. Samples of the coronary effluent were collected before ischemia and during reperfusion at indicated time points to measure the LDH activity. The activity of LDH was assessed by an enzymatic method as previously described (30) with minor modifications. After the corresponding protocols, the hearts were used to isolate mitochondria.

Isolation of mitochondria

To isolate mitochondria, the ventricles were cut, weighed, and homogenized with a Polytron homogenizer at 1500 rpm for 5 s in ice-cold sucrose buffer containing 300 mM sucrose, 10 mM Tris–HCl, and 2 mM EGTA, at pH 7.4 (29). Mitochondria were isolated from the homogenate by centrifugation at 2000 g for 2 min in the sucrose buffer containing 0.5% BSA in a benchtop centrifuge to remove cell debris, followed by centrifugation of the supernatant at 10,000g for 5 min to sediment the mitochondrial suspension. The pellet was then washed two times at 10,000 g for 5 min in 40 ml of sucrose buffer (BSA free). The final pellet was resuspended in 300 μ l of the sucrose buffer.

The yield of mitochondria was 12.1 ± 0.3 mg/ml. A 100 μ l sample of the suspension was then used for measurement of mitochondrial PTP opening, ROS production, and respiration rate. A 200 μ l sample was mixed with protease and phosphatase

inhibitor cocktails (Sigma-Aldrich), then immediately frozen in liquid nitrogen, and stored at -80°C for gel analysis.

Measurement of PTP opening

Swelling of mitochondria as an indicator of PTP opening in the presence or absence of Ca^{2+} was determined by monitoring the decrease in light scattering at 525 nm as described previously (29). Freshly isolated mitochondria (0.4 mg/ml) were incubated at 37°C in 0.1 ml of incubation buffer containing 200 mM sucrose, 10 mM Tris–MOPS, 5 mM α -ketoglutarate, 2 mM malate, 1 mM P_i , 10 μ M EGTA–Tris, pH 7.4. The absorbance was monitored for 5 min without Ca^{2+} (basal swelling) and then with Ca^{2+} (Ca^{2+} -induced swelling). The rates of swelling were calculated as the change of absorbance induced by Ca^{2+} .

Measurement of mitROS production

Freshly isolated mitochondria were incubated at 37°C in 0.1 ml of the incubation buffer containing 200 mM sucrose, 10 mM Tris–MOPS, 5 mM α -ketoglutarate, 2 mM malate, 1 mM P_i , 10 μ M EGTA–Tris, pH 7.4. Production of H_2O_2 as an indicator of ROS was measured in isolated mitochondria with Amplex Red in the medium containing 0.1 mM Amplex Red (Invitrogen), 50 mM sodium phosphate, pH 7.4, and 0.2 U/ml HRP. ROS production was monitored in the presence or absence of Ca^{2+} at excitation of 560 nm and emission at 590 nm.

Measurement of mitochondrial respiration rates

Rates of mitochondrial respiration were measured at 37°C using a YSI Oxygraph (Yellow Springs, OH) model 5300 equipped with a Clark-type oxygen electrode (29). Mitochondria were incubated in a buffer containing (in mM) 125 KCl, 20 MOPS, 10 Tris, 0.5 EGTA, and 2 KH_2PO_4 , at pH 7.2, supplemented with 2.5 mM 2-oxoglutarate and 1 mM L-malate to measure the rate of oxygen consumption at complex I. Respiration rates were measured in the absence (state 2) and presence (state 3) of 1 mM ADP. At the end of each run, 0.5 μ M antimycin A, followed by 10 mM ascorbate and 0.3 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), was added, and the new rate of respiration was recorded (Supplementary Fig. S2E). Respiration charts were analyzed using Chart5 (PowerLab) and expressed in nmols O_2 per min to mg of protein.

Analysis of mitochondrial ETC SCs

Mitochondrial ETC SCs were analyzed by BNAGE as previously described with modifications (22,62,63). Briefly, 120 μ g of mitochondrial proteins was dissolved with 100 μ l of solubilization buffer (50 mM NaCl, 50 mM imidazole–HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA) supplemented with 4 μ l of 20% digitonin (or 1.7 μ l of 20% DDM), 1 μ l protease and phosphatase inhibitor cocktails (Sigma-Aldrich), and 25 U of Benzonase. Samples were incubated on ice for 20 min and then centrifuged for 20 min at 20,000 g. Supernatants were collected and mixed with 30 μ l of sample buffer (50 mM NaCl, 10% glycerol, 0.001% ponceau S, 50 mM Tris–HCl, pH 7.2). BNAGE was conducted as per the manufacturer's recommendations (Invitrogen). After electrophoresis, gels were stained by Coomassie brilliant blue G250 and then scanned with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). The resulting images were analyzed with ImageJ (NIH).

For second-dimension SDS-PAGE, the lanes were cut from native gels after running and incubated with loading buffer (50 mM Tris, pH 8.8, 50 mM DTT, 2% SDS, and 0.01% bromophenol blue) for 5 min. The lanes were loaded into 15% SDS-PAGE gel and sealed with 0.5% agarose. Second-dimension run and subsequent immunoblotting were conducted as per the manufacturer's recommendations (Bio-Rad). The antibody used for the analysis was the Total OXPHOS Rodent WB Antibody Cocktail (Abcam). They were used as per the manufacturer's recommendations. The signals were visualized by VersaDoc 4000 Gel Imaging System (Bio-Rad) and analyzed by ImageJ (NIH).

Analysis of the activity of ETC complexes

Isolated mitochondria were dissolved 0.1 $\mu\text{g}/\mu\text{l}$ in the buffer containing 2 mM EDTA and 0.01% Triton X-100 and then freeze-thawed two times to give the substrate access to the IMM. All assays were performed using SpectraMax M3 at 37°C as previously described (25,31).

Complex I activity was determined by measuring the rotenone-sensitive decrease of the absorbance of NADH at 340 nm. The assay was performed in phosphate buffer containing 25 mM KH_2PO_4 , pH 7.4, 0.5 mM KCN, 5 mM MgCl_2 , 2 mg/ml BSA, 2.4 $\mu\text{g}/\text{mL}$ antimycin, 1 mM NaN_3 , and 0.24 mM CoQ_1 . After stabilization, the reaction was started by adding 0.15 mM NADH, and then, rotenone was added to calculate the rotenone-sensitive (complex I) activity. Molar extinction coefficient of NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for calculation.

Complex II activity was measured as the velocity of 2,6-dichlorophenolindophenol (DCPIP) reduction, which corresponded to a decrement of absorbance at 600 nm. The assays were performed in the buffer containing 50 mM KH_2PO_4 , 2 mg/ml BSA, 5 mM KCN, 20 mM succinate, and 4 mM antimycin A. After preincubation with the reaction mixture (17), 50 μM DCPIP was added and the absorbance was measured at 600 nm. Molar extinction coefficient of DCPIP ($36 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for calculation.

Complex III activity was measured as rotenone-sensitive increment of absorbance at 550 nm (Complex I + III). The assays were performed in the buffer containing 50 mM KH_2PO_4 , 2 mg/ml BSA, 0.5 mM KCN, 1 mM NaN_3 , and 0.12 mM cytochrome c. After stabilization, the reaction was started by adding 5 mM NADH, and then, rotenone was added to calculate the rotenone-sensitive activity. The molar extinction coefficient of cytochrome c ($28 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for calculation.

Complex IV activity was measured as oxidation of cytochrome c as the decrease of absorbance at 550 nm. The assays were performed in the buffer containing 10 mM Tris-HCl, pH 7.4, 12 mM KCl. After stabilization, the reaction was started by adding reduced cytochrome c to 0.3 mg/ml. The molar extinction coefficient of cytochrome c ($28 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for calculation.

Citrate synthase activity was assayed spectrophotometrically by measuring coenzyme A formation at 412 nm as described previously (6). The molar extinction coefficient of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) ($14.15 \text{ mM}^{-1} \text{ cm}^{-1}$) was used for calculation.

Analysis of protein carbonylation and 4-HNE adducts

Analysis of carbonylated proteins was conducted as described previously with modifications (41,60). Samples

treated with 0.25 U/ μl of Benzonase[®] underwent derivatization using freshly prepared 1 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid. Resulting mixtures were loaded onto 10% SDS-PAGE gels and underwent standard immunoblot procedure. Anti-DNP antibody (Sigma-Aldrich) was used as per the manufacturer's recommendations. For 4-HNE adduct, Anti-HNE-Michael Adducts antibody (Millipore) was used as per the manufacturer's recommendations.

Analysis of CL content by high-resolution mass spectrometry

The CL content was analyzed (in duplicate) in mitochondria isolated from hearts in five groups (P60, I25, IR60, IR60+SfA, and IR60+XJB; $n=3$ per group). After isolation, the mitochondria were kept at -80°C in sucrose buffer containing 300 mM sucrose, 10 mM Tris-HCl, and 2 mM EGTA, pH 7.4. Butylated hydroxytoluene (50 μM) was added to the samples to prevent spontaneous lipid peroxidation. Mitochondrial fraction (7.5 μl), containing 10–20 μg protein/ μl , was added to ACN:water (3:1, 475 μl) to give a total volume of 482.5 μl , which was vortexed for 5 min and then centrifuged in an Eppendorf centrifuge 5702 at 4400 rpm and 20°C for 30 min. The supernatant (400 μl) was separated from the protein pellet for further analysis.

Cardiolipin content was quantified on a Thermo Scientific Exactive mass spectrometer using an electrospray ionization source. Samples of the supernatant solution (5 μl injections, five injections per sample) were directly injected into the probe head (-60 V , 350°C). Relative abundances of combined nonoxidized (intact) CL (m/z 723.5 and 1447.9) and combined oxidized CL (m/z 747.5, 760.5, and 790.5) species were determined with Thermo Xcalibur software. Results were averaged across the five injections per each sample and the ratio of oxidized CL to nonoxidized CL was calculated.

Statistical analysis

Values are presented as mean $\pm$ SE. Data were analyzed using ANOVA with normality test (Shapiro-Wilk) and pairwise multiple comparison procedures (Holm-Sidak method) in addition to Student's t -test. $p < 0.05$ was considered statistically significant.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

2D = two dimensional
 BNGE = blue native gel electrophoresis
 CL = cardiolipin
 CsA = cyclosporine A
 CyP-D = cyclophilin D
 DDM = dodecyl maltoside
 ETC = electron transport chain
 IMM = inner mitochondrial membrane
 IR = ischemia–reperfusion
 KO = knockout
 LDH = lactate dehydrogenase
 LVDP = left ventricular developed pressure
 LVEDP = left ventricular end diastolic pressure
 LVSP = left ventricular systolic pressure
 mitROS = mitochondrial ROS
 PTP = permeability transition pore
 ROS = reactive oxygen species
 RPP = rate pressure product
 SC = supercomplex
 SfA = sanglifehrin A
 TazKD = tafazzin knockdown
 WT = wild type
 XJB = XJB-5-131