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H_2O_2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system[1]

Katrin Staniek, Hans Nohl *

Institute of Pharmacology and Toxicology, Veterinary University of Vienna, Veterinärplatz 1, 1210 Vienna, Austria

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Abstract

Evaluation of the existence of superoxide radicals ($O_2^{\bullet-}$), the site of generation and conditions required for one-e^- transfer to oxygen from biological redox systems is a prerequisite for the understanding of the deregulation of O_2 homeostasis leading to oxidative stress. Mitochondria are increasingly considered the major $O_2^{\bullet-}$ source in a great variety of diseases and the aging process. Contradictory reports on mitochondrial $O_2^{\bullet-}$ release prompted us to critically investigate frequently used $O_2^{\bullet-}$ detection methods for their suitability. Due to the impermeability of the external mitochondrial membrane for most constituents of $O_2^{\bullet-}$ detection systems we decided to follow the stable dismutation product H_2O_2. This metabolite was earlier shown to readily permeate into the cytosol. With the exception of tetramethylbenzidine none of the chemical reactants indicating the presence of H_2O_2 by horseradish peroxidase-catalyzed absorbance change were suited due to solubility problems or low extinction coefficients. Tetramethylbenzidine-dependent H_2O_2 detection was counteracted by rereduction of the dye through e^- carriers of the respiratory chain. Although the fluorescent dyes scopoletin and homovanillic acid were found to be suited for the detection of mitochondrial H_2O_2 release, fluorescence change was strongly affected by mitochondrial protein constituents. The present study has resolved this problem by separating the detection system from H_2O_2-producing mitochondria.

Keywords: Mitochondria; Hydrogen peroxide; Scopoletin; Homovanillic acid; Tetramethylbenzidine

1. Introduction

The recognition of the involvement of oxygen radicals in an ever increasing number of diseases has focused the scientific interest on mitochondria as the putative source of oxygen activation [1–7]. There are a couple of peculiarities which strongly favor these organelles as the most likely candidates for the establishment of oxidative stress in the cell. Mitochondria are present in almost all cells where they are packed in high density. They consume over 90% of total cellular oxygen. The various one-electron carriers which form the respiratory chain are suited for an e^- transfer onto the peculiar electron configuration of dioxygen which prevents the acceptance of a pair of electrons. When a redox couple between any of these electron carriers with oxygen is estab-

Abbreviations: $O_2^{\bullet-}$, superoxide radical; SMP, submitochondrial particles; SOD, superoxide dismutase; HRP, horseradish peroxidase; ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); BSA, bovine serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine·2HCl; HVA, homovanillic acid; RHM, rat heart mitochondria; DH_2, hydrogen donor; P_i, inorganic phosphate; DETAPAC, diethylenetriaminepentaacetic acid

* Corresponding author. Fax: +43 (1) 250774491; E-mail: hans.nohl@vu-wien.ac.at

[1] Dedicated to Prof. E. Elstner, on the occasion of his 60th anniversary.

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lished single e^- will leak out of the respiratory chain (provided that the free energy change favors this pathway) and superoxide ($O_2^{\bullet-}$) radicals are formed [2,8–10]. Such a leakage of electrons giving rise to permanent $O_2^{\bullet-}$ release as a side product of respiration was assumed to occur in mitopathies associated with various neurological diseases, in most myopathies, anthracycline cardiotoxicity and also in the biological process of aging [7,11–14]. Also in reperfusion injury mitochondria were suggested to contribute to oxidative tissue damage by a dramatic increase of $O_2^{\bullet-}$ radical release [1,3,15]. In recent time $O_2^{\bullet-}$ radicals were also recognized to be involved in signaling activities of physiological relevance. An evaluation of mitochondria as a potential physiological and pathophysiological source of $O_2^{\bullet-}$ is therefore required together with the understanding of the regulator system which triggers the leakage of e^- from the respiratory chain to cytosolic oxygen. So far it was assumed that in mitochondria when respiring without ATP production (state IV) electrons escape at the level of redox-cycling ubisemiquinone and reduce O_2 to $O_2^{\bullet-}$. However, conflicting results on the putative role of mitochondria as a permanent, or as an occasional physiological/pathophysiological $O_2^{\bullet-}$ generator exist in the literature. This may be due to the use of inadequate detection methods for $O_2^{\bullet-}$ and undefined variations of the intactness of the mitochondrial preparations.

It is unequivocally accepted that the mitochondrial respiratory chain can be made to generate $O_2^{\bullet-}$ radicals when antimycin A is added to succinate-respiring submitochondrial particles (SMP) [5,16]. SMP are methodologically suited for $O_2^{\bullet-}$ detection since in contrast to intact mitochondria also detection systems with high molecular weight compounds such as cytochrome *c* and superoxide dismutase (SOD) for testing the implication of $O_2^{\bullet-}$ radicals have free access to sites of oxygen activation. In addition, mitochondrial SOD which may interfere with $O_2^{\bullet-}$ radicals of the respiratory chain can be removed from SMP preparations. However, results obtained from SMP do not allow conclusions to be made on in vivo conditions of intact mitochondria where respiration switches between state IV (resting state) and state III (active ATP-generating state). Thus, an evaluation on the formation of $O_2^{\bullet-}$ radicals during physiological activities of mitochondria requires studies with coupled mitochondria in the resting and active state. Superoxide detection methods suited for SMP do not give reliable information on $O_2^{\bullet-}$ radical formation of intact mitochondria. Uncertainties with respect to the evaluation of $O_2^{\bullet-}$ in mitochondria may be due to an insufficient accessibility of the detection system to $O_2^{\bullet-}$ radicals or insufficient quenching by SOD. SOD sensitivity of the detection system is, however, the only evidence that $O_2^{\bullet-}$ radicals were responsible for the dye formation. In addition, matrix-bound SOD of mitochondria together with the spontaneous dismutation of $O_2^{\bullet-}$ in the intermembranous space decrease steady-state levels of $O_2^{\bullet-}$ if generated.

Looking for a more reliable method in order to assess mitochondrial $O_2^{\bullet-}$ formation we decided to follow the formation of the dismutation product H_2O_2 because of its free diffusion through the mitochondrial membrane [17]. A standardized mitochondrial H_2O_2 source was used to generate reproducible amounts of $O_2^{\bullet-}$ derived H_2O_2. Frequently applied H_2O_2 detection methods were investigated for their qualitative and quantitative suitability as indicators of H_2O_2 release from the standardized mitochondrial source. The present study shows that results derived from mitochondria in direct contact with the H_2O_2 detection system are unreliable due to direct interactions with constituents of the respiratory chain or other shortcomings. A non-invasive procedure based on H_2O_2 measurement was developed which avoids the uncertainties mentioned above.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (HRP, EC 1.11.1.7, type VI, approx. 1000 ABTS units per mg solid), essentially fatty acid-free bovine serum albumin (BSA, fraction V), 3,3′,5,5′-tetramethylbenzidine·2HCl (TMB), 3,3′-dimethoxybenzidine·2HCl, *o*-phenylenediamine·2HCl, homovanillic acid (HVA), antimycin A and myxothiazol were purchased from Boehringer (Mannheim, Germany). Triethanolamine·HCl and succinic acid were obtained from Fluka (Buchs, Switzerland); 3,3′-diaminobenzidine·4HCl 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 lab of our institute (open system, PEC-type cages No. IV, 2–3 animals per cage, Altromin pellets No. 1324FF from Marek, Vienna, feeding and tap water ad libitum, natural night and day cycle). Rats were killed by cervix dislocation and decapitation. 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 [18] 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 standard.

2.3. Standardized mitochondrial H_2O_2 source

It is unequivocally accepted that succinate-respiring mitochondria generate $O_2^{\bullet-}$ derived H_2O_2 when the e^- flux through components of the respiratory chain is inhibited by antimycin A [2,5]. H_2O_2 is readily formed by SOD-catalyzed dismutation of $O_2^{\bullet-}$ radicals migrating into the matrix space and spontaneous dismutation of the small $O_2^{\bullet-}$ radical fraction diffusing into the intramembranous space [17]. Iron-catalyzed degradation of H_2O_2 was excluded through chelation of iron by diethylenetriaminepentaacetic acid (DETAPAC). H_2O_2 formation rates are governed by the supply of succinate-dependent reducing equivalents and the oxygen tension. Throughout all experiments in this study in which the various H_2O_2 detection systems were investigated conditions required for standardized H_2O_2 release from the respiratory chain were identical.

2.4. H_2O_2 detection by means of UV-vis spectroscopy

Spectrophotometric measurements were performed with a double beam/dual wavelength DW-2000 UV-vis spectrophotometer (SLM Aminco, Rochester, NY, USA). Conditions for determination of the spectrophotometric parameters of hydrogen-donating dyes were: split beam mode, wavelength scan (240–640 nm), 2 nm slit width, 5 nm/s scanning rate, 25°C, buffer consisting of 50 mM potassium phosphate, pH 7.4, 1 mM DETAPAC. The concentrations of HRP and the hydrogen donor were 10 U/ml and 20 μM, respectively. The reaction was started by the addition of 0–20 μM H_2O_2, the concentration of which was determined at 240 nm using a molar extinction coefficient of 43.6 [19].

The suitability of the hydrogen donor TMB for the detection of H_2O_2 in mitochondrial samples was tested under the following conditions: dual wavelength mode, 465 nm/575 nm, 2 nm slit width, quartz cuvettes with magnetic stirrer. RHM (final protein concentration 0.56 mg/ml) were resuspended in the incubation buffer (0.3 M sucrose, 20 mM triethanolamine, 1 mM DETAPAC, pH 7.4, 25°C) and supplemented with 2 mM inorganic phosphate (P_i) and 10 mM succinate. 10 U HRP/ml and 10 μM TMB were added as the H_2O_2 detection system. In order to calibrate the system 5 μM H_2O_2 were used as the standard.

2.5. H_2O_2 detection by means of 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 to make possible 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 nm and 312–420 nm, respectively. Stock solution of scopoletin was prepared in acetonitrile and stored in the dark on ice. Calibration curves (with known concentrations of H_2O_2) and H_2O_2 liberation from mitochondrial samples (0.5 mg mitochondrial protein per ml, final concentration) were estimated in the mitochondrial incubation buffer supplemented with 0.5 mg BSA/ml.

3. Results

All H_2O_2 detection methods applied in biological studies are based on the enzymatic reduction of H_2O_2 to water using horseradish peroxidase as the catalyst and the indicator probe as the hydrogen donor (DH_2) according to:

$$H_2O_2 + HRP \rightarrow H_2O_2 - HRP\ [\text{compound I}] \quad (1)$$

$$H_2O_2 - HRP + DH_2 \rightarrow HRP + 2H_2O + D \quad (2)$$

Table 1 presents the various hydrogen donors generally used for the estimation of H_2O_2 generation. The quantitative analysis is based on the 1:1 stoichiometry of H_2O_2 and the hydrogen donor so that H_2O_2 formation could be readily calculated from the absorption change of the oxidized dye. All compounds are water-soluble in their original (reduced) form but only TMB and *o*-phenylenediamine remain soluble following oxidation through the HRP-H_2O_2 system. Due to the 12-fold higher extinction coefficient of TMB in comparison to *o*-phenylenediamine we started our studies on mitochondrial oxygen activation with TMB.

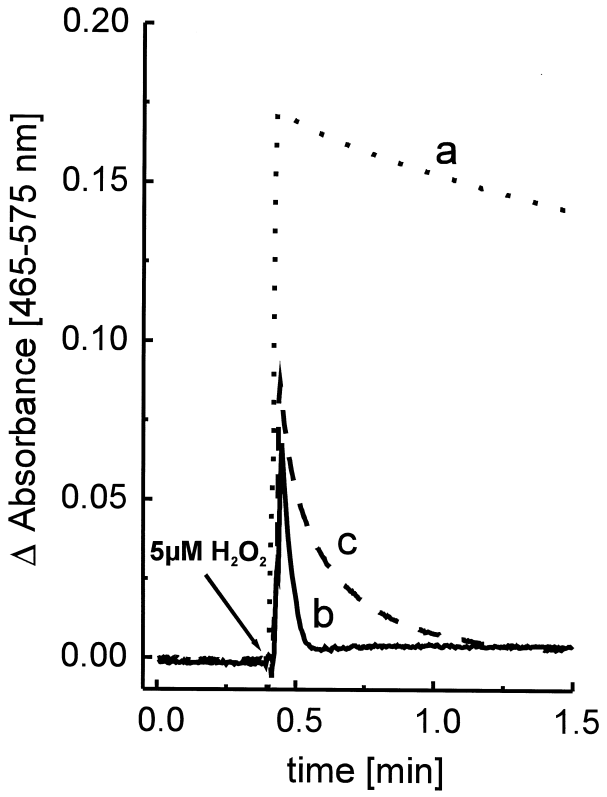

Fig. 1. HRP-catalyzed H_2O_2-dependent TMB oxidation in the mitochondrial incubation buffer. Final concentrations used: 10 U HRP/ml, 10 μM TMB, 2 mM P_i, 10 mM succinate, 0.56 mg RHM protein/ml. The reaction was started by the addition of 5 μM H_2O_2. Curve a, TMB oxidation in the absence of RHM; curve b, TMB oxidation in the presence of succinate-respiring mitochondria; curve c, TMB oxidation in the presence of non-substrate-supplemented mitochondria.

Calibration of H_2O_2-related TMB absorbance changes was performed with the addition of 5 μM H_2O_2 to the mitochondrial incubation buffer containing HRP and TMB as detection system (Fig.

Table 1
Spectrophotometric parameters and water solubility of different hydrogen donors generally used for H_2O_2 detection

Hydrogen donor	Absorption maximum of the oxidized form λ (nm)	ε (mM^{-1} cm^{-1})	Water solubility of the	
			reduced form	oxidixed form
3,3′,5,5′-Tetramethylbenzidine·2HCl	465	37.1	+	+
3,3′-Dimethoxybenzidine·2HCl	435	10.2	+	−
o-Phenylenediamine·2HCl	456	3.1	+	+
3,3′-Diaminobenzidine·4HCl	440	2.2	+	−

1). Absorbance increase of the dye compound formed occurred immediately after the addition of H_2O_2 followed by a slight decline (0.029 ΔE/min; Fig. 1a). The presence of mitochondria had a great impact both on the quantitative yield and the stability of the oxidized form of TMB. The effect was clearly stronger with mitochondria supplemented with succinate and inorganic phosphate (Fig. 1b) but still significant with intact mitochondria respiring endogenous substrates (Fig. 1c). From these observations it appears that oxidized TMB will interact with e^- carriers of the respiratory chain being readily reduced to the original form which does not absorb light at 465 nm. Support for this assumption comes from the fact that the disappearance of the dye compound is more profound when e^- carriers of the respiratory chain exhibit higher reduction states (in the presence of succinate; Fig. 1b). This finding reveals that TMB is not suited for the assessment of mitochondrial H_2O_2 generation.

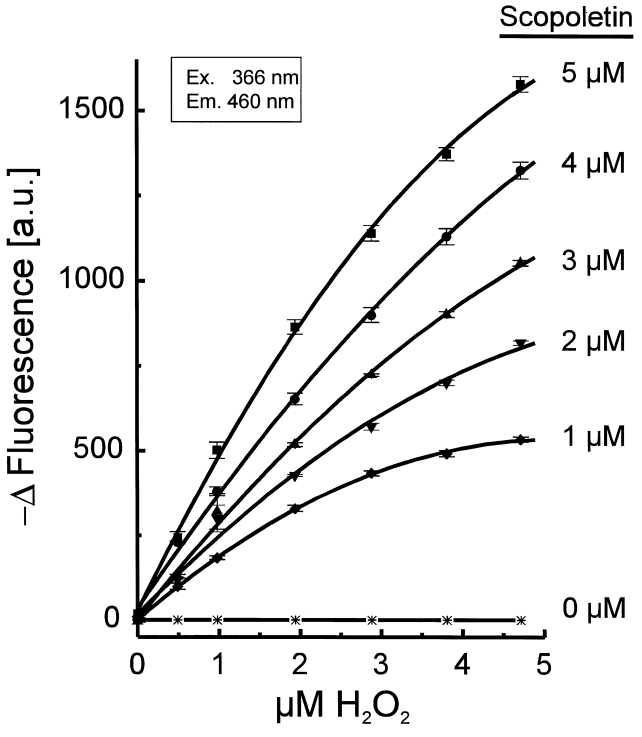

Fig. 2. H_2O_2 calibration curves performed in the mitochondrial incubation buffer (plus 0.5 mg BSA/ml, 10 U HRP/ml). H_2O_2 and scopoletin concentrations were varied each from 0 to 5 µM (final concentrations). The reaction was initiated by the addition of the respective amount of H_2O_2. Curves were adapted by polynomial regression fitting.

We therefore examined the suitability of scopoletin for mitochondrial H_2O_2 detection. Fluorescence decrease of scopoletin following oxidation in the presence of H_2O_2 and HRP was often used to assess H_2O_2 release from intact mitochondria [20,21].

According to Eqs. 1 and 2 it can be expected that 1 mole of H_2O_2 consumes 1 mole of reduced scopoletin. Fig. 2 shows that the intensity of fluorescence change in the presence of HRP does not linearly follow the amount of H_2O_2 added to the reaction system. In contrast, oxidation of scopoletin was both a function of scopoletin and H_2O_2 concentration. This indicates that substrate and co-substrate do not saturate for full activity of HRP.

Calibration of scopoletin-fluorescence decline was performed with H_2O_2 concentrations adjusted to mitochondrial generation activities as reported in the literature [1–3,5,16,22–24]. The concentration of 5 µM scopoletin was found to be optimal for calibration of the H_2O_2 range of interest although full linearity was not obtained. A further increase of scopoletin in the calibration system did not result in a linear correlation between fluorescence change and H_2O_2 concentration due to intermolecular quenching effects.

When HRP and scopoletin are applied for mitochondrial H_2O_2 measurement, the complete detection set is usually brought into direct contact with mitochondria and fluorescence change is monitored directly from the reaction system.

Addition of mitochondria to scopoletin resulted in a strong fluorescence decrease (Fig. 3A). The extent of non-H_2O_2-linked fluorescence decline was a function of the concentration of RHM in the reaction system. The same effect was observed with RHM kept at 50°C over a period of 4 h. Thus, fluorescence decrease which is normally taken as evidence of scopoletin oxidation through H_2O_2 in the presence of HRP is due to light absorption through constituents of mitochondria which are resistant to heat inactivation. In addition also the redox states of the mitochondrial e^- carriers were found not to be critical for fluorescence decrease (Fig. 3B). Considering the high amount of flavoenzymes in mitochondria exhibiting absorption maxima in the range of light emission from scopoletin [25] one can assume that a great fraction of light emitted from reduced scopoletin is

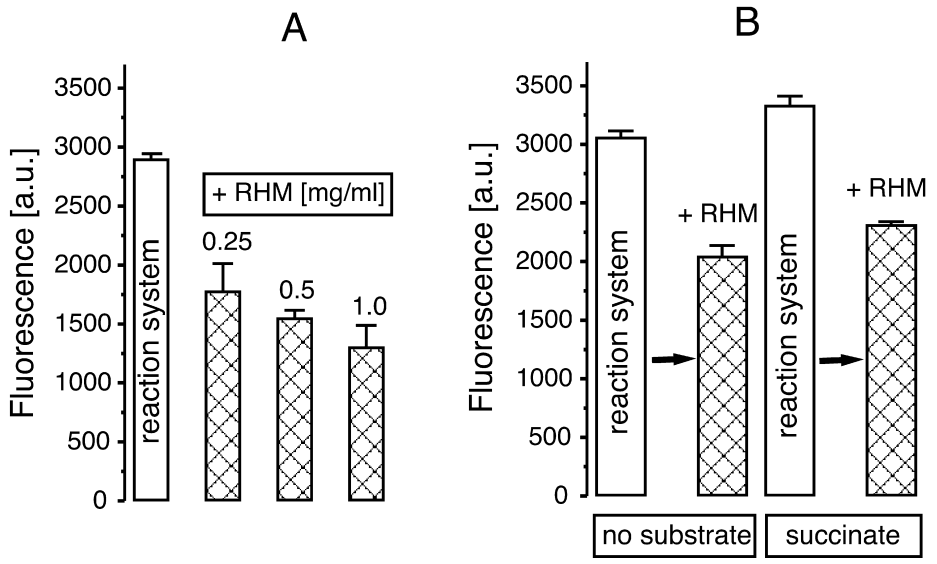

Fig. 3. (A) Intensity change of scopoletin fluorescence following the addition of mitochondrial protein. Reaction system = mitochondrial incubation buffer containing 5 μM scopoletin. (B) Influence of the mitochondrial redox state on fluorescence intensity of scopoletin. Reaction system = mitochondrial incubation buffer supplemented with 0.5 mg/ml BSA, 4 mM P_i, 5 μM scopoletin, 10 U/ml HRP. Additions were 0.5 mg/ml RHM protein and 10 mM succinate, respectively.

absorbed by the various constituents of the respiratory chain.

Recently homovanillic acid has been increasingly used instead of scopoletin for H_2O_2 detection in mitochondria [26,27]. In contrast to the fluorescent scopoletin which indicates the presence of H_2O_2 by a fluorescence decrease, HVA becomes a fluorescent through H_2O_2-induced oxidation in the presence of HRP (Fig. 4, calibration curve). Like scopoletin, light emission of the fluorescent HVA (oxidized form) was decreased in the presence of mitochondria (Fig. 5). The fluorescence quench was a function of the amount of mitochondria in the system. The effect was established irrespectively of whether HVA was oxidized to the respective fluorescent prior to mixing with mitochondria or in the presence of mitochondria. The decrease of HVA-mediated fluorescence was also not linked to the reduction state of e^- carriers of the respiratory chain since transition from state II (not shown) to state IV was not critical. We therefore conclude that light emitted from the excited HVA product is partially absorbed by mitochondrial constituents.

Fig. 6 gives details on a non-invasive procedure which bypasses undesired interactions of the H_2O_2 detection systems with mitochondria. The idea behind this procedure is the determination of the stable $O_2^{\bullet-}$ dismutation product H_2O_2 in the supernatant

Table 2
Inhibitory effect of catalase on H_2O_2 detection applied in non-invasive and invasive assays

Assay	Catalase activity applied (U/ml)	% inhibition of H_2O_2 detection
Non-invasive		
Homovanillic acid	725	100
Scopoletin	725	100
Invasive		
Homovanillic acid	725	38
	1450	58
	2900	67
Scopoletin	725	34
	1450	57
	2900	70

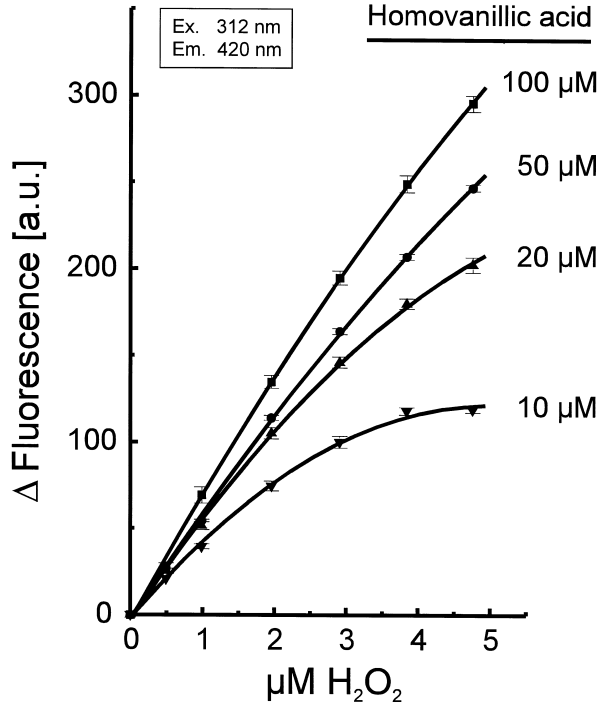

Fig. 4. Calibration curves of HVA-related H_2O_2 detection. Measurements were performed in the mitochondrial incubation buffer (plus 0.5 mg BSA/ml; 10 U HRP/ml). The final concentrations of H_2O_2 and HVA range from 0 to 5 µM and 0 to 100 µM, respectively. The reaction was initiated by the addition of the respective amount of H_2O_2. Curves were adapted by polynomial regression fitting.

following separation from the oxygen-activating system of the respiratory chain.

RHM (0.5 mg protein/ml) were suspended in the mitochondrial incubation buffer and supplemented with 4 mM P_i and 10 mM succinate. The reaction was started with antimycin A (2 µg/ml) to stimulate the production of reactive oxygen species. Catalase (725 U/ml) was added to prove the specificity of the detection system for H_2O_2. The samples (in closed Eppendorf tubes) were vortex-mixed for 10 s and allowed to equilibrate for 3 min at 25°C. After this the suspension was immediately centrifuged at $9000 \times g$ for 5 min (at 25°C) in order to separate mitochondria from the supernatant. 900 µl of the supernatant containing accumulated H_2O_2 were placed in a fluorescence cuvette and supplemented with 5 µM scopoletin or 100 µM HVA, respectively. The amount of H_2O_2 formed was inferred from HRP-catalyzed (10 U/ml) fluorescence change calculated on the basis of the respective calibration curves of Fig. 2 or Fig. 4. To prove the stability of H_2O_2 released from mitochondria into the incubation buffer, control experiments were performed under identical conditions in the absence of mitochondria with concentrations in the range of mitochondrial H_2O_2 production (half-maximal: 2.17 ± 0.12 µM; maximal 4.53 ± 0.10 µM). H_2O_2 concentrations were deter-

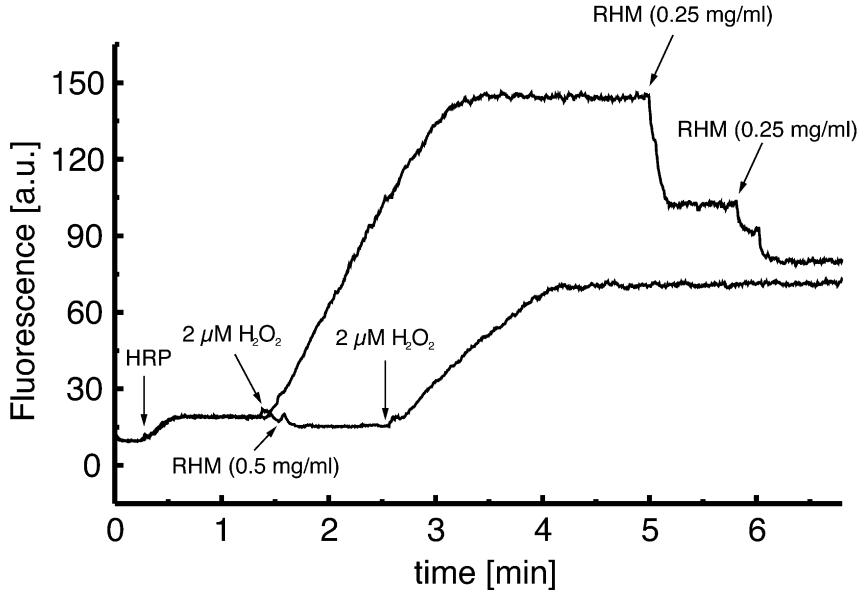

Fig. 5. Influence of respiring mitochondria (mitochondrial incubation buffer, 0.5 mg/ml BSA, 4 mM P_i, 10 mM succinate) on time scans of HVA-related (100 µM) fluorescence (addition: 10 U/ml HRP).

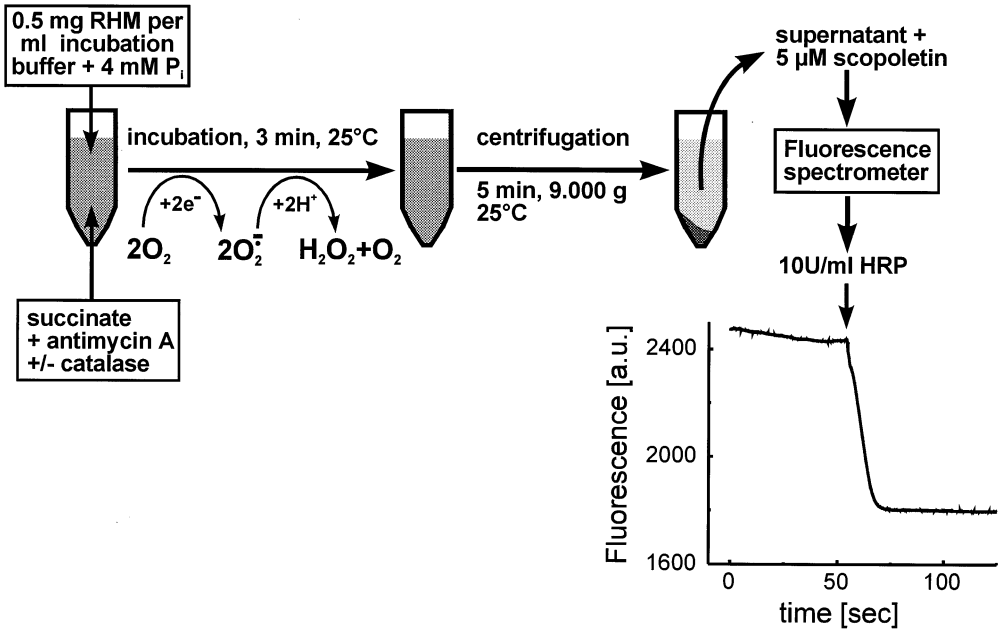

Fig. 6. Schematic presentation of processing non-invasive H_2O_2 determination.

mined after 0.25, 1, 3, 5, 7.5 and 10 min incubation and following centrifugation. After all indicated time intervals H_2O_2 concentrations were the same as those initially added to the incubation buffer (non-significant differences between the mean values; $P > 0.05$).

With this procedure it was possible to follow the formation and accumulation of H_2O_2 under the various metabolic conditions without affecting mitochondria through the detection system itself and vice versa. The sensitivity of the detection system

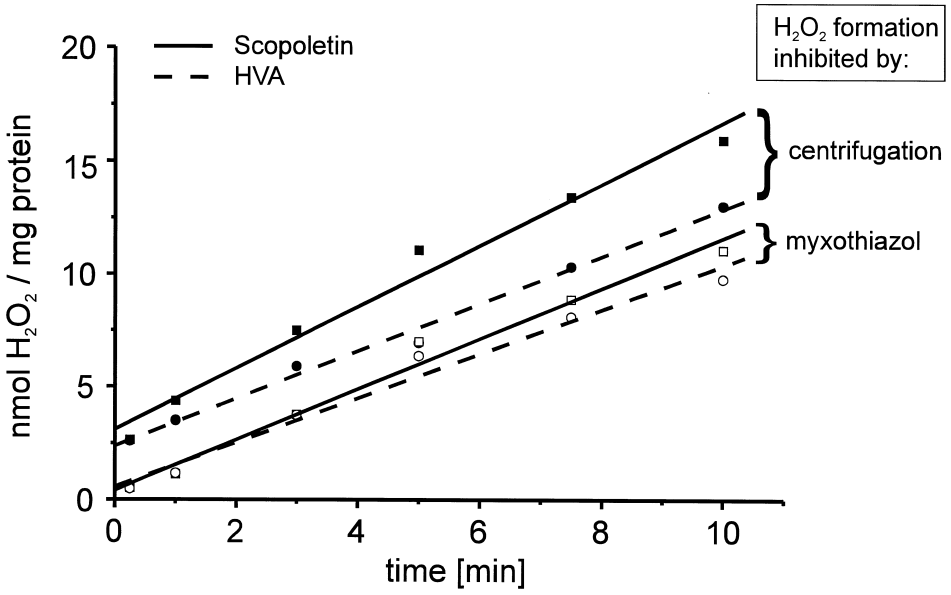

Fig. 7. Antimycin A-induced H_2O_2 formation rates detected with the non-invasive method using scopoletin (5 µM) or HVA (100 µM). H_2O_2 production was terminated either by centrifugation only or by the addition of myxothiazol to the mitochondrial incubation system followed by centrifugation. Data represent means of three independent mitochondrial preparations. For other details, see explanations to Fig. 6.

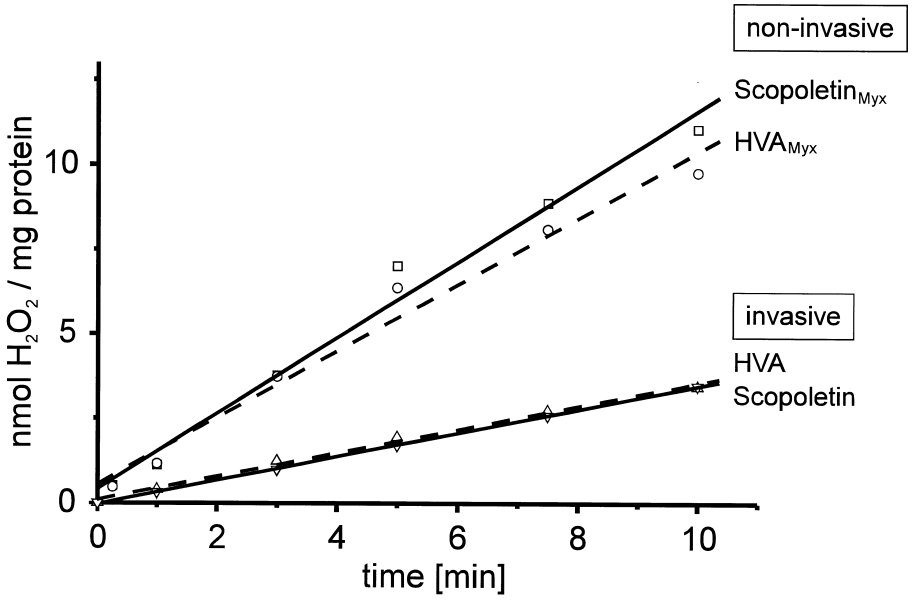

Fig. 8. Detection of mitochondrial H_2O_2 using the non-invasive assay in comparison to the invasive method. Data represent means of three independent mitochondrial preparations.

was thereby increased. In addition, when accumulated H_2O_2 is determined in the supernatant (non-invasive) catalase can be used as a control for mitochondrial H_2O_2 formation. Since HRP is not present while mitochondria generate H_2O_2 the latter is totally metabolized by catalase. In contrast, when the complete detection system is in direct contact with H_2O_2-generating mitochondria catalase removes H_2O_2 in competition with HRP only. Table 2 shows that in this case total inhibition of fluorescence change is not possible with any catalase activities applied.

The suitability of the non-invasive detection method for a reliable quantitative determination of $O_2^{\bullet-}$ related H_2O_2 formation rates was tested with succinate-respiring mitochondria in the presence of antimycin A (Fig. 7). To exclude ongoing H_2O_2 release from mitochondria during the separation procedure of the supernatant we used myxothiazol which was earlier shown to totally inhibit mitochondrial oxygen activation [28]. With this method it was possible to obtain H_2O_2 values at any time desired. As before H_2O_2 contents were determined from fluorescence changes after the addition of HRP using the respective calibration curves (Fig. 2 for scopoletin, Fig. 4 for homovanillic acid). The results show that irrespective of whether scopoletin or homovanillic acid was used as fluorescent dye H_2O_2 liberation followed a linear kinetic. From the slopes it was possible to calculate the rates of H_2O_2 release. H_2O_2 formation rates measured were clearly higher when accumulation of H_2O_2 was terminated by rapid centrifugation only. In this case scopoletin values were clearly higher than homovanillic acid values and ordinate intercepts were in the range of 3 nmol H_2O_2/mg protein indicating that mitochondrial H_2O_2 release continues during centrifugation. Thus, the determination of real H_2O_2 formation rates is not possible in this way. However, when myxothiazol (2 μg/ml) was used to stop H_2O_2 formation by interaction with the one-e^- reductant of oxygen, more realistic H_2O_2 formation rates were calculated by exclusion of H_2O_2 production during the separation period of mitochondria from the supernatant. Under these conditions both scopoletin and HVA gave identical values for H_2O_2 release (approx. 1 nmol H_2O_2/min/mg protein). The intercepts with the abscissa and ordinate at point zero in the presence of myxothiazol revealed that H_2O_2 production was not continued during centrifugation.

H_2O_2 formation rates observed when the detection system was in direct contact with H_2O_2-generating mitochondria were linear (Fig. 8). Linearity was obtained when applying the respective calibration

curves of Fig. 2 and Fig. 4. The values measured with scopoletin or HVA were identical. This was also the case when applying the non-invasive detection system although the slopes of the curves were by a factor of 3.2 higher as compared to the invasive H_2O_2 detection system.

4. Discussion

The contradictory reports on the role of mitochondria in cellular $O_2^{\bullet-}$ formation and the conditions required for the formation of this side product can be explained by the use of inadequate detection methods. While it is generally accepted that $O_2^{\bullet-}$ when generated in mitochondria have to be assessed from the release of the SOD-catalyzed dismutation product H_2O_2, little attention has been focused on undesired interactions of the respective detection systems with other compounds of respiring mitochondria. The present study analyzes the suitability of the various H_2O_2 detection methods for the assessment of mitochondrial oxygen reduction out of sequence. All H_2O_2 detection systems are based on the supply of reducing equivalents to HRP compound I. Prerequisite conditions for an optimal measurement of mitochondrial oxygen activation are the following: (i) absorption maxima of the respective dyes should be clearly different from those of the various mitochondrial constituents, (ii) high extinction coefficients of the dyes are requested to ensure a high sensitivity, (iii) reactants applied and reaction products formed which indicate the presence of H_2O_2 should be water-soluble. Although among all H_2O_2-sensitive dyes only TMB was found to correspond to these conditions it turned out that the oxidation product which indicates the presence of H_2O_2 was readily reduced when it came into contact with respiring mitochondria. Reduction of oxidized TMB could be slowed down when e^- carriers of the respiratory chain were in the oxidized state indicating a direct e^- transfer from components of the respiratory chain to the TMB dye.

Scopoletin which is the most commonly used indicator of mitochondrial H_2O_2 formation [20,21] exhibited a strong fluorescence decrease also when brought into contact with non-respiring mitochondrial preparations. Oxidation of scopoletin by compounds of the respiratory chain can be excluded since fluorescence activity was totally restored following removal from mitochondria. However, the emission spectrum of excited scopoletin is in the range of the absorption maxima of flavoproteins which form a great part of respiratory compounds. Thus, the degree of non-H_2O_2-linked fluorescence quench of excited scopoletin which was found to increase with mitochondrial protein concentration most likely results from the partial absorption through mitochondrial flavoproteins.

HVA-related light emission which is increasingly applied for the detection of mitochondrial H_2O_2 [26,27] was also found to interfere with mitochondrial components in resonance with the wavelength of light emitted from the indicator.

The concept presented in this study bypasses these uncertainties by a separate interaction of mitochondrial H_2O_2 with the detection system in the absence of mitochondria. Accumulation of H_2O_2 in the suspension medium was made safe by removing adventitious iron in order to prevent chemical degradation of H_2O_2. Determination of the amount of H_2O_2 in the supernatant from which mitochondria were removed not only excluded an interference in the excitation procedure and light emission, but also prevented any possible interaction of the reduced or oxidized fluorescents with the various redox systems of the respiratory chain.

The interception of the time profile of mitochondrial H_2O_2 formation at the origin irrespective of whether scopoletin or HVA was used shows that myxothiazol inhibition prior to non-invasive H_2O_2 detection provides a method for the reliable determination of mitochondrial H_2O_2 formation rates. In contrast, kinetic analysis of mitochondrial H_2O_2 obtained when the detection system was in direct contact with mitochondria revealed the existence of uncontrolled interactions with mitochondrial constituents causing a dramatic loss of H_2O_2-induced fluorescence change (Fig. 8).

We have work in progress showing that the application of this non-invasive method for cellular and subcellular H_2O_2-producing systems is more sensitive and gives more reliable results.

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