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Reverse electron flow-mediated ROS generation in ischemia-damaged mitochondria: Role of complex I inhibition vs. depolarization of inner mitochondrial membrane

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Abstract

Background—The reverse electron flow-induced ROS generation (RFIR) is decreased in ischemia-damaged mitochondria. Cardiac ischemia leads to decreased complex I activity and depolarized inner mitochondrial membrane potential (Ψ) that are two key factors to affect the RFIR in isolated mitochondria. We asked if a partial inhibition of complex I activity without alteration of the Ψ is able to decrease the RFIR.

Methods—Cardiac mitochondria were isolated from mouse heart (C57BL/6) with and without ischemia. The rate of H_2O_2 production from mitochondria was determined using amplex red coupled with horseradish peroxidase. Mitochondria were isolated from the mitochondrial-targeted STAT3 overexpressing mouse (MLS-STAT3E) to clarify the role of partial complex I inhibition in RFIR production.

Results—The RFIR was decreased in ischemia-damaged mouse heart mitochondria with decreased complex I activity and depolarized Ψ . However, the RFIR was not altered in the MLS-STAT3E heart mitochondria with complex I defect but without depolarization of the Ψ . A slight depolarization of the Ψ in wild type mitochondria completely eliminated the RFIR.

Conclusions—The mild uncoupling but not the partially decreased complex I activity contributes to the observed decrease in RFIR in ischemia-damaged mitochondria.

General significance—The RFIR is less likely to be a key source of cardiac injury during reperfusion.

Keywords

Ischemia; Electron transport chain; STAT3; Reactive oxygen species

1. Introduction

The mitochondrial electron transport chain (ETC) is a key source of reactive oxygen species (ROS) [1]. Complexes I, II and III are established sites that generate ROS [2–4]. The generation of ROS from complex I can increase in two ways: by a blockade of forward electron flow through NADH-linked substrates via inhibition of complex I activity [2,3,5–8]; or by induction of reverse electron flow from complex II to complex I using succinate as a substrate [2,9–11]. Although the exact site of ROS generation within complex I remains unclear, the redox centers, including flavin mononucleotide (FMN) [3,5,6,12,13], Fe–S clusters [7], and ubiquinone (Q)-binding site, [8,11,12] are potential sites. In isolated heart mitochondria, inhibition of complex I has a minimal effect on the net release of H₂O₂ produced in the presence of complex I substrates [2,14]. In contrast, the H₂O₂ release is dramatically increased in isolated mitochondria when succinate is used as a substrate—a condition that favors the occurrence of the reverse electron flow [11,12]. The reverse flow-induced ROS generation (RFIR) has been observed mainly *in vitro* when succinate is used as a sole substrate for the ETC [10,15]. However, both NADH linked complex I substrates and FADH₂-linked complex II substrates are simultaneously present in intact cells. In this study, we hence asked if RFIR still occurred in isolated mitochondria when both complex I and complex II substrates were simultaneously present.

Uncontrolled ROS formation from the ETC contributes to myocardial injury during ischemia–reperfusion [1,2]. The RFIR occurs in intact mitochondria oxidizing succinate [4]. The altered metabolic environment of hypoxia or ischemia may favor RFIR [4]. On the other hand, ischemia could lead to decreased RFIR in that damage of the ETC during ischemia can result in a decrease in complex I activity and a depolarization of inner mitochondrial membrane potential (Ψ), two key factors that affect the RFIR in isolated mitochondria [10,11]. The complex I activity is decreased in many pathological conditions including cardiac ischemia–reperfusion [16]. A complete inhibition of complex I using rotenone prevents the RFIR in isolated heart and brain mitochondria [15]. However, cardiac ischemia–reperfusion leads only to a partial inhibition of complex I activity [16,17]. We therefore asked whether the partial inhibition of complex I alters RFIR in cardiac mitochondria. Ischemia–reperfusion not only decreases complex I activity, but also impairs the inner mitochondrial membrane, leading to depolarized Ψ that could limit the RFIR [18]. In order to differentiate the role of the partial decrease in complex I activity from the role of Ψ on the RFIR, a special model that carried a mitochondrial complex I defect without alteration to Ψ had to be used. A complex I defect is present in certain transgenic mouse models. For instance, over-expression of mitochondrial-targeted-STAT3 (MLS-STAT3E) in the heart leads to decreased complex I activity with preserved Ψ [19]. This model allows the study whether partial inhibition of complex I alone is sufficient to decrease the RFIR.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade, purchased from Sigma Chemical (St. Louis, MO). Supplies for the amplex red assay and membrane potential measurement were obtained from Molecular Probes (Invitrogen, Eugene, OR).

2.2. Mitochondria isolation

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University (VCU) and the McGuire Department of Veterans Affairs Medical Center. Wild type C57BL/6 mice (male, 2–3 month old) were purchased from Harlan (Indianapolis, Indiana). MLS-STAT 3E mice (male, 2–3 month old) were bred in-house in the VCU animal facility. A single combined population of heart mitochondria was isolated from mouse heart [19]. Briefly, cardiac tissue was homogenized in cold buffer A (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, and 1 mM ATP pH 7.4) with a polytron (Kinematica, Switzerland) at 10,000 rpm for 2.5 s and incubated with trypsin (5 mg/g) for 15 min at 4 °C. Trypsin was used to enhance mitochondrial protein yield [19]. Cold buffer B (buffer A+ 0.2% bovine serum albumin) was then added into the homogenate to dilute trypsin action. The homogenate was centrifuged at 500 g for 10 min, and the supernatant was further centrifuged at 3000 g for 10 min to pellet mitochondria. The mitochondrial pellet was washed and suspended in 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA. Protein concentration was measured using the Lowry method with bovine serum albumin used as standard [20].

2.3. Detection of H₂O₂ release

The rate of H₂O₂ production from mitochondria was determined using the oxidation of the fluorogenic indicator amplex red in the presence of horseradish peroxidase (HRP) [2,14,21]. Isolated mitochondria (0.1 mg/ml) were incubated in a reaction buffer that contained chelex-treated 160 mM KCl, 10 mM potassium phosphate, 1 mM EGTA, 25 μM Amplex red, 0.1 U/ml of HRP, pH 7.4 at 30 °C. The rate of fluorescence intensity change was converted to a H₂O₂ generation rate based on the standard curve. Glutamate (5 mM) and malate (2.5 mM) were used as the complex I substrate, and succinate (5 mM) was used as complex II substrate. Rotenone (2.4 μM) and antimycin A (10 μM) were used to inhibit the activities of complex I and complex III, respectively. Dinitrophenol (DNP) was used to depolarize inner membrane potential.

Determination of inner mitochondrial membrane potential (ψ): Mitochondrial inner membrane potential was measured using the fluorogenic indicator TMRM (tetramethylrhodamine, methyl ester) [22,23]. The ψ was reflected as the ADP-stimulated change of the ratio of fluorescence intensity of TMRM (emission at 590 nm, excitation at two wavelengths: 573 nm and 546 nm, ratio = value of 573/ 546). Freshly isolated mitochondria (0.2 mg/ml) were incubated in a single cuvette at 30 °C and glutamate (10 mM) + malate (5 mM) or succinate (10 mM) was used as complex I or complex II substrates, respectively. [23].

2.4. Mouse heart ischemia in vitro

Mice (C57BL/6) were anesthetized with sodium pentobarbital (100 mg/kg) and heparin (1000 units/kg). Mouse hearts were excised and subjected to 30 min global ischemia at 37 °C [19]. Cardiac mitochondria were isolated at the end of ischemia. Mitochondrial oxidative phosphorylation was measured using a Clark-type oxygen electrode at 30 °C to reflect the condition and quality of isolated mitochondria [20].

2.5. Statistical analyses

Data were expressed as mean $\pm$ standard error of the mean (SEM). Student t-test (unpaired) was used for statistical analysis. One-way ANOVA analysis of variance with repeated measurements was used to assess the difference in more than two groups. A difference of $p < 0.05$ was considered significant.

3. Results

3.1. Reverse electron flow-mediated H₂O₂ generation in isolated mitochondria

The rate of H₂O₂ generation was markedly increased in mitochondria when succinate alone was used as a substrate (Fig. 1A and D). Subsequent inhibition of complex I using rotenone decreased H₂O₂ generation (Fig. 1A and D), indicating that the increased H₂O₂ generation was from the RFIR and not from the forward electron flow from complex II into complex III. As previously reported, antimycin A increased the H₂O₂ generation from complex III using succinate + rotenone as substrates (Fig. 1A). H₂O₂ generation was scavenged by catalase, supporting that the dominant ROS detected by amplex red was H₂O₂. The RFIR was also prevented in the presence of a protonophore, dinitrophenol (DNP) (Fig. 1B and D), consistent with the notion that the RFIR was sensitive to the mitochondrial ψ .

In addition, we found that the RFIR was decreased in the presence of DIDS (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid), a VDAC (voltage dependent anion channel) inhibitor [24] (Fig. 1C and D). Since DIDS was first dissolved in DMSO, we tested if DMSO (0.001%) alone had an effect on the RFIR. DMSO alone had no effect on RFIR, but DIDS inhibited the RFIR. These results suggest that inhibition of VDAC decreases the RFIR in isolated heart mitochondria. Since the alteration of the complex I activity and the ψ affected the RFIR, we investigated the effect of DIDS on the complex I activity and ψ in the isolated mitochondria. DIDS did not alter the rate of oxidative phosphorylation in mitochondria oxidizing complex I or complex II substrates (Table 1), indicating that the activities of complex I and complex II were not significantly affected by DIDS treatment. However, DIDS induced a slight depolarization (about 10%) of the ψ when glutamate + malate or succinate was used as substrates for complex I or II, respectively [mean $\pm$ SEM, $n = 4$ in each group: glutamate + malate: 0.267 ± 0.004 (vehicle) vs. 0.245 ± 0.004 (DIDS, 50 μ M) $p < 0.05$; succinate: 0.364 ± 0.001 (vehicle) vs. 0.324 ± 0.004 (DIDS, 50 μ M) $p < 0.05$]. These data suggest that the depolarization of the ψ by DIDS contributes to decreased RFIR.

3.2. Forward electron flow-mediated H₂O₂ production in isolated mitochondria

Mitochondria only release a minimal amount of H₂O₂ in the absence of inhibitors when glutamate was used as a complex I substrate (Fig. 2A and B). Inhibition of complex I using rotenone increased background fluorescence intensity (Fig. 2A), but the rate of H₂O₂ generation was not altered compared to glutamate + malate alone (Fig. 1B), consistent with previous observations [2]. The rate of H₂O₂ generation by forward electron flow was also not affected in the presence of DNP (Fig. 2A and B), indicating that the loss of the proton gradient across the inner membrane (uncoupling) did not affect forward electron flow mediated net H₂O₂ generation from complex I. DIDS did not affect the rate of H₂O₂ generation using complex I substrate (Fig. 2C and D), further supporting that a DIDS-mediated slight decrease in Ψ did not impact the forward electron flow-mediated ROS production.

3.3. The RFIR occurs in the presence of complex I substrate

In the presence of complex I substrate, addition of succinate still dramatically increased the net release of H₂O₂ from wild type mitochondria (Fig. 3A, B). The generation of H₂O₂ in the presence of combined complex I and complex II substrates was inhibited either by rotenone or by DNP-induced uncoupling (Fig. 3A, B, and C). These results indicated that the increased ROS generation after addition of succinate was from the RFIR. Thus, RFIR can occur in the simultaneous presence of complex I and complex II substrates.

3.4. The RFIR is decreased in mitochondria following ischemia

Cardiac ischemia results in damage to the ETC [20,25]. In the present study, the Ψ was depolarized in mitochondria isolated from ischemic hearts and subsequently studied (Fig. 4A). Since the Ψ was measured in isolated mitochondria incubated with oxygenated buffer with ETC substrates, the observation of a depolarized Ψ during these conditions supported the presence of persistent damage to the inner membrane. The complex I activity was decreased in mouse heart mitochondria following ischemia compared to controls (Fig. 4B), consistent with previous findings [17,19,26]. The RFIR was markedly decreased in ischemia-damaged mitochondria (Fig. 4C).

3.5. The RFIR is not altered in mitochondria isolated from MLS-STAT3E mouse heart

The overexpression of mitochondria-targeted transcriptionally inactive STAT3 in the murine heart decreases complex I activity, but the Ψ is not depolarized compared to wild type [19]. Interestingly, in parallel to Ψ , the RFIR was also not changed in the MLS-STAT3E mitochondria compared to wild type controls (Fig. 4D). These findings suggested that the MLS-STAT3E-mediated partial blockade of complex I is not sufficient to block the RFIR and it would probably also require a change in Ψ .

3.6. The RFIR is abolished in MLS-STAT3E mouse heart mitochondria following ischemia

Ischemia significantly decreased the complex I activity in wild type mouse heart mitochondria, whereas the complex I activity was maintained in MLS-STAT3E mitochondria following ischemia [19]. There were no differences in RFIR between wild type and MLS-STAT3E mitochondria following ischemia when succinate was used as

substrate [mean $\pm$ SEM, 33 ± 1 (pmol/min/mg, wild type) vs. 28 ± 5 (MLS-STAT3E), $p = \text{NS}$, $n = 4$ in each group].

4. Discussion

The RFIR is known to occur when succinate is used as a sole substrate for the ETC in isolated mitochondria [10]. Surprisingly, the RFIR can still occur in the presence of both complex I and complex II substrates available simultaneously to isolated heart mitochondria. Cardiac ischemia leads to decreased complex I activity as well as inner membrane defects that result in a partially depolarized ψ [16,18]. The RFIR is markedly decreased in ischemia-damaged mouse heart mitochondria. The MLS-STAT3E-mediated partial inhibition of complex I alone without depolarization of ψ [19] does not alter the RFIR compared to wild type control. In contrast, a slightly depolarized ψ , achieved by the addition of DIDS to the wild type heart mitochondria, completely abolishes the RFIR. Thus, a slightly depolarized Ψ , rather than partially inhibited complex I activity, leads to decreased RFIR in heart mitochondria following ischemia, indicating that RFIR is unlikely to be a major source of the ROS generated during early reperfusion [16].

A polarized ψ is required to form the RFIR at complex I [10]. In the current study, depolarization of the ψ using DNP abolishes the RFIR, in line with the proposal that the RFIR is sensitive to depolarized ψ [10]. In contrast, the superoxide generated by the forward flow at complex I is not affected by DNP, suggesting that the forward flow-induced superoxide generation is not dependent on the ψ [27]. The RFIR in the presence of combined complex I and complex II substrates is still sensitive to depolarized ψ . In addition, an inhibition of complex I with rotenone also decreases the superoxide generation when substrates of both complexes are present. These results indicate that the superoxide generated by combined complex I and complex II substrates mainly originates from the reverse electron flow in that the forward flow-mediated superoxide generation from complex I is not sensitive to the depolarized ψ . Rotenone inhibition only decreases superoxide generated by the reverse flow but not by the forward electron flow from succinate through complex II into complex III. Thus, the RFIR occurs in the presence of both complex I and complex II substrates, indicating that the RFIR may occur *in vivo* when both complex I and complex II substrates are used to feed electrons into the ETC. However, the RFIR is less likely to occur *in vivo* in non-pathological condition in that the fully polarized ψ likely does not exist because energy from the proton gradient across inner mitochondrial membrane is constantly used to phosphorylate ADP that leads to a partially depolarized ψ [28].

DIDS prevents the release of superoxide, generated by complex III, from the mitochondrial intermembrane space into cytosol by blocking the VDAC channel [24]. In the present study, DIDS treatment decreases the RFIR in the isolated mouse heart mitochondria. These results suggest that DIDS leads to decreased RFIR by inhibiting VDAC. However, superoxide generated by the RFIR is released into mitochondrial matrix, away from VDAC which is located on outer mitochondrial membrane. Therefore, inhibition of VDAC should have limited effect on the RFIR. Thus, we studied if DIDS can decrease the RFIR by affecting the complex I activity or the Ψ in isolated mitochondria. The rate of oxidative phosphorylation

using complex I substrate is not altered in DIDS-treated mitochondria, indicating that DIDS treatment does not affect the complex I activity. In contrast, DIDS treatment does lead to slightly depolarized Ψ . These results suggest that the mild uncoupling of mitochondrial Ψ by DIDS attenuates the RFIR in the mouse heart mitochondria, consistent with a previous report [10]. DIDS does not decrease the forward flow-mediated ROS generation at complex I, further supporting that blockade of VDAC as the sole mechanism cannot account for the decrease in superoxide production from complex I.

In addition to complex I, complex II has recently been reported as a site of superoxide generation in isolated mitochondria when succinate is used as substrate [4]. However, the superoxide generated from complex II is dependent on the concentration of succinate. The peak of RFIR occurs when 0.5 mM succinate is used in isolated mitochondria in the presence of rotenone and myxothiazol [4]. The superoxide generated by complex II is markedly decreased in the presence of 5 mM succinate [4], the concentration that is used in the present study. Thus, the RFIR detected in the present study is less likely from complex II.

Cardiac ischemia damages the ETC and increases the generation of superoxide from forward electron flow [1,16,29] but decreases the RFIR from isolated heart mitochondria [11]. Ischemia also leads to increased permeability of the inner mitochondrial membrane that limits the ability to form Ψ in isolated mitochondria [30]. Thus, the lack of RFIR observed in mitochondria isolated from ischemic hearts could be due to a decrease in complex I activity or the partial uncoupling of Ψ . Transgenic models were used to investigate these possibilities. In the MLS-STAT3E mouse heart mitochondria, the complex I activity is decreased, and the defect is likely located within Fe-S protein clusters [19,31]. However, the overexpression of mitochondrial STAT3 does not lead to depolarized ψ [19]. The decreased complex I activity in MLS-STAT3E mouse heart mitochondria does not increase the forward electron flow-induced superoxide generation. Overexpressing mitochondrial STAT-3 does not affect the mitochondrial antioxidants [19]. In the present study, the RFIR is also not decreased in MLS-STAT3E mouse heart mitochondria, indicating that partial inhibition of complex I alone is not sufficient to decrease the RFIR. Moreover, ischemia decreased the RFIR in MLS-STAT3E mitochondria to the same extent as in wild type controls (Fig. 4D) whereas complex I activity was preserved during ischemia in MLS-STAT3E in contrast to wild type [19], further supporting that complex I activity is not the main factor affecting RFIR. These data strongly support that the decreased RFIR in the ischemia-damaged heart mitochondria is due to depolarization of ψ but not to the decrease in complex I activity [10].

The increased generation of ROS contributes to myocardial injury during ischemia–reperfusion [1,32]. Identification of the sources of the ROS generation during ischemia–reperfusion is a critical step in the development of efficient strategies to decrease cardiac injury by targeting the ROS generation. RFIR has been proposed as a mechanism of increased ROS generation during hypoxia or at the onset of reperfusion, in part due to the increase in succinate content [33]. The decreased RFIR in the ischemia-damaged mitochondria deemphasizes the role of RFIR as a significant contributor to cardiac injury during early reperfusion. The decrease in ability to fully polarize mitochondria due to

defects in the inner membrane limit RFIR generated from mitochondria that have been damaged by ischemia. Thus, strategies to decrease cardiac injury during early reperfusion need to focus on approaches to attenuate forward flow-mediated ROS generation during early reperfusion.

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Abbreviations

ETC	mitochondrial electron transport chain
ROS	reactive oxygen species
RFIR	reverse electron flow-induced ROS production
Ψ	inner mitochondrial membrane potential
STAT3	signal transducer and activator of transcription 3
MLS-STAT3E	mitochondrial-targeted transcriptionally inactive STAT3 overexpressing mouse
H₂O₂	hydrogen peroxide
FMN	flavin mononucleotide
Q	ubiquinone
NADH	nicotinamide adenine dinucleotide
FADH₂	flavin adenine dinucleotide
DNP	dinitrophenol
DIDS	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid
VDAC	voltage dependent anion channel
TMRM	tetramethylrhodamine

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Fig. 1.

The RFIR (reverse flow-induced ROS generation) in the isolated heart mitochondria. Panel A: A representative original tracing of the RFIR. When succinate (5 mM) is used to energize the mitochondria, the net release of H_2O_2 is markedly increased. Addition of rotenone leads to increased fluorescence intensity of background (FIB, bracketed short straight line), but the rate of H_2O_2 generation (slope of the line) is significantly decreased compared to the absence of rotenone. Antimycin A (AA) further increases the ROS generation, whereas catalase dramatically decreases the H_2O_2 generation. Panel B: The effect of DIDS on the RFIR. Succinate alone increases the RFIR in isolated heart mitochondria. DMSO has no effect on the RFIR, but DIDS almost entirely inhibits the RFIR. Panel C: The effect of dinitrophenol (DNP) on the RFIR. DNP is an uncoupler that is used to depolarize the ψ . Addition of DNP completely eliminates the RFIR in the isolated mitochondria, suggesting that the RFIR is sensitive to the depolarized ψ . Panel D: Summarized results. The RFIR is decreased by rotenone inhibition, DNP, and DIDS treatment. Mean $\pm$ SEM. *p < 0.05 vs. succinate alone n = 4 in each group.

Fig. 2.

The forward flow-mediated ROS generation. Panel A: A representative tracing of H_2O_2 generation from isolated mitochondria using complex I substrate. Addition of complex I substrate does not dramatically increase the ROS generation. Rotenone inhibition also increases the fluorescence background, but it does not increase the ROS generation. DNP does not affect the H_2O_2 generation using complex I substrates. Panel B: Summarized results. Rotenone inhibition and DNP treatment have minimal effects on ROS generation by forward electron flow in isolated heart mitochondria. Panels C & D: DIDS treatment does not significantly alter the forward flow-induced ROS generation in the isolated mitochondria. Mean $\pm$ SEM. $p = NS$ $n = 4$ in each group.

Fig. 3.

The RFIR in the isolated mitochondria in the presence of both complex I and complex II substrates. Panel A: Addition of complex I substrate alone does not substantially increase the ROS generation. However, addition of succinate following glutamate increases the ROS generation. The increased H_2O_2 generation by succinate is inhibited by rotenone. Panel B: The increased H_2O_2 generation is also decreased when DNP is used to depolarize the ψ in the presence of both complex I and complex II substrates. Panel C: Bar graphs summarize that the H_2O_2 production in the presence of combined substrates remains sensitive to complete inhibition of complex I by rotenone and to the DNP-mediated uncoupling of inner mitochondrial membrane potential. Mean $\pm$ SEM. * $p < 0.05$ vs. substrates alone $n = 4$ in each group.

Fig. 4.

The RFIR in wild-type ischemia-damaged heart mitochondria and MLS-STAT3E mouse heart mitochondria. Panel A: Cardiac ischemia leads to depolarized ψ compared to control mitochondria. Panel B: Cardiac ischemia decreases complex I enzyme activity vs. non-ischemic time control. Panel C: The RFIR is decreased in wild-type mitochondria isolated following ischemia compared to non-ischemic controls. Panel D: The RFIR is not altered in the MLS-STAT3E mouse heart mitochondria in the baseline state compared to littermate controls. Mean $\pm$ SEM. * $p < 0.05$ vs. control mitochondria $n = 5$ in each group.

Table 1

DIDS does not alter oxidative phosphorylation in isolated heart mitochondria.

	<u>Glutamate</u>		<u>Succinate + rotenone</u>	
	ADP (2 mM)	DNP (0.3 mM)	ADP (2 mM)	DNP (0.3 mM)
DIDS (0 μ M)	388 $\pm$ 35	390 $\pm$ 38	386 $\pm$ 26	345 $\pm$ 24
DIDS (50 μ M)	366 $\pm$ 29	373 $\pm$ 26	378 $\pm$ 19	343 $\pm$ 15

Data are expressed as mean $\pm$ SEM. p = NS, n = 4 in each group. Unit of respiration: nAO/mg/min. DIDS (0 μ M): DMSO (0.01%) was included in medium without DIDS.