Published in final edited form as:

Arch Biochem Biophys. 2009 April 1; 484(1): 87–93. doi:10.1016/j.abb.2009.01.006.

Mitochondrial superoxide production and respiratory activity: biphasic response to ischemic duration.[†]

Satoshi Matsuzaki, Luke I. Szweda, and Kenneth M. Humphries^{*}

Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

SUMMARY

Long bouts of ischemia are associated with electron transport chain deficits and increases in free radical production. In contrast, little is known regarding the effect of brief ischemia on mitochondrial function and free radical production. This study was undertaken to examine the relationship between the duration of ischemia, effects upon electron transport chain activities, and the mitochondrial production of free radicals. Rat hearts were subjected to increasing ischemic durations, mitochondria were isolated, and superoxide production and electron transport chain activities were measured. Results indicate that even brief ischemic durations induced a significant increase in superoxide production. This rate was maintained with ischemic durations less than 15 min, and then increased further with longer ischemic times. Mechanistically, brief ischemia was accompanied by an increase in NADH oxidase activity, reflected by a specific increase in complex IV activity. In contrast, longer ischemic durations were accompanied by a decrease in NADH oxidase activity, reflected by deficits in complexes I and IV activities.

Keywords

cardiac ischemia; ischemic preconditioning; mitochondria; electron transport chain; free radicals; superoxide

INTRODUCTION

Cardiac ischemia leads to declines in mitochondrial respiratory function that can be exacerbated upon reperfusion [1–14]. Depending on the model and specific experimental conditions, prolonged ischemia has been associated with decreases in the activities of each of the electron transport chain complexes [2,3,6,9,10,13,15,16]. Although mechanisms by which these events occur have yet to be fully defined, a likely consequence would be an increase in free radical production. Decreased respiratory activity extends the half-life of reduced components of the electron transport chain that would be expected to promote superoxide production upstream of the deficit [17–23]. Thus, loss of electron transport chain activity

[†]The project described was supported by Grant Number R01AG016339 (LS) from the National Institute on Aging and Grant Number 0625752Z (SM) from the American Heart Association. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Aging or the National Institutes of Health.

^{*}Correspondence Address: Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, Oklahoma, 73104, Phone: (405) 271-7584, Fax: (405) 271-1437, Email: E-mail: Kenneth-Humphries@omrf.org.

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

induced by ischemia likely contributes to mitochondrial free radical production upon reperfusion.

In striking contrast to prolonged ischemia, short and repeated bouts of ischemia, termed ischemic preconditioning (IPC), are known to protect from various indices of damage evident upon subsequent extended durations of ischemia/reperfusion (e.g. hemodynamic dysfunction, apoptosis) [24,25]. Paradoxically, evidence suggests the production of free radicals is a necessary component of the protective effects of ischemic preconditioning [26–33]. Increased levels of free radical species have been observed in intact hearts during preconditioning, and infusion of antioxidants has been reported to prevent the phenomenon [31,33]. Mechanistically, cell signaling events, such as activation of PKG and PKC ϵ [34], are believed to converge upon mitochondria and modulate free radical production. Increased production has been attributed to mild uncoupling via opening of K_{ATP} channels and/or activation of UCP and ANT [35]. Nevertheless, little is known regarding neither the effects of brief periods of ischemia on electron transport chain activity nor the sites of mitochondrial superoxide production as a function of ischemic duration. To gain insight into the mechanisms by which mitochondria produce free radicals in a beneficial versus deleterious manner, temporal relationships between changes in respiratory function, the activities of electron transport chain components, and the rate of superoxide production as a function of ischemic duration must be defined.

In the present study, using a Langendorff model of perfusion, hearts were subjected to different times of ischemia, mitochondria were isolated, and the rates of NADH-linked electron transport chain activities and superoxide production were measured. Results indicate that mitochondria increase capacity to produce superoxide in a biphasic manner as a function of ischemic duration. Increased superoxide production was associated with an increase and subsequent decrease in electron transport chain activity. Mitochondria therefore appear capable of increasing superoxide production by distinct mechanisms and at specific sites within the electron transport chain dependent on ischemic conditions.

MATERIALS AND METHODS

Reagents and Animals

Antimycin A, cytochrome *c*, decyl ubiquinone, myxothiazol, NADH, ubiquinone-1, and superoxide dismutase (CuZn-SOD), were purchased from Sigma. Hydroethidine was purchased from Invitrogen/Molecular Probes. Male Sprague-Dawley rats (250–300 g) were obtained from Harlan Laboratories.

Preparation and Perfusion of Isolated Rat Hearts

Male Sprague-Dawley rats were anesthetized and hearts were excised and placed in 37°C Krebs-Henseleit buffer (120 mM NaCl, 4.8 mM KCl, 2.0 mM CaCl₂, 1.25 mM MgCl₂, 1.25 mM KH₂PO₄, 25 mM NaHCO₃ and 10 mM glucose) to remove blood. Extraneous tissue was rapidly removed, the aorta was cannulated, and the heart was perfused in retrograde fashion according to Langendorff with modified Krebs-Henseleit buffer, at 37°C, saturated with 95% O₂/5% CO₂. Hearts were placed in a water-jacketed chamber (37°C) and the perfusion rate was maintained at 10 mL/min. Elapsed time between isolation of the heart and perfusion was approximately 1.0 min. Experiments consisted of the following protocols: (a) a 30min normoxic perfusion, or (b) a 30 min perfusion followed by a 3.75 min, 7.5 min, 15 min, 22.5 min, or 30 min no-flow global ischemia.

Isolation of Mitochondria

Subsarcolemmal mitochondria were isolated from hearts as previously described [36]. The isolation protocol was completed at 5°C and in the absence of respiratory substrates, which

would be expected to minimize oxygen-induced changes following the ischemic period. Briefly, following each perfusion protocol, hearts were immersed, and rinsed in ice-cold isolation buffer containing 210 mM Mannitol, 70 mM Sucrose, 10 mM MOPS, and 1.0 mM EDTA at pH 7.4. The hearts were then minced with scissors followed by homogenization in 20 mL of the isolation buffer with a polytron homogenizer (3×2 sec passes). Homogenate was then centrifuged at 500g for 5.0 min and supernatant was collected. The supernatant was then filtered through cheese cloth and divided into two equal fractions, both of which were then centrifuged at 10,000g for 10 min. The resulting mitochondria pellets were either washed and resuspended in isolation buffer for determination of respiratory activity, or resuspended in 10 mM MOPS at pH 7.4 and immediately snap-frozen in liquid N₂ for analysis of superoxide anion production and electron transport chain activities. Protein concentrations were determined by the BCA method (Pierce), with BSA as a standard.

Mitochondrial Respiratory Activity

Mitochondria were diluted (0.25 mg/mL) in a buffer containing 210 mM mannitol, 70 mM sucrose, 5.0 mM KH₂PO₄, and 10 mM MOPS, pH 7.4. Respiratory activity was measured in the presence of 10 mM glutamate, 2 mM malate using a Clark style oxygen electrode (Instech). State 3 respiration was initiated by addition of 0.25 mM ADP.

Electron Transport Chain Enzymatic Analyses

All assays were performed in 25 mM KCl, 10 mM MOPS at pH 7.4 (25°C) with 25 µg/mL frozen-thawed mitochondria except where noted. NADH Oxidase Activity: NADH oxidase activity, an assessment of overall electron transport through complexes I, III, and IV, was measured spectrophotometrically as the rate of NADH oxidation ($\epsilon_{340} = 6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$) following addition of 200 µM NADH. Activity was completely inhibited by either rotenone or antimycin A. Complex I: Following addition of 50 nM antimycin A and 100 µM ubiquinone-1, complex I activity was initiated with NADH (200 µM). Activity was measured spectrophotometrically as the rate of NADH oxidation ($\epsilon_{340} = 6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Activity required the presence of ubiquinone-1 and was inhibited by rotenone indicating that NADH utilization was dependent on complex I activity. Complex III–IV combination: Flow of electrons from complex III through complex IV was initiated upon addition of ubiquinol-1 (100 µM). Activity was measured spectrophotometrically as the rate of ubiquinol-1 oxidation to ubiquinone-1 ($\epsilon_{280} = 13700 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Antimycin A or CN⁻ inhibited activity indicating transport of electrons from complex III through complex IV. Complex III: Mitochondria were diluted to a protein concentration of 5.0 µg/mL in MOPS buffer (pH 7.4). Following addition of 0.3 mM dodecyl maltoside, 2.0 mM CN⁻, and 25 µM cytochrome *c* (oxidized), complex III activity was initiated with 50 µM decyl ubiquinol. Activity was measured spectrophotometrically as the rate of cytochrome *c* reduction ($\epsilon_{550} = 18500 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Antimycin A inhibited activity indicating that cytochrome *c* reduction was dependent on complex III activity. Complex IV: Mitochondria were diluted to a protein concentration of 5.0 µg/mL in buffer containing 25 mM KCl, 10 mM MOPS (pH 7.4). Complex IV activity was initiated with reduced cytochrome *c* (25 µM) and measured spectrophotometrically as the rate of cytochrome *c* oxidation ($\epsilon_{550} = 18500 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Activity was inhibited by CN⁻ indicating that cytochrome *c* oxidation was dependent on complex IV activity.

Superoxide Anion Production Measurements

The NADH-supported rate of O₂⁻ production was measured as described previously [37,38]. Briefly, the oxidation of hydroethidine by O₂⁻ to the fluorescent product 2-hydroxyethidium was measured. The fluorescent signals were recorded (excitation, 480 nm; emission, 567 nm) over time utilizing 10 µM hydroethidine and 500 µM NADH. CuZn-SOD (8.0 U/mL) was used to test the specificity of the measurement for superoxide anion. Where indicated, the complex

III inhibitor, myxothiazol (50 nM), was included. It should be noted that oxidation of hydroethidine by $O_2^{\cdot -}$ resulted in a product with properties (reverse phase HPLC elution profile and fluorescence properties) consistent with 2-hydroxyethidium and not ethidium as recently reported [37,38]. Nevertheless, the structurally related compound ethidium was used as a standard to estimate the relative rate of $O_2^{\cdot -}$ production due to the absence of commercially available 2-hydroxyethidium.

Statistical Analysis

Data are presented as means $\pm$ SE. The data were evaluated utilizing a two-tailed *t* test. Statistical significance was assigned for $p \leq 0.05$, as indicated.

RESULTS

NADH-Supported Superoxide Anion Production as a Function of Ischemic Time

Rat hearts were subjected to varying durations of ischemia. Following each experimental protocol, mitochondria were isolated and analyzed for respiratory activity. Utilizing NADH linked substrates, glutamate and malate, no changes in state 3 respiration or RCR were seen as a function of perfusion time, or with ischemic durations less than 15 minutes (Table 1). In contrast, 30 minutes of ischemia induced a significant decrease in state 3 respiration. This indicates brief periods of ischemia do not induce overt disruptions in oxidative phosphorylation or loss of mitochondrial integrity.

To assess free radical production as a function of ischemic duration, superoxide anion production was examined by fluorometrically measuring the NADH supported rate of hydroethidine (HE) oxidation [37–39]. Experiments were performed using frozen/thawed mitochondria to allow for direct assessment of electron transport chain derived superoxide production while minimizing interference of mitochondrial antioxidants. An additional advantage of this method is that, unlike assays employing intact mitochondria, dismutation of superoxide to hydrogen peroxide and diffusion out of the matrix is not required. Moreover, inclusion of reagents that can complicate free radical measurements, such as horseradish peroxidase, is not necessary. Finally, we have found that superoxide production measurements in disrupted mitochondria offer unparalleled sensitivity and reproducibility as compared to the use of similar fluorescent reagents in intact mitochondria. As shown in Fig 1A, the observed rates of superoxide production are linear for an extended duration. This indicates that under the conditions of the assay and mitochondrial isolation procedure, superoxide production remains stable. While brief periods of ischemia do not impair respiratory capacity, Fig. 1B illustrates that durations as brief as 3.75 min induce significant increases in superoxide production relative to perfused controls. Further increases in superoxide production with longer durations of ischemic time were not evident until ischemia was extended beyond 15 min. This biphasic response appears to involve distinct sites of free radical production within the electron transport chain. As shown in Table 2, rates of superoxide production observed after 15 min and 30 min ischemia are 30% and 62% greater than that seen in perfused controls, respectively. Notably, superoxide production measured in the presence of myxothiazol, a complex III inhibitor that blocks electron flow prior to entry into the Q cycle (Table 2) [40], demonstrates differential sensitivity to inhibitor induced modulation of superoxide production that is dependent upon the ischemic duration. Superoxide production measured in the presence of myxothiazol was unchanged following 15 min ischemia, but significantly increased after 30 min ischemia, as compared to perfused controls. Because myxothiazol prevents superoxide production downstream of the cytochrome *b-c1* in complex III [40], this result suggests that brief ischemia promotes superoxide generation within complex III-IV. However, longer times of ischemia also stimulate superoxide production upstream of complex III.

Ischemia-Induced Alterations in Electron Transport Chain Activity

Increased mitochondrial free radical production has traditionally been attributed to deficits in electron transport chain function. Nevertheless, increased rates of superoxide production with ischemic times of less than 15 min were not accompanied by loss in mitochondrial respiration (Table 1). However, respiration in intact mitochondria may not reflect subtle changes in electron transport chain activity. NADH oxidase activity, a direct measurement of electron transfer from complex I to III and then IV, was therefore assessed. As shown in Fig. 1B, NADH oxidase activity was significantly higher ($p < .05$) following brief ischemia (< 15 min) as compared to perfused controls. The maximal NADH oxidase rate was 17% higher than the control value at 3.75 min ischemia. Interestingly, deficits in NADH oxidase activity were not apparent until hearts were exposed to greater than 15 min of ischemia. The rate of NADH oxidase activity after 30 minutes of ischemia was 17% less than the rate in mitochondria isolated from perfused controls.

To gain insight into the specific electron transport complexes responsible for these changes, individual complex activities were examined. As shown in Fig. 2A, complex I activity was unaffected by ischemic durations of less than 15 min, but was significantly inhibited upon extension of ischemic times. Complex III activity steadily increased, and was elevated at ischemic times greater than or equal to 15 min (Fig. 2C). Nevertheless, complex III activity did not follow the biphasic response to increasing ischemic duration as observed for NADH oxidase activity and the rate of superoxide production. In contrast, the combined activities of complex III-IV (Fig. 2B) closely followed a biphasic pattern (Fig. 1B). Complex III-IV activity was significantly increased with brief ischemia, with maximal activity detected at 3.75 min of ischemia (118% of control, $p < 0.05$). Longer durations of ischemia reduced activity to that of perfused controls, significantly below the maximal activity determined at 3.75 min. Complex IV activity (Fig. 2D) paralleled the biphasic patterns of complex III-IV and NADH oxidase activities. This result suggests that modulation of complex IV activity may be responsible for the overall tone of NADH-linked respiratory activity and influences free radical production upstream of this complex.

In Vitro Analysis of Superoxide Anion Production Relative to NADH Oxidase Activity

The results presented in Fig. 1B demonstrate that the increased rate of superoxide production that occurs as a function of brief ischemia (< 15 min) parallels the increase in NADH oxidase activity. This result was unexpected because ischemia-induced increases in free radical production are typically associated with deficits in electron transport chain activity. *In vitro* experiments were therefore undertaken to mechanistically examine the relationship between NADH activity and superoxide production. As a model system, perfused, control mitochondria were prepared and NADH oxidase activity was examined as a function of ionic strength. Under these conditions, NADH oxidase activity increased linearly as ionic strength was increased from 0 to 15 mM KCl (Fig. 3). Importantly, it was found that this increase in activity induced a concurrent increase in superoxide production (Fig. 3). Therefore, an increase in electron transport chain activity can be accompanied by an increase in superoxide production.

The increase in NADH oxidase activity as a function of ionic strength is due to specific activation of complex IV. As shown in Fig. 4, the activities of complex I (Fig. 4A) and complex III (Fig. 4C) were unaffected by an increase in potassium chloride concentration from 0 to 25 mM. In contrast, complex III-IV showed a salt-dependent increase in activity (Fig. 4B) that closely corresponded to the change in NADH oxidase activity (Fig. 3). Similar to NADH oxidase, complex III-IV activity decreased with concentrations of potassium chloride above 15 mM. The biphasic response of NADH oxidase and complex III-IV activities versus increasing potassium chloride concentration is explained by the effect of ionic strength on *cytochrome c* [41]. Both assays were measured without addition of exogenous *cytochrome c*,

and as previously shown, addition of potassium chloride above 15 mM begins to disassociate endogenous cytochrome *c* from the membrane and lower activity [41]. In contrast to complexes I and III, complex IV activity was stimulated in response to increasing concentrations of potassium chloride (Fig. 4D). This is attributed to enhanced cycling of the highly basic protein cytochrome *c*, which is exogenously added for this assay, at the complex IV interface. This represents a unique observation that *in vitro*, an increase in electron transport, specifically within complex III-IV activity, is associated with an increase in free radical production and this same pattern of activity is observed following brief ischemia (Fig. 1B).

DISCUSSION

It is well established that cardiac ischemia promotes conditions that predispose mitochondria to produce oxygen-derived free radicals at elevated rates upon reperfusion [42–46]. The majority of studies examined relatively long durations of ischemia where electron transport chain deficiencies were evident [2,3,6,9,10,13,15,16]. Inhibition extends the half-life of reduced components of the chain thus increasing the likelihood that an electron will be donated to O₂ resulting in incomplete reduction to superoxide anion [17–19,21–23]. A unique aspect of the current study is the demonstration that within the first few minutes of ischemia (3.75 min), the capacity of mitochondria to produce superoxide increases substantially, reaching approximately 50% of that observed upon prolonged ischemia. In addition, increases in superoxide production induced by a short bout of ischemia are accompanied by stimulation of electron transport chain activity rather than inhibition.

The biphasic response of superoxide production to ischemic duration is indicative of separate and distinguishable mechanisms. This is supported by evidence that NADH oxidase activity initially increases with short durations of ischemia and then declines with prolonged bouts. Brief periods of ischemia were associated with an increase in complex III-IV activity that closely mimicked the overall change in NADH oxidase activity. In contrast, longer durations of ischemia were accompanied by a drop in complex I and IV activities (Fig. 2). It has been well documented that deficits in complex I activity provides a source of superoxide [6,9,13, 15]. In addition, pharmacological evidence presented here also indicates that distinct sites of superoxide production exist with brief versus prolonged ischemia. Ischemic-induced increase in superoxide production occurs downstream of the Q-cycle with brief ischemia. Longer durations of ischemia, where marked deficits in complex I activity are observed, result in free radical production upstream of the Q-cycle. As the ischemic duration increases and electron transport chain deficits begin to arise, it is likely that overlapping modes of superoxide production occur.

In vitro studies were undertaken to provide insight into the relationship between NADH oxidase activity, individual electron transport chain complex activities, and superoxide production. Specifically, the sensitivity of NADH oxidase to changes in ionic strength was exploited to demonstrate the relationship between increased electron flux and superoxide production. Similar to what is seen with brief bouts of ischemia, *in vitro* activation of NADH oxidase activity was attributable to complex III-IV activation. In fact, relatively small increases in NADH oxidase activity were sufficient to increase the rate of superoxide formation as was observed following a brief period of ischemia. The mechanism by which complex IV is activated in response to brief episodes of ischemia is currently unknown, but there are several possibilities. Phosphorylation of complex IV by PKC ϵ *in vitro* results in stimulation of complex IV activity [47,48]. Furthermore, selective disassociation of subunit VIb from complex IV *in vitro* has been shown to increase activity [49]. However, the effects of each of these potential events on superoxide production are not currently known. It is also important to note that the site of superoxide production is not likely directly within complex IV. Structural alterations to protein complexes and/or the mitochondrial inner membrane may enhance cycling of the highly

basic protein cytochrome *c* at the complex IV interface enhancing cytochrome *c* catalyzed superoxide production. It should be noted the overall content of cytochrome *c*, determined by Western blot, did not change as a function of ischemic duration (data not shown). Cytochrome *c* is found in multiple forms, with one population tightly bound to the inner mitochondrial membrane through hydrophobic interactions and another population bound more loosely through ionic interactions with cardiolipin [50]. Subtle changes in this distribution may modulate the production of superoxide by the complex III-IV segment of the electron transport chain. Clearly, future studies must delineate the mechanism(s) whereby stimulation of NADH oxidase activity enhances superoxide production.

Oxygen derived free radical species and pro-oxidants do not simply catalyze oxidative damage, but are also capable of regulating mitochondrial and cellular processes through the reversible modulation of protein function [51–58]. This is evident when considering ischemic preconditioning, in which free radical production has been suggested to be a prerequisite for cardioprotection [26–33]. The implication is that the regulated and reversible production of free radicals by mitochondria can induce prosurvival events, including the activation of kinases and the prevention of the opening of the mitochondrial permeability pore [29]. The current study provides important direction for understanding how these free radicals are produced. Further mechanistic evidence may lead to more appropriate therapeutic options. It will also be important to expand upon the current studies to determine the effects of repeated bouts of ischemia and reperfusion, which induces ischemic preconditioning, on the reversible modulation of electron transport chain activity and superoxide production.

LIST OF ABBREVIATIONS

NADH

reduced nicotinamide adenine dinucleotide

SOD

superoxide dismutase

References

1. Czerski LW, Szweida PA, Szweida LI. *J Biol Chem* 2003;278:34499–34504. [PubMed: 12813053]
2. Duan J, Karmazyn M. *Can J Physiol Pharmacol* 1989;67:704–709. [PubMed: 2548694]
3. Hardy L, Clark JB, Darley-Usmar VM, Smith DR, Stone D. *Biochem J* 1991;274(Pt 1):133–137. [PubMed: 1900416]
4. Lesnefsky EJ, Tandler B, Ye J, Slabe TJ, Turkaly J, Hoppel CL. *Am J Physiol* 1997;273:H1544–1554. [PubMed: 9321848]
5. Jennings RB, Ganote CE. *Circ Res* 1976;38:I80–91. [PubMed: 1269093]
6. Lucas DT, Szweida LI. *Proc Natl Acad Sci U S A* 1999;96:6689–6693. [PubMed: 10359773]
7. Lucas DT, Szweida LI. *Proc Natl Acad Sci U S A* 1998;95:510–514. [PubMed: 9435222]
8. Murfitt RR, Stiles JW, Powell WJ Jr, Sanadi DR. *J Mol Cell Cardiol* 1978;10:109–123. [PubMed: 204792]
9. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM. *Circ Res* 2004;94:53–59. [PubMed: 14656928]
10. Piper HM, Sezer O, Schleyer M, Schwartz P, Hutter JF, Spieckermann PG. *J Mol Cell Cardiol* 1985;17:885–896. [PubMed: 4046049]
11. Sadek HA, Humphries KM, Szweida PA, Szweida LI. *Arch Biochem Biophys* 2002;406:222–228. [PubMed: 12361710]
12. Sadek HA, Nulton-Persson AC, Szweida PA, Szweida LI. *Arch Biochem Biophys* 2003;420:201–208. [PubMed: 14654058]

13. Veitch K, Hombroeckx A, Caucheteux D, Pouleur H, Hue L. *Biochem J* 1992;281(Pt 3):709–715. [PubMed: 1346958]
14. Venditti P, Masullo P, Di Meo S. *Cell Mol Life Sci* 2001;58:1528–1537. [PubMed: 11693531]
15. Rouslin W. *Am J Physiol* 1983;244:H743–748. [PubMed: 6305212]
16. Ylitalo K, Ala-Rami A, Vuorinen K, Peuhkurinen K, Lepojarvi M, Kaukoranta P, Kiviluoma K, Hassinen I. *Biochim Biophys Acta* 2001;1504:329–339. [PubMed: 11245796]
17. Boveris A. *Methods Enzymol* 1984;105:429–435. [PubMed: 6328196]
18. Boveris A, Cadenas E. *FEBS Lett* 1975;54:311–314. [PubMed: 236930]
19. Boveris A, Chance B. *Biochem J* 1973;134:707–716. [PubMed: 4749271]
20. Cadenas E, Boveris A. *Biochem J* 1980;188:31–37. [PubMed: 7406888]
21. Cadenas E, Boveris A, Ragan CI, Stoppani AO. *Arch Biochem Biophys* 1977;180:248–257. [PubMed: 195520]
22. Cadenas E, Davies KJ. *Free Radic Biol Med* 2000;29:222–230. [PubMed: 11035250]
23. Chance B, Williams GR. *Adv Enzymol Relat Subj Biochem* 1956;17:65–134. [PubMed: 13313307]
24. Yellon DM, Downey JM. *Physiol Rev* 2003;83:1113–1151. [PubMed: 14506302]
25. Murry CE, Jennings RB, Reimer KA. *Circulation* 1986;74:1124–1136. [PubMed: 3769170]
26. Yaguchi Y, Satoh H, Wakahara N, Katoh H, Uehara A, Terada H, Fujise Y, Hayashi H. *Circ J* 2003;67:253–258. [PubMed: 12604877]
27. Novalija E, Hogg N, Kevin LG, Camara AK, Stowe DF. *J Cardiovasc Pharmacol* 2003;42:593–600. [PubMed: 14576506]
28. Kevin LG, Camara AK, Riess ML, Novalija E, Stowe DF. *Am J Physiol Heart Circ Physiol* 2003;284:H566–574. [PubMed: 12414448]
29. Halestrap AP, Clarke SJ, Khaliuln I. *Biochim Biophys Acta* 2007;1767:1007–1031. [PubMed: 17631856]
30. da Silva MM, Sartori A, Belisle E, Kowaltowski AJ. *Am J Physiol Heart Circ Physiol* 2003;285:H154–162. [PubMed: 12623788]
31. Baines CP, Goto M, Downey JM. *J Mol Cell Cardiol* 1997;29:207–216. [PubMed: 9040035]
32. Ambrosio G, Tritto I, Chiariello M. *J Mol Cell Cardiol* 1995;27:1035–1039. [PubMed: 7563100]
33. Tritto I, D'Andrea D, Eramo N, Scognamiglio A, De Simone C, Violante A, Esposito A, Chiariello M, Ambrosio G. *Circ Res* 1997;80:743–748. [PubMed: 9130455]
34. Costa AD, Pierre SV, Cohen MV, Downey JM, Garlid KD. *Cardiovasc Res* 2008;77:344–352. [PubMed: 18006449]
35. Otani H. *Antioxid Redox Signal* 2008;10:207–247. [PubMed: 17999631]
36. Humphries KM, Yoo Y, Szveda LI. *Biochemistry* 1998;37:552–557. [PubMed: 9425076]
37. Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. *Free Radic Biol Med* 2003;34:1359–1368. [PubMed: 12757846]
38. Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, Kalyanaraman B. *Proc Natl Acad Sci U S A* 2005;102:5727–5732. [PubMed: 15824309]
39. Matsuzaki S, Szveda LI. *Biochemistry* 2007;46:1350–1357. [PubMed: 17260964]
40. Thierbach G, Reichenbach H. *Biochim Biophys Acta* 1981;638:282–289. [PubMed: 6274398]
41. Jacobs EE, Sanadi DR. *J Biol Chem* 1960;235:531–534. [PubMed: 14406362]
42. Das DK, George A, Liu XK, Rao PS. *Biochem Biophys Res Commun* 1989;165:1004–1009. [PubMed: 2558644]
43. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, et al. *J Biol Chem* 1993;268:18532–18541. [PubMed: 8395507]
44. Churchill EN, Szveda LI. *Arch Biochem Biophys* 2005;439:194–199. [PubMed: 15963450]
45. Otani H, Tanaka H, Inoue T, Umemoto M, Omoto K, Tanaka K, Sato T, Osako T, Masuda A, Nonoyama A, et al. *Circ Res* 1984;55:168–175. [PubMed: 6086177]
46. Ueta H, Ogura R, Sugiyama M, Kagiya A, Shin G. *J Mol Cell Cardiol* 1990;22:893–899. [PubMed: 2172558]
47. Ogbi M, Johnson JA. *Biochem J* 2006;393:191–199. [PubMed: 16336199]

48. Barnett M, Lin D, Akoyev V, Willard L, Takemoto D. *Exp Eye Res.* 2007
49. Weishaupt A, Kadenbach B. *Biochemistry* 1992;31:11477–11481. [PubMed: 1332762]
50. Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. *Proc Natl Acad Sci U S A* 2002;99:1259–1263. [PubMed: 11818574]
51. Barrett WC, DeGnore JP, Keng YF, Zhang ZY, Yim MB, Chock PB. *J Biol Chem* 1999;274:34543–34546. [PubMed: 10574916]
52. Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, Zhang ZY, Yim MB, Chock PB. *Biochemistry* 1999;38:6699–6705. [PubMed: 10350489]
53. Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G, Szweda LI. *Proc Natl Acad Sci U S A* 2005;102:5987–5991. [PubMed: 15840721]
54. Nulton-Persson AC, Szweda LI. *J Biol Chem* 2001;276:23357–23361. [PubMed: 11283020]
55. Nulton-Persson AC, Starke DW, Mieyal JJ, Szweda LI. *Biochemistry* 2003;42:4235–4242. [PubMed: 12680778]
56. Droge W. *Physiol Rev* 2002;82:47–95. [PubMed: 11773609]
57. Finkel T. *FEBS Lett* 2000;476:52–54. [PubMed: 10878249]
58. Rhee SG. *Exp Mol Med* 1999;31:53–59. [PubMed: 10410302]

A. **B.** **Figure 1.**

NADH oxidase activity and superoxide production as a function of ischemic time. Rat hearts were subjected to ischemic times indicated on the abscissa. Hearts were then homogenized and mitochondria prepared as described in the Materials and Methods. **A.** Superoxide production was measured fluorometrically as the rate at which disrupted mitochondria oxidize hydroethidine in the presence of NADH. Representative traces demonstrate the increase in the rate of superoxide production as a function of ischemic duration and is given as the relative change in fluorescence intensity over time. **B.** NADH oxidase activity (open circles) was measured spectrophotometrically as the rate at which disrupted mitochondria oxidized NADH. The NADH-supported rate of superoxide production (closed circles) is shown as a function of

ischemic duration. Perfused control mitochondria had an NADH oxidase rate of 754 ± 35 nmol NADH $\times$ min⁻¹ $\times$ mg⁻¹ and produced superoxide at a rate of 1.245 ± 0.052 nmol ethidium $\times$ min⁻¹ $\times$ mg⁻¹. Values at each ischemic time point are expressed as percentages of these rates. All NADH oxidase and superoxide production measurements were rotenone and SOD sensitive, respectively. Values represent means $\pm$ S.E. (n=5). Symbols (*) indicate statistical significance ($p < 0.05$) between the indicated time point and perfused controls using a student t-test.

Figure 2.

Electron transport chain activities as a function of ischemic time. Rat hearts were subjected to ischemic times indicated on the abscissa. Hearts were then homogenized and mitochondria prepared as described in the Materials and Methods. **A.** Complex I activity was measured spectrophotometrically as the rotenone-sensitive oxidation of NADH in the presence of antimycin A and ubiquinone-1. Perfused control mitochondria had a specific activity of 336 ± 10 nmol NADH $\times$ min⁻¹ $\times$ mg⁻¹. **B.** Complex III-IV activity was measured spectrophotometrically as the rate of ubiquinol-1 oxidation. Perfused control mitochondria had a specific activity of 542 ± 15 nmol ubiquinol $\times$ min⁻¹ $\times$ mg⁻¹. **C.** Complex III was measured spectrophotometrically as the rate of cytochrome *c* reduction in the presence of dodecyl maltoside, decyl ubiquinol and cyanide. Perfused control mitochondria had a specific activity of 2705 ± 104 nmol cytochrome *c* $\times$ min⁻¹ $\times$ mg⁻¹. **D.** Complex IV was measured

spectrophotometrically as the rate of cytochrome *c* oxidation. Perfused control mitochondria had a specific activity of 2253 ± 85 nmol cytochrome *c* $\times$ min⁻¹ $\times$ mg⁻¹. Values represent means $\pm$ S.E. (n=5). Symbols (*) indicate statistical significance ($p < 0.05$) between the indicated time point and perfused controls using a student t-test.

Figure 3.

NADH oxidase and superoxide production as a function of potassium chloride concentration. Mitochondria were prepared from perfused, control hearts. NADH oxidase activity (open circles) and superoxide production (closed circles) measurements were performed at potassium chloride concentrations indicated on the abscissa as described in the legend for Fig. 1B and in Materials and Methods. All NADH oxidase and superoxide production measurements were rotenone and SOD sensitive, respectively. Hydroethidine fluorescence was unaffected by a change in potassium chloride concentration from 0 to 25 mM. Values are expressed as a percent change from activities in the absence of KCl, and represent means $\pm$ S.D (n=3).

Figure 4.

Electron transport chain activities as a function of potassium chloride concentration. Mitochondria were prepared from perfused, control hearts. **A.** Complex I, **B.** Complex III-IV, **C.** Complex III, and **D.** Complex IV activities were measured with the potassium chloride concentrations indicated on the abscissa with methodology described in the legend for Fig. 2 and the Materials and Methods. Values are expressed as a percent change from activities in the absence of KCl, and represent means $\pm$ S.D (n=3).

Table 1

Mitochondria do not exhibit respiratory deficits with brief (< 15 min) ischemic durations. Rat hearts were subjected to indicated durations of ischemia, mitochondria isolated, and respiratory activity assessed polarographically using glutamate (10 mM) and malate (2.5 mM) as substrates. Values are expressed as $\text{nmol O}_2 \times \text{min}^{-1} \times \text{mg}^{-1}$. Values represent means $\pm$ S.E. (n=4), Like symbols (*) indicate statistical significance ($p < 0.05$) between compared samples using a student t-test.

	Perfused	5 min Ischemia	15 min Ischemia	30 min Ischemia
State 3	72.4 $\pm$ 4.3*	70.2 $\pm$ 3.0	72.5 $\pm$ 2.7	47.2 $\pm$ 5.7*
State 4	11.2 $\pm$ 1.1	10.4 $\pm$ 2.0	10.9 $\pm$ 1.3	10.9 $\pm$ 1.3

Table 2

The biphasic production of superoxide as a function of ischemic time involves distinct sites of free radical production within the electron transport chain. Superoxide production was measured, as described in Figure 1, without electron transport chain inhibitor (basal) and in the presence of 50 nM myxothiazol. Values are expressed as the relative change between each experimental condition and the perfused control. All superoxide production measurements were SOD sensitive. Values represent means $\pm$ S.E. (n=3).

	Perfused	15 min Ischemia	30 min Ischemia
Basal	100.0 $\pm$ 6.1%	129.9 $\pm$ 4.8%	161.7 $\pm$ 15.4%
Myxothiazol	283.4 $\pm$ 6.3%	280 $\pm$ 13.8%	311.8 $\pm$ 5.7%