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Are mitochondria a permanent source of reactive oxygen species?

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Abstract

The observation that in isolated mitochondria electrons may leak out of the respiratory chain to form superoxide radicals ($O_2^{\bullet-}$) has prompted the assumption that $O_2^{\bullet-}$ formation is a compulsory by-product of respiration. Since mitochondrial $O_2^{\bullet-}$ formation under homeostatic conditions could not be demonstrated in situ so far, conclusions drawn from isolated mitochondria must be considered with precaution. The present study reveals a link between electron deviation from the respiratory chain to oxygen and the coupling state in the presence of antimycin A. Another important factor is the analytical system applied for the detection of activated oxygen species. Due to the presence of superoxide dismutase in mitochondria, $O_2^{\bullet-}$ release cannot be realistically determined in intact mitochondria. We therefore followed the release of the stable dismutation product H_2O_2 by comparing most frequently used H_2O_2 detection methods. The possible interaction of the detection systems with the respiratory chain was avoided by a recently developed method, which was compared with conventional methods. Irrespective of the methods applied, the substrates used for respiration and the state of respiration established, intact mitochondria could not be made to release H_2O_2 from dismutating $O_2^{\bullet-}$. Although regular mitochondrial respiration is unlikely to supply single electrons for $O_2^{\bullet-}$ formation our study does not exclude the possibility of the respiratory chain becoming a radical source under certain conditions.

Keywords: Mitochondrion; Heart; Superoxide radical; Hydrogen peroxide; Fluorescence; Scopoletin; Homovanillic acid

1. Introduction

Since the recognition of the pathophysiological significance of oxygen radicals in a number of clinical diseases mitochondria have increasingly been a focus of interest for scientists looking for cellular sites of oxygen activation and the underlying trigger mechanisms. In recent times, superoxide radicals ($O_2^{\bullet-}$) have also been assumed to be involved in signaling activities of physiological relevance [1–5]. In addition, the dismutation product of $O_2^{\bullet-}$, H_2O_2, has been shown to exert a variety of physiological activities [1–4,6–9]. An evaluation of the putative role of $O_2^{\bullet-}$ and H_2O_2 in normal cellular functions requires both the demonstration of the site(s) transferring electrons to dioxygen and the conditions which regulate this pathway of electrons.

The suggested role of the mitochondrial respiratory chain as a cellular source for $O_2^{\bullet-}$ was deduced from the finding that antimycin A-inhibited respiration unequivocally results in the release of $O_2^{\bullet-}$. The general conception of the origin of mitochondrial one-electron transfer to dioxygen was the assumption

Abbreviations: SOD, superoxide dismutase; HRP, horseradish peroxidase; BSA, bovine serum albumin; HVA, homovanillic acid; RHM, rat heart mitochondria; DETAPAC, diethylenetriaminepentaacetic acid; $SQ^{\bullet-}$, ubisemiquinone

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that single e^- are derived from redox-cycling ubisemiquinone ($SQ^{\bullet -}$) or cytochrome b_{566} [10–12]. Conditions required to run this redox couple were reported to exist during state IV respiration where mitochondrial respiration is not used for ATP synthesis. Under these conditions the proton-motive force, the membrane potential and the reduction states of possible oxygen reductants all exhibit the highest values. Transition of this respiration state (state IV) to the active state (state III) which is characterized by ATP synthesis was assumed to inhibit $O_2^{\bullet -}$ generation [11,13,14]. Recently, $O_2^{\bullet -}$ release from complex I was reported to occur as a side product of normal respiration which was found to be independent of the state of respiration [15]. In contrast, the authors observed even higher formation rates when respiration switched from state IV to the active state.

Convincing experimental evidence which proves the existence of a permanent mitochondrial source of $O_2^{\bullet -}$ is still lacking due to the inadequacy of existing detection methods. Problems arise from the existence of the outer membrane which is a permeation barrier for compounds used to detect and to prove the existence of $O_2^{\bullet -}$, e.g. superoxide dismutase (SOD)-mediated inhibition of $O_2^{\bullet -}$-indicating dye formation is compelling proof that $O_2^{\bullet -}$ was involved. $O_2^{\bullet -}$ expected to be released from the inner mitochondrial membrane into the intermembraneous space is not accessible for SOD externally added. In addition, matrix-bound SOD of mitochondria together with the spontaneous dismutation of $O_2^{\bullet -}$ will decrease steady state levels of $O_2^{\bullet -}$ when generated. To exclude these uncertainties submitochondrial particles were used when mitochondria were studied for their capability to generate $O_2^{\bullet -}$. However, this artificially established respiratory chain does not represent bioenergetic conditions of intact mitochondria.

The lack of reliable methods for the detection of $O_2^{\bullet -}$ release from intact mitochondria prompted us to focus on the detection of the dismutation product H_2O_2. H_2O_2 was earlier shown to readily diffuse from its site of generation across the outer membrane into the extramitochondrial space [16,17] where it can be easily detected by adequate methods. To exclude any interferences of the H_2O_2 detection system with regular mitochondrial respiration we applied a non-invasive H_2O_2 detection system recently developed in our institute. This method excludes any undesired interference of the H_2O_2 detection system with mitochondrial constituents. It therefore provides more reliable results than would be obtained when the detection system is in direct contact with mitochondria [18].

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (HRP, EC 1.11.1.7., type VI, approximately 1000 ABTS units per mg solid), essentially fatty acid-free bovine serum albumin (BSA, fraction V), homovanillic acid (HVA), L-malic acid and rotenone were purchased from Sigma-Aldrich Handels (Vienna, Austria). Pyruvate, ADP (potassium salt) and antimycin A were from Boehringer (Mannheim, Germany). Triethanolamine-HCl and succinic acid were obtained from Fluka (Buchs, Switzerland) and scopoletin from Serva-Feinbiochemica (Heidelberg, Germany). Other chemicals came from Merck (Darmstadt, Germany).

2.2. Preparation of mitochondria

Rat heart mitochondria (RHM) were prepared from male Sprague–Dawley rats (Him:OFA/SPF) weighing 300–400 g. The animals were obtained from the Research Institute for Laboratory Breeding in Himberg (Austria) and allowed to acclimatize for at least 2 weeks in the animal laboratory of our institute (open system, PEC-type cages No. IV, 2–3 animals per cage, Altromin pellets No. 1324FF from Marek Co., Vienna, feeding and tap water ad libitum, natural day–night cycle). Rats were killed by cervix dislocation and decapitated. The hearts were quickly excised and plunged into ice-cold isolation buffer. Vessels and auricles as well as remaining blood were removed. The tissue was chopped into small pieces with scissors and washed several times with buffer to reduce the contaminating blood. The isolation of mitochondria was performed as described by Mela and Seitz [19] with the following modifications: the isolation buffer contained 0.3 M sucrose, 20 mM triethanolamine and 1 mM EDTA–

Na_2. The pH was adjusted to 7.4 with KOH. Nagarse was omitted. The tissue homogenate was centrifuged at 2500 rpm for 10 min (SS34 rotor, Sorvall RC5B centrifuge); the supernatant was filtered through cheesecloth and immediately spun down at 9000 rpm for 10 min. The resulting pellets were gently resuspended using a 15 ml capacity Potter–Elvehjem tissue homogenizer driven manually. After a 10 min centrifugation period at 9000 rpm this washing procedure was repeated once more. The final mitochondrial pellet was resuspended carefully in the isolation buffer using an Eppendorf pipette. The protein content of the mitochondrial suspension (40–50 mg protein per ml) was measured by the biuret method using BSA as a standard.

2.3. Mitochondrial oxygen consumption

Respiratory parameters were determined at 25°C in the mitochondrial incubation buffer consisting of 0.3 M sucrose, 20 mM triethanolamine, 1 mM diethylenetriaminepentaacetic acid (DETAPAC), 0.5 g fatty acid-free BSA/l, pH 7.4 with a Clark-type oxygen electrode of our own design. State IV respiration was stimulated by the addition of 3.6 mM inorganic phosphate and one of the following respiratory substrates: 5 mM glutamate/5 mM malate, 2.5 mM pyruvate/2.5 mM malate or 10 mM succinate in the presence of 5 μM rotenone. State III conditions were established by the addition of 250 μM ADP. The bioenergetic intactness of mitochondria used in this study was ensured by the determination of respiratory control values (6.62 ± 0.16 for glutamate/malate, 6.13 ± 0.38 for pyruvate/malate).

2.4. Detection of H_2O_2 using fluorescence spectroscopy

Fluorescence measurements were performed using a Hitachi F4500 spectrofluorimeter at 700 V PMT voltage and 5 nm slit widths, both for excitation and emission. The instrument was equipped with a Hitachi microsampling accessory allowing external additions during the time scans. The assays were performed at 25°C in 3 ml fluorimeter quartz cuvettes containing a magnetic stirrer. Excitation and emission wavelengths for scopoletin and HVA were 366–460 and 312–420 nm, respectively. A stock solution of scopoletin was prepared in acetonitrile and stored in the dark on ice. Incubations and measurements were performed in the mitochondrial incubation buffer at 25°C. The system was calibrated with known concentrations of H_2O_2 in the presence of 10 U/ml HRP and 5 μM scopoletin or 100 μM HVA. The concentration of H_2O_2 was determined at 240 nm using a molar extinction coefficient of 43.6 [20].

2.5. Mitochondrial H_2O_2 liberation

RHM (0.5 mg protein/ml) were suspended in the mitochondrial incubation buffer and supplemented with 4 mM inorganic phosphate and 10 mM succinate, 5 mM glutamate/5 mM malate or 2.5 mM pyruvate/2.5 mM malate to start state IV respiration. Transformation to state III respiration was started with the addition of 500 μM ADP. $O_2^{\bullet -}$ formation following the addition of antimycin A (2 μg/ml) was taken as a control for the evaluation of spontaneous $O_2^{\bullet -}$-derived H_2O_2 release in the absence of inhibitors [17]. Catalase (725 U/ml) was added to prove the specificity of the detection system. Uncoupled mitochondria were obtained from diluted mitochondrial suspensions (10 mg protein/ml) by freezing for 10 min in liquid nitrogen and subsequent thawing at 25°C. This procedure was repeated three times.

H_2O_2 liberation rates were determined either invasively, when RHM were in direct contact with the respective detection system, or non-invasively, when H_2O_2 was accumulated in the suspension medium and analyzed after separation from respiring mitochondria [18]. In the latter case mitochondrial samples were supplemented with complex I or complex II substrates, inhibitors as indicated or ADP and allowed to equilibrate for 3 min at 25°C. After this the suspensions were immediately centrifuged at $9000 \times g$ for 5 min (at 25°C) to sediment the mitochondrial fraction which otherwise will absorb the emitted light during the following fluorescence detection. 900 μl of the supernatants containing the accumulated H_2O_2 was placed in a fluorescence cuvette and supplemented with 5 μM scopoletin or 100 μM HVA. The amount of fluorescence decrease (scopoletin) or increase (HVA) following HRP-catalyzed reaction (10 U/ml) of H_2O_2 with the respective detection system was quantified using calibration curves. The linearity of the applied H_2O_2 detection

methods was verified by using standardized conditions of mitochondrial H_2O_2 formation (succinate plus antimycin A).

3. Results

In order to exclude any direct interference of the detection system with mitochondrial constituents and vice versa, H_2O_2 was determined in the supernatant of mitochondrial suspensions [18] respiring under the various conditions reported to be associated with the release of H_2O_2. The accumulation of H_2O_2 in the suspension was terminated after an incubation period of 3 min by centrifugation. The supernatant was subjected to analysis for H_2O_2 possibly accumulated during the test period of respiration (Fig. 1).

3.1. H_2O_2 release from succinate-driven respiration

Fig. 1 shows the results obtained with coupled and uncoupled mitochondria during succinate respiration. In contrast to the general assumption that mitochondria deviate e^- to oxygen out of sequence when bioenergetic conditions with high proton gradients and high reduction states of their e^- carriers are established (state IV, Fig. 1a), H_2O_2 was not formed in the resting state unless antimycin A was added (Fig. 1b). The amount of H_2O_2 accumulated during the incubation period was considerably higher when antimycin A-inhibited mitochondria were uncoupled by freezing in liquid nitrogen and subsequent thawing (Fig. 1c) while in the absence of antimycin A no H_2O_2 was formed.

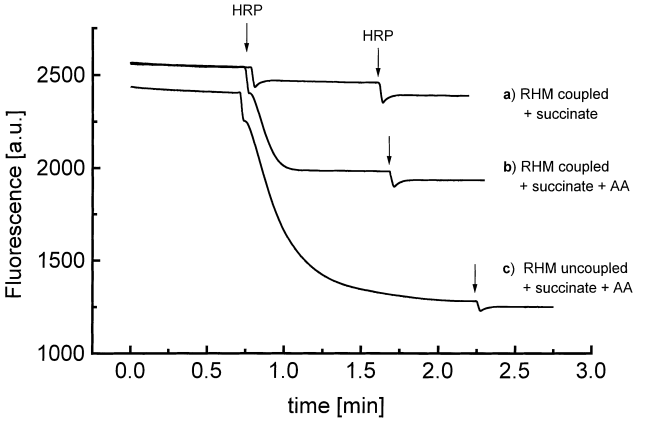

Fig. 1. Succinate-induced H_2O_2 release from RHM determined non-invasively with scopoletin. Experimental conditions: 0.3 M sucrose, 20 mM triethanolamine, 1 mM DETAPAC, 0.5 mg BSA/ml, pH 7.4 (incubation buffer), 4 mM inorganic phosphate, 10 mM succinate, 2 μg/ml antimycin A (AA), 0.5 mg/ml mitochondrial protein. RHM were made to produce H_2O_2 in the absence of the detection system for 3 min at 25°C and separated by centrifugation (5 min; $9000 \times g$). The supernatant was placed in a fluorescence cell, 5 μM scopoletin was added and HRP-catalyzed (10 U/ml) fluorescence decrease (excitation: 366 nm, emission: 460 nm) was monitored. RHM were uncoupled by three subsequent freezing and thawing procedures. Curves represent mean values of time scans obtained from 11 independent mitochondrial preparations.

3.2. H_2O_2 release from complex I-driven respiration

Recently it was reported that mitochondria generate H_2O_2 when supplemented with pyruvate/malate or other complex I substrates [15]. H_2O_2 was suggested to stem from $O_2^{\bullet -}$ released from constituents of complex I. In contrast to succinate-related oxygen activation, H_2O_2 release from complex I was reported even to increase during transition from state IV (resting state) to state III (active state). Fig. 2A shows that with the scopoletin method [13,21,22], modified for non-invasive H_2O_2 detection, H_2O_2 release could not be found with any combination of complex I substrates irrespective of whether mitochondria were made to respire in the resting (IV) or in the active state (III). H_2O_2 release from complex I was also not observed when rotenone was used to increase the reduction states of e^- carriers associated with complex I to maximal values (not shown). However, complex I substrates induced H_2O_2 formation when e^- flow through the Q cycle was inhibited by antimycin A. H_2O_2 release in the presence of antimycin A was small with glutamate/malate and rather negligible with pyruvate/malate. It was rotenone-sensitive and correlated quantitatively with the flow rate of reducing equivalents from the substrates to cytochrome oxidase in the absence of inhibitors (see Fig. 3).

The results obtained from non-invasive H_2O_2 determination (Fig. 2A) were qualitatively confirmed when applying the conventional detection method where the complete set of the detection system was in direct contact with mitochondria (Fig. 2B). Under conditions of state IV and state III respiration, scopoletin fluorescence slightly increased instead of

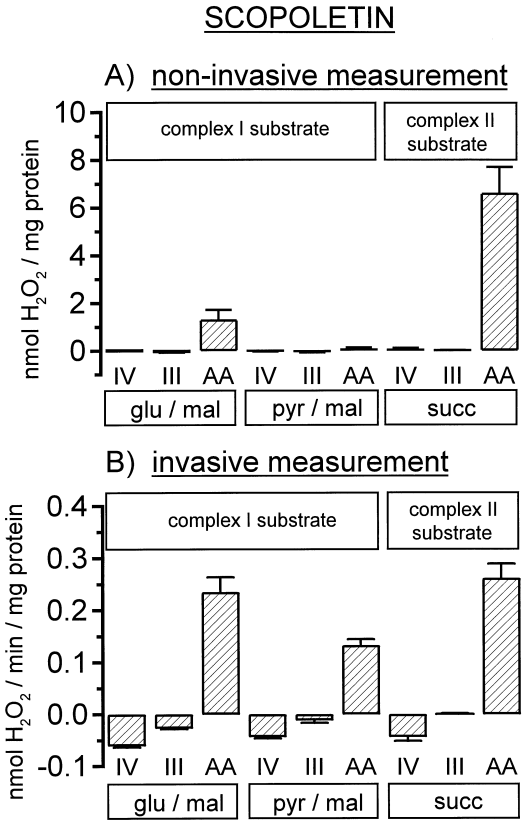

Fig. 2. Determination of H_2O_2 release from RHM respiring under various metabolic conditions, e.g. state IV (substrate only), state III (substrate plus ADP) or antimycin A-inhibited respiration. H_2O_2 release was detected either non-invasively (A) or invasively, e.g. when the detection system was in direct contact with H_2O_2-releasing RHM (B). Experimental conditions: 5 mM glutamate/5 mM malate, 2.5 mM pyruvate/2.5 mM malate. Other details as in Fig. 1. Data represent means ± S.E.M. of 3–9 independent mitochondrial preparations.

being decreased as is to be expected if H_2O_2 is involved. This effect may result from mitochondrial pyridine nucleotides which have excitation and emission properties similar to scopoletin. Additional evidence for this interpretation comes from the fact that mitochondria, when exposed to the same excitation wavelength in the absence of scopoletin, respond with light emission in the range of scopoletin fluorescence (not shown). Although the conventional method normally applied for mitochondrial H_2O_2 detection gave identical qualitative results as obtained from the non-invasive detection method, H_2O_2 formation rates assessed with the latter method were higher (approximately two times for glutamate/malate and nine times for succinate).

As a control for H_2O_2 formation based on scopoletin fluorescence change we performed the same type of experiments with HVA as a fluorescence probe for H_2O_2 (Fig. 4). In contrast to the fluorescent scopoletin which indicates the presence of H_2O_2 by a fluorescence decrease, HVA becomes fluorescent through H_2O_2-induced oxidation. Using antimycin A-inhibited succinate respiration as a standard for mitochondrial H_2O_2 generation HVA fluorescence indicated the presence of almost identical amounts of H_2O_2 as obtained with scopoletin. This was the case both when the fluorescence dyes were in direct contact with mitochondria (Fig. 4B) and when they were added after mitochondria were separated from the supernatant (Fig. 4A). As for scopoletin it was found that HVA was also a more sensitive H_2O_2 probe (approximately four times using glutamate/malate and 10 times using succinate as respiratory substrates) when mitochondria were separated from the suspension medium prior to H_2O_2 detection. H_2O_2 release was also not observed with HVA in mitochondria respiring under state IV or state III conditions irrespective of whether mitochondria

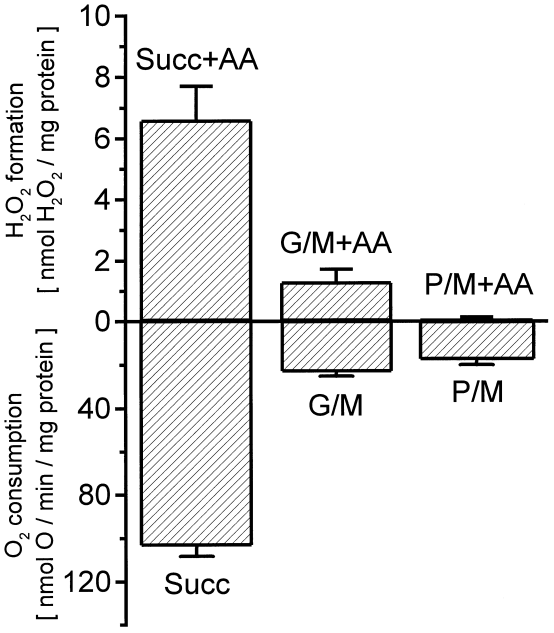

Fig. 3. Correlation between antimycin A-stimulated H_2O_2 release from coupled RHM and oxygen consumption rates during state IV respiration using either 10 mM succinate, 5 mM glutamate/5 mM malate or 2.5 mM pyruvate/2.5 mM malate as substrates. H_2O_2 release was determined as described in Fig. 1 in the presence of antimycin A. Data represent means ± S.E.M. of four independent mitochondrial preparations.

were supplemented with succinate or complex I substrates (Fig. 4A). It was remarkable that, in contrast to H_2O_2 patterns obtained from the use of scopoletin, HVA fluorescence increase was observed with succinate when the detection system was operating in direct contact with mitochondria. This particular succinate-induced fluorescence increase was only slightly sensitive to catalase. Total inhibition was not possible even at extremely high catalase concentrations (7250 U/ml) which makes the existence of a native mitochondrial H_2O_2 source unlikely.

4. Discussion

Applying the recently developed non-invasive H_2O_2 detection method, the absence of HRP and scopoletin or HVA, respectively, during state IV and state III respiration ensures that any undesired effect on these complex bioenergetic activities possibly simulating mitochondrial H_2O_2 generation is excluded. Thus, H_2O_2 analyzed in the supernatants of the various experimental sets of mitochondrial preparations can be considered to realistically reflect whether or not e^- deviation to oxygen out of sequence occurred. Comparison of the conventional H_2O_2 detection methods with the indirect non-invasive method revealed a considerably higher sensitivity of the latter. Thus, the indirect method applied in this study is more suited for the detection of even low rates of mitochondrial oxygen activation. Our results show that inhibition of respiration through antimycin A is a prerequisite for branching off single e^- from the respiratory chain to oxygen. The presence of antimycin A has an impact on the redox potentials of the various e^- carriers taking part in the Q cycle. It was suggested that $SQ^{\bullet -}$, formed at the cytosolic face of complex III, will shift to more negative redox potentials under conditions where both *b*-type cytochromes exist in the reduced state [23,24]. This change establishes thermodynamic conditions which make an electron flow to dioxygen possible. In addition there is evidence that one-electron reduction of O_2 also requires an impact on the regular interaction of redox-cycling ubiquinone with the protein complex where e^- are bifurcated for the subsequent reduction of cytochrome b_{566} and Rieske iron–sulfur protein (Nohl and Gille, unpublished results, 2000). Accordingly, H_2O_2 formation was observed to considerably increase when the mitochondrial membrane was changed by freezing and thawing. When mitochondria respire under state IV conditions the proton-motive force shifts *b*-type cytochromes versus the reduced state, thereby imposing similar changes on the reduction potential of $SQ^{\bullet -}$ as antimycin A does. Nevertheless, our experiments have shown that direct e^- leakage to oxygen does not occur during this physiological state of respiration. This may support the idea that the establishment of a redox couple between mitochondrial e^- donors and dioxygen requires an impediment of the regular e^- bifurcation at complex III.

We have recently demonstrated that autoxidation of $SQ^{\bullet -}$ requires both the adjustment of thermody-

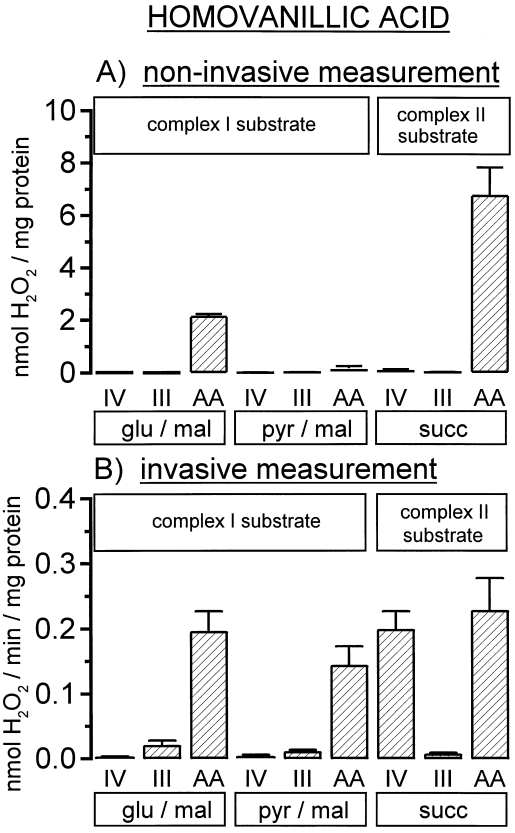

Fig. 4. Comparison of H_2O_2 release from RHM under different metabolic states. Conditions were the same as described in Fig. 2 with the exception of HVA (100 μM; excitation: 312 nm, emission: 420 nm) which was used as the hydrogen donor instead of scopoletin. Data represent means ± S.E.M. of 3–9 independent mitochondrial preparations.

namically adequate redox potentials and the disturbance of the kinetic facilitation of regular e^- transfer to *b*-type cytochromes [25,26]. Impairment of the hydrophobic interaction of $SQ^{\bullet-}$ with its natural oxidants using toluene pretreatment was found to result in a derangement of regular e^- flow from redox-cycling $SQ^{\bullet-}$ to complex III (bifurcation of e^- flow to Rieske iron–sulfur protein and cytochrome b_{566}) [25,26]. A similar situation is expected to occur when mitochondrial membranes are subjected to liquid nitrogen for uncoupling. It was recently shown that even carefully isolated RHM were contaminated with 3–5% of fragmented mitochondria [27]. This fraction does not contribute to ATP synthesis due to the loss of coupling properties. Thus, it can be assumed that the addition of antimycin A to the suspension of freshly isolated mitochondria preferentially induces autoxidation of $SQ^{\bullet-}$ associated with membranes of fragmented mitochondria. This is quantitatively in line with the generally reported percentage of dioxygen utilized by mitochondria undergoing one-e^- reduction (1–4%) [11,28]. Further support for this assumption comes from the observation that H_2O_2 release was a function of the coupling state of mitochondria in the suspension (see Fig. 1). While it is becoming increasingly clear that electrons of the Q-cycle do not leak to oxygen out of sequence under physiological conditions [25,26,29,30], complex I was recently suggested to produce $O_2^{\bullet-}$ as a natural by-product of respiration [15]. We have extensively studied this possibility by applying conventional as well as indirect H_2O_2 detection methods using both scopoletin fluorescence quench and HVA fluorescence development. Results obtained confirm that H_2O_2 release requires the presence of antimycin A while oxygen activation did not occur when intact mitochondria were made to respire complex I or complex II substrates in the absence of antimycin A.

We do not exclude autoxidation of Fe–S proteins of complex I when they equilibrate in an unphysiologically high reduction state. This can be expected following functional fragmentation of complex I from the natural oxidants, a situation which was shown by Turrens, Boveris and others to result from high Ca^{2+} levels [31,32]. Ischemia/reperfusion-linked decompartmentation of Ca^{2+} was suggested to be the trigger mechanism of complex I-related oxygen activation [31,33–35]. At 37°C complex I-derived reactive oxygen species were also observed when e^- flow to the Q pool was inhibited by rotenone [36].

Uncertainties about the mitochondrial respiratory chain as a permanent physiological radical generator may be due to the use of inadequate detection methods or destabilizing mitochondrial preparation methods which affect kinetic and thermodynamic conditions of regular e^- flow through complex I and the Q cycle. A good example in this respect are the inconsistent results on the apparent H_2O_2 generation in succinate-respiring mitochondria. Positive results were obtained only when HVA was allowed to directly interact with mitochondria supplemented with succinate under state IV conditions. The assumption that the observed fluorescence change of HVA does not reflect native mitochondrial H_2O_2 in this case is supported by the impossibility of suppressing this effect by catalase together with the fact that scopoletin under otherwise identical conditions did not show any fluorescence change. Accordingly succinate-related fluorescence change of HVA was also not seen when the H_2O_2 detection system was not in direct contact with respiring mitochondria. That result was consistent with scopoletin which did not change fluorescence either in contact with mitochondria or when added to the supernatant of succinate-respiring mitochondria. Conformity exists on the putative sites of the respiratory chain involved in the supply of single e^- to oxygen out of sequence. However, conditions required for an activation of these radical sources are not consistent with cellular homeostasis. Uncontrolled e^- leakage from the respiratory chain decreases the efficiency of energy gain and would affect a cascade of secondary effects including opening of the phase-transition pore, calcium decompartmentation and apoptosis [37–39]. These events would in turn affect the regular e^- flow of the respiratory chain in a way which further stimulates autoxidation of e^- carriers accounting for $O_2^{\bullet-}$ release [40,41]. The strong control of e^- transfer along natural redox couples of cardiac mitochondria is in line with the high energy request of the working heart. In addition, mitochondrial respiration seems not to be required as a permanent $O_2^{\bullet-}$ source for possible physiological activities such as signaling. It is more likely that if $O_2^{\bullet-}$ is involved in regulatory mechanisms, it is generated in the close vicinity of the respective regulator in order to exclude harmful alter-

native pathways. For instance, there are many cofactors such as tetrahydropterin which directly cooperate as activators of molecular oxygen with monooxygenases represented by nitric oxide synthase [42]. Considering the consequences of a non-compensated permanent production of activated oxygen species during respiration our findings excluding the respiratory chain as a compelling radical source is in harmony with homeostasis of orderly cell function. This is also valid in the case of small amounts of H_2O_2 which may escape detection when decomposed by matrix-bound catalase [43,44]. Although the method applied in this study is more sensitive by one order of magnitude than conventional methods used earlier [18], the lack of H_2O_2 stresses the significance of catalase in preserving homeostasis by the removal of H_2O_2 from the Fenton-type pathway.

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References

[1] Y.J. Suzuki, H.J. Forman, A. Sevanian, Free Radic. Biol. Med. 22 (1997) 269–285.
[2] M. Saran, C. Michel, W. Bors, Z. Naturforsch. C 53 (1998) 210–227.
[3] T. Finkel, Curr. Opin. Cell Biol. 10 (1998) 248–253.
[4] J.T. Hancock, Br. J. Biomed. Sci. 54 (1997) 38–46.
[5] V. Darley-Usmar, R. White, Exp. Physiol. 82 (1997) 305–316.
[6] M. Zheng, G. Storz, Science 279 (1998) 1718–1721.
[7] X. Wang, J.L. Martindale, Y. Liu, N.J. Holbrook, Biochem. J. 333 (1998) 291–300.
[8] J. Duranteau, N.S. Chandel, A. Kulisz, Z. Shao, P.T. Schumacker, J. Biol. Chem. 273 (1998) 11619–11624.
[9] J. Zhang, N. Jin, Y. Liu, R.A. Rhoades, Am. J. Respir. Cell Mol. Biol. 19 (1998) 324–332.
[10] H. Nohl, W. Jordan, Biochem. Biophys. Res. Commun. 138 (1986) 533–539.
[11] A. Boveris, N. Oshino, B. Chance, Biochem. J. 128 (1972) 617–630.
[12] A. Boveris, E. Cadenas, A.O. Stoppani, Biochem. J. 156 (1976) 435–444.
[13] G. Loschen, L. Flohe, B. Chance, FEBS Lett. 18 (1971) 261–264.
[14] E. Cadenas, A. Boveris, Biochem. J. 188 (1980) 31–37.
[15] A. Herrero, G. Barja, J. Bioenerg. Biomembr. 29 (1997) 241–249.
[16] H. Nohl, W. Jordan, Eur. J. Biochem. 111 (1980) 203–210.
[17] J.F. Turrens, Biosci. Rep. 17 (1997) 3–8.
[18] K. Staniek, H. Nohl, Biochim. Biophys. Acta 1413 (1999) 70–80.
[19] L. Mela, S. Seitz, Methods Enzymol. LV (1979) 39–46.
[20] J. Duley, R.S. Holmes, Anal. Biochem. 69 (1975) 164–169.
[21] W.A. Andreae, Nature 175 (1955) 859–860.
[22] H. Perschke, E. Broda, Nature 190 (1961) 257–258.
[23] B. Chance, R.F. Wilson, P.L. Dutton, M. Erecinska, Proc. Natl. Acad. Sci. USA 66 (1970) 1175–1182.
[24] M.K.F. Wikström, J.A. Berden, Biochim. Biophys. Acta 283 (1972) 403–420.
[25] H. Nohl, K. Stolze, Free Radic. Res. Commun. 16 (1992) 409–419.
[26] H. Nohl, L. Gille, K. Schönheit, Y. Liu, Free Radic. Biol. Med. 20 (1996) 207–213.
[27] K. Schönheit, H. Nohl, Arch. Biochem. Biophys. 372 (1996) 319–323.
[28] A. Boveris, B. Chance, Biochem. J. 134 (1973) 707–716.
[29] H.J. Forman, A. Azzi, FASEB J. 11 (1997) 374–375.
[30] R.E. Beyer, K. Nordenbrand, L. Ernster, in: K. Kolkers, Y. Yamamura (Eds.), Biomedical and Clinical Aspects of Coenzyme Q, Vol. 5, Elsevier, Amsterdam, 1986, pp. 17–24.
[31] J.F. Turrens, M. Beconi, J. Barilla, U.B. Chavez, J.M. McCord, Free Radic. Res. Commun. 12–13 (1991) 681–688.
[32] J.F. Turrens, A. Boveris, Biochem. J. 191 (1980) 421–427.
[33] C.D. Malis, J.V. Bonventre, J. Biol. Chem. 261 (1986) 14201–14208.
[34] H.M. Piper, T. Noll, B. Siegmund, Cardiovasc. Res. 28 (1994) 1–15.
[35] J. Hardy, J.B. Clark, V.M. Darley-Usmar, D.R. Smith, D. Stone, Biochem. J. 274 (1991) 133–137.
[36] H. Nohl, in: C.M. Caldarerea, P. Harris (Eds.), Advances in Studies on Heart Metabolism, CLUEB, Bologna, 1982, pp. 413–421.
[37] H.R. Lotscher, K.H. Winterhalter, E. Carafoli, C. Richter, Proc. Natl. Acad. Sci. USA 76 (1979) 4340–4344.
[38] D. Carbonera, G.F. Azzone, Biochim. Biophys. Acta 943 (1988) 245–255.
[39] C. Richter, Biosci. Rep. 17 (1997) 53–66.
[40] T. Paraidathathu, H. de Groot, J.P. Kehrer, Free Radic. Biol. Med. 13 (1992) 289–297.
[41] E. Chacon, D. Acosta, Toxicol. Appl. Pharmacol. 107 (1991) 117–128.
[42] J.B. Fukuto, B. Mayer, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, John Wiley and Son, Chichester, 1998, pp. 147–160.
[43] H. Nohl, D. Hegner, FEBS Lett. 89 (1978) 126–130.
[44] R. Radi, J.F. Turrens, L.Y. Chang, K.M. Bush, J.D. Crapo, B.A. Freeman, J. Biol. Chem. 266 (1991) 22028–22034.