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Sites of generation of reactive oxygen species in homogenates of brain tissue determined with the use of respiratory substrates and inhibitors

Alexei P. Kudin [a,b], Dominika Malinska [c], Wolfram S. Kunz [a,b,d,*]

[a] Division of Neurochemistry, Department of Epileptology, University of Bonn Medical Center, Germany
[b] Platform NeuroCognition, Life & Brain Center, Bonn, Germany
[c] Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Warsaw, Poland
[d] Institute of Biochemistry II, Friedrich-Schiller-University, Jena, Germany

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ABSTRACT

Reactive oxygen species (ROS) have been widely implicated in the pathogenesis of various neurological diseases and aging. But the exact sites of ROS generation in brain tissue remained so far elusive. Here, we provide direct experimental evidence that at least 50% of total ROS generation in succinate-oxidizing homogenates of brain tissue can be attributed to complex I of mitochondrial respiratory chain. Applying quantitative methods for ROS detection we observed in different preparations from human, rat and mouse brain (digitonin-permeabilized tissue homogenates and isolated mitochondria) a linear relationship between rate of oxygen consumption and ROS generation with succinate as mitochondrial substrate. This quantitative relationship indicates, that under the particular conditions of oxygen saturation about 1% of the corresponding respiratory chain electron flow is redirected to form superoxide. Since we observed in mouse and rat brain mitochondria a unique dependency of both forward and reverse electron flow-dependent mitochondrial H_2O_2 production on NAD redox state, we substantiated previous evidence that the FMN moiety of complex I is the major donor of electrons for the single electron reduction of molecular oxygen.

1. Introduction

It is commonly accepted that reactive oxygen species (ROS — H_2O_2, $O_2^{-\cdot}$ and $OH^{\cdot}$) play a significant role in pathogenesis of various neurodegenerative diseases (for comprehensive reviews see Refs. [1,2]). Despite the progress in characterising ROS effects on lipids (resulting in peroxidation), proteins (resulting in SH-group oxidation and formation of carbonyls) and DNA (formation of 8-OH guanosine and of single and double strand breaks) [3,4], the particular impact of the potential sites relevant for cellular superoxide and hydrogen peroxide generation in brain tissue is less clear. Molecular oxygen is a triplet species that can accept only single electrons from potential donors [5]. This prevents oxygen (the midpoint potential of the O_2/O_2^{-}·. couple is −0.33 V [6]) from spontaneously oxidising reduced biomolecules with appropriate redox potentials, such as NAD(P)H, which are obligate two-electron donors. Potential single electron donor sites with matching redox potentials for the reduction of molecular oxygen are located within the mitochondrial respiratory chain which transfers electrons to oxygen. Within the respiratory chain complex I the FMN moiety [7,8], iron sulphur clusters [9,10] and semiquinones [11], all of which are competent for univalent redox reactions, have been suggested to be responsible for mitochondrial superoxide production. For respiratory chain complex III the semiquinone at center 'o' of the Q-cycle being stabilized by antimycin A treatment has been identified as an additional site of mitochondrial superoxide production [12], which in contrast to complex I releases superoxide to the intermembrane space [13,14]. However, the relevance of this site under the conditions of uninhibited electron flow is not clear [14,15]. In addition to the respiratory chain, several flavoproteins in the mitochondrial matrix space, like the α-lipoamide dehydrogenase moiety of the α-ketoglutarate dehydrogenase complex [16,17] or the electron transfer flavoprotein of the β-oxidation pathway [13] are possible candidate sites for mitochondrial ROS production.

In brain tissue, apart from mitochondria and the monamine oxidases of outer mitochondrial membrane involved in catecholamine breakdown [18], certain potentially relevant ROS producers have no mitochondrial localization. These are plasma membrane NADPH oxidases [19,20], cytochromes P450 [21], and also catecholamine-derivatives [22]. Moreover, oligodendrocytes and astrocytes contain considerable amounts of peroxisomes, which potentially also can contribute to overall ROS production in brain tissue. For these particular reasons and due to artificially high estimates of the mitochondrial ROS production in the classical literature [23], the previously widely accepted viewpoint of a predominant mitochondrial origin of cellular ROS *in vivo* (cf. Ref. [23]) has been questioned [2,24–27].

* Corresponding author. Division of Neurochemistry, NeuroCognition, Life & Brain Center, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. Tel.: +49 2286885290; fax: +49 2286885295.
E-mail address: wolfram.kunz@ukb.uni-bonn.de (W.S. Kunz).

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In this work, we performed an experimental quantification of the ROS production in homogenates of mouse, rat and human brain tissue. We applied digitonin-permeabilised brain homogenates which under the particular conditions of an accessible mitochondrial compartment [28,29] allow to obtain an estimate of the potential of mitochondria to contribute to whole tissue ROS production. We compared the obtained results on digitonin-treated homogenates with mitochondrial preparations of different purity: isolated mitochondria and purified mitochondrial inner membrane preparations (SMP). Using this approach we substantiated the evidence that apart from a rather small extramitochondrial ROS generation, potentially relevant mitochondrial ROS generating sites in brain are (i) the FMN moiety of mitochondrial respiratory chain complex I which with succinate as substrate contributes to at least 50% of overall ROS production, and (ii) the center 'o' of mitochondrial respiratory chain complex III.

2. Material and methods

2.1. Solutions

In the experiments the following solutions were used: MSE solution (for homogenate and mitochondria isolation) containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, and 1 mg/ml essential fatty acid free BSA (pH 7.4); medium A (for SMP isolation) containing 15 mM $MgCl_2$ and 50 mM potassium phosphate (pH 7.4); MTP medium (standard measurement medium) containing 10 mM KH_2PO_4, 60 mM KCl, 60 mM Tris–HCl, 110 mM mannitol, 5 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.4).

2.2. Homogenate preparation and isolation of mitochondria and submitochondrial particles (SMP)

Rat, mouse and human brain homogenates were prepared according to the following procedure (cf. Ref. [28]). One male Wistar II rat (~80 days old) or one C57BL6 mouse (~50 days old) was euthanized, and the brain was rapidly removed. Tissue samples from human parahippocampal gyrus were obtained from 5 patients (3 female and 2 male) with therapy-resistant temporal lobe epilepsy, who underwent epileptic surgery. The whole rat or mouse brain, or the human brain tissue was washed and rapidly placed into ice-cold MSE medium. After isolation of the subsequent brain areas (rat hippocampus, total mouse brain or human parahippocampal gyrus), about 200 mg wet tissue was homogenized twice for 20 s at 8000 rpm using an ultra-turrax homogenizer T 25 (IKA, Staufen, Germany) in 1 ml ice-cold MSE medium and stored on ice. The respiratory control indices of the homogenate preparations with 5 mM pyruvate + 5 mM malate as respiratory substrates were routinely better than 5 (cf. [28]).

Rat, mouse and human brain mitochondria were isolated according to the protocol originally described by Rosental et al. with small modification that allowed to obtain mitochondria with much better functional characteristics [8,14]. The respiratory control index with 10 mM glutamate and 5 mM malate as respiratory substrates with all preparations was routinely better than 6; the specific active state respiration rate of mouse brain mitochondria was 321±14 nmol O_2/min/mg protein (n=5), of rat brain mitochondria was 210±7 nmol O_2/min/mg protein (n=4) and of human brain mitochondria was 136±35 nmol O_2/min/mg protein (n=4).

Rat brain SMP were prepared according to Cino and Del Maestro [30] with following modifications. Isolated mitochondria from 8 rat brains were pooled and stored in liquid nitrogen before use (usually one or two days). After thawing on ice, the mitochondrial suspension was centrifuged at 10,000 g for 10 min and the pellet was re-suspended in medium A to a final volume of 10 ml. After sonication (70 W, 3 times 15 s) centrifugation at 25,000 g for 10 min was performed. The supernatant was centrifuged at 48,400 g for 87 min. The resulting pellet was re-suspended in MSE solution without BSA and centrifuged again at 48,400 g for 87 min. The pellet was again re-suspended in medium A and centrifuged at 25,000 g for 15 min. The SMP in supernatant was washed twice in medium A with following centrifugation at 48,400 g for 87 min. The final pellet was suspended in medium A to a concentration of 8–10 mg protein/ml, divided into aliquots and frozen in liquid nitrogen before use. Every aliquot was used only during one day, re-freezing and repeated use of the SMP aliquots led to lowered rates of superoxide generation.

2.3. Respiration measurements

Mitochondria, submitochondrial particles and homogenates were incubated in air gassed MTP medium at 30 °C. The oxygen consumption was determined with a PC-supported Oroboros high resolution oxygraph [8,28].

2.4. Measurement of H_2O_2 and superoxide generation

H_2O_2 generation in isolated mitochondria was measured by monitoring the change in fluorescence of 200 μM p-hydroxyphenylacetic acid (pHPAA) (λ_{ex}=317 nm, λ_{em}=390 nm) catalyzed by 20 U/ml horseradish peroxidase [7,8]. To detect H_2O_2 in brain homogenates and for control experiments with isolated mitochondria we applied the Amplex red/peroxidase-coupled method (1 μM Amplex red (λ_{ex}=560 nm, λ_{em}=590 nm)+20 U/ml horseradish peroxidase in the additional presence of 15 U/ml SOD), since we observed nonspecific reactions of pHPAA applying brain homogenates and certain substrates (cf. [14]).

Since NADH reacts with peroxidase [31] we found that it is not possible to use peroxidase-coupled fluorescent methods in the presence of SOD excess for reliable quantitative superoxide generation measurements in SMP when NADH is used as

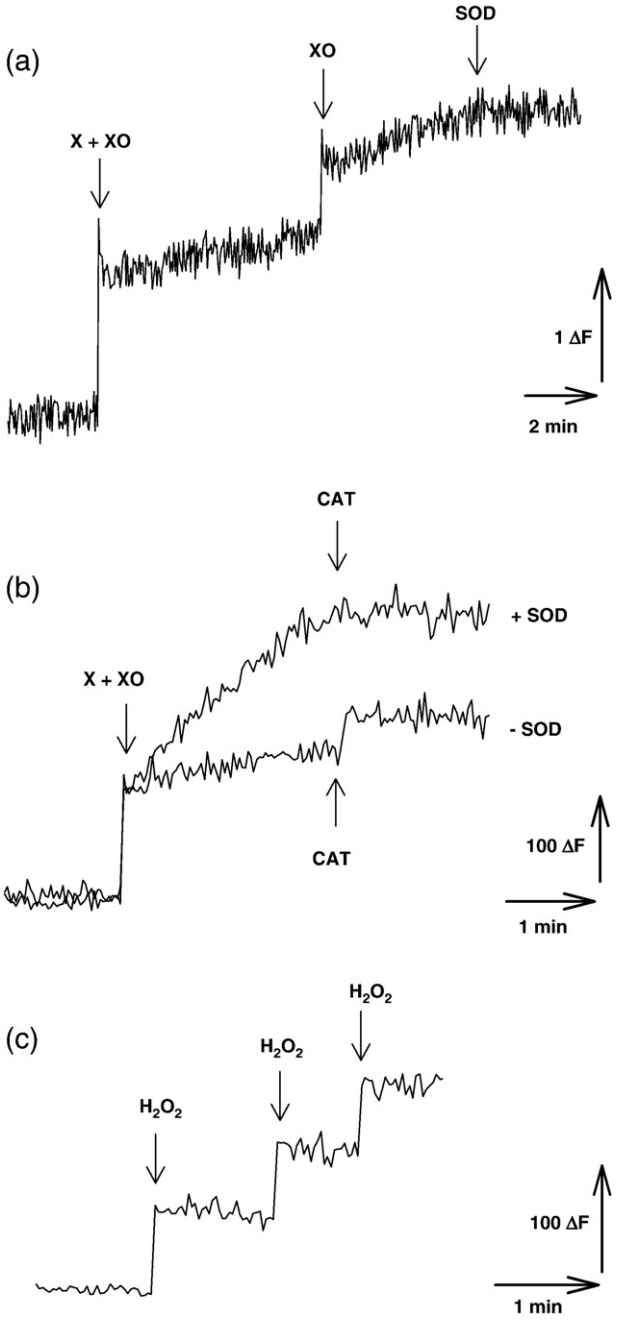

Fig. 1. Calibration experiment for determination of superoxide production rates. Trace a: In oxygen saturated MTP medium the slope of the dihydroethidium fluorescence increase (λ_{ex}=470 nm, λ_{em}=585 nm) was determined in the presence of 500 μM xanthine (X) and xanthine oxidase (XO), which was added in two titration steps (final concentration: 4.08 and 6.12 mU/ml). Addition of 45 U/ml SOD prevented further oxidation of dihydroethidium. Trace b: The pHPAA oxidation (λ_{ex}=317 nm and λ_{em}=390 nm) in the presence of 20 U/ml horseradish peroxidase and 176 U/ml SOD was followed at after the addition of 500 μM xanthine and 6.12 mU/ml of xanthine oxidase. This fluorescence increase was catalase-sensitive (CAT, 13,800 U/ml). The same experiment was repeated in the absence of SOD (lower trace). For quantification of superoxide generation the slope observed in the absence of SOD was subtracted from the slope measured in the presence of SOD. Trace c: The pHPAA fluorescence signal at λ_{ex}=317 nm, λ_{em}=390 nm was calibrated by three additions of 140 pmol H_2O_2.

substrate for Complex I. Therefore, we determined the SOD-sensitive reduction of 100 μg/ml acetylated cytochrome *c* at λ_{abs}=550 nm [32]. Superoxide generation in brain homogenates was measured following the slope in fluorescence increase after addition of 3.17 μM dihydroethidium (λ_{ex}=470 nm, λ_{em}=585 nm) when steady state level of fluorescence increase was reached. The dihydroethidium stock was prepared according to [14]. The superoxide production was calibrated in H_2O_2 equivalents with the use of the xanthine/xanthine oxidase system with the pHPAA/peroxidase method in the presence of SOD excess. A typical calibration experiment for superoxide production rates is shown in Fig. 1. First, the slope of the dihydroethidium fluorescence increase at λ_{ex}=470 nm and λ_{em}=585 nm after addition of 500 μM xanthine plus 6.12 mU/ml xanthine oxidase was determined to be 0.116 ΔF/min (trace a). In the second experiment the same amount of xanthine/xanthine oxidase was used to measure the fluorescence increase of 70 ΔF/min in presence of 200 μM pHPAA, 20 U/ml horseradish peroxidase and 176 U/ml SOD at λ_{ex}=317 nm and λ_{em}=390 nm (trace b). And finally, the pHPAA fluorescence signal at λ_{ex}=317 nm and λ_{em}=390 nm is calibrated by additions of 140 pmol H_2O_2 (trace c) which result in an average signal change of 54 ΔF. Therefore, the slope of the trace b, equal to 70 ΔF/min, is equivalent to a production of (70/54)*140=181.5 pmol H_2O_2/min. Therefore, the 0.116 ΔF/min in the dihydroethidium experiment correspond to this production of 181.5 pmol H_2O_2 equivalents/min. The linearity of dihydroethidium response was checked with titration of xanthine oxidase in the range from 4.1 to 8.2 mU/ml. In these experiments the 1.5 and 2-fold increase in the xanthine oxidase amount resulted in 1.64±0.11 and 2.14±0.14-fold increase in the dihydroethidium fluorescence slope, respectively (n=3).

All measurements of ROS generation were performed at 30 °C in oxygen saturated MTP medium (10 mM KH_2PO_4, 60 mM KCl, 60 mM Tris–HCl, 110 mM mannitol, 0.5 mM EDTA, pH 7.4) [8,14].

2.5. Measurements of the redox state of the NAD system

To determine the NAD redox state, the NAD(P)H fluorescence (λ_{ex}=340 nm, λ_{em}=450 nm) of isolated mouse or rat brain mitochondria (0.2 mg protein/ml) in the presence of different mitochondrial substrates or in the presence of succinate and different concentrations of malonate was measured. To calculate the redox state we determined the maximal NAD(P)H fluorescence F_{max} in the presence of 10 mM glutamate, 5 mM malate and 4 mM KCN and the minimal NAD(P)H fluorescence F_{min} in the presence of 1 μM TTFB and in the absence of substrates. The actual NAD redox state R (in %) at the determined NAD(P)H fluorescence value F is then:

$$R = (F-F_{min})/(F_{max}-F_{min})*100$$

3. Results

3.1. Origin of H_2O_2 generation in brain tissue homogenates

At the cellular level ROS (H_2O_2 and superoxide) originate both from mitochondrial or from cytoplasmic competent one-electron donors for reduction of molecular oxygen (cf. Ref. [2]). In order to obtain a quantitative estimate for the potential ROS production in brain tissue and to calculate the approximate contribution of the different cellular compartments we used in this study brain tissue homogenates and subcellular fractions of these homogenates which allow to apply quantitative fluorimetric methods. Since brain tissue homogenates contain rather high activities of Cu/Zn- and Mn-superoxide dismutases [1], direct superoxide production measurements yield in nonpermeabilized preparations rather low values. We therefore decided to determine H_2O_2 generation rates, and added SOD in excess to the homogenate, to ensure that all superoxide was converted to H_2O_2. For quantification of H_2O_2 production in homogenates we used the very sensitive Amplex red/peroxidase-coupled method. Interfering activities of the relatively low activities of catalase (cf. Ref. [33]) and glutathione peroxidase would lead to underestimation of the total H_2O_2 production rates. To account for this problem we performed the assay calibration with H_2O_2 in the presence of the homogenate. First, we performed experiments with rat hippocampal homogenates using glucose as substrate. As can be seen in Fig. 2a, addition of 10 mM

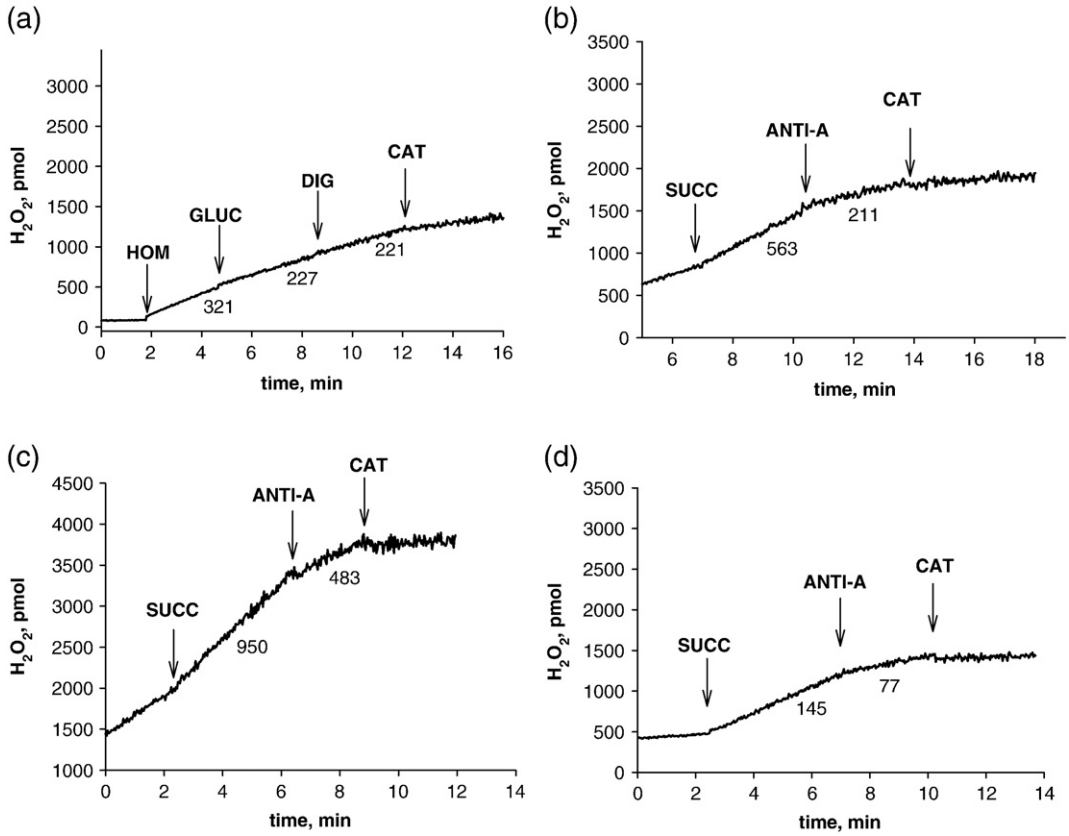

Fig. 2. Experimental traces of H_2O_2 generation by rat hippocampal (a,b), whole mouse brain (c), and human parahippocampal gyrus (d) homogenates. The hydrogen peroxide production was detected by monitoring of Amplex red/peroxidase-coupled fluorescence increase (cf. Materials and methods). (a, b) — rat hippocampal homogenate (0.4 mg protein/ml), (c) — whole mouse brain homogenate (0.5 mg protein/ml), (d) — human parahippocampal gyrus homogenate (0.7 mg protein/ml). SUCC — 10 mM succinate, ANTI-A — 0.5 μM antimycin A, HOM — homogenate, GLUC — 10 mM glucose, DIG — 100 μg/ml digitonin (in b, c and d already initially present), CAT — 13,800 U/ml catalase. The numbers at the traces are hydrogen peroxide generation rates in pmol/min/mg protein.

glucose to the homogenate even diminished the baseline production of H_2O_2. That indicates that cytosolic enzymes entrapped in synaptosomes do not appear to have a considerable contribution to the ROS generation of homogenates. An inhibition of H_2O_2 generation was also observed after the addition of 1 μM TTFB, an uncoupler of oxidative phosphorylation (data not shown, cf. Table 1). The addition of 100 μg/ml digitonin to permeabilize cholesterol-containing membranes, like residual cell membranes and synaptosomal membranes, and to ensure optimal mitochondrial accessibility (cf. [28,29]), did not have an additional effect on the H_2O_2 generation rate. The H_2O_2 generation was abolished by catalase excess (for quantification the small remaining catalase-insensitive fluorescence increase was always subtracted). As shown in Fig. 2b, in contrast to the effect of glucose, the addition of the mitochondrial substrate succinate to digitonin-treated rat hippocampal homogenates resulted in a substantial increase of H_2O_2 generation. This effect is very likely related to complex I-dependent H_2O_2 generation by reversed electron flow, since uncoupler or antimycin additions resulted in inhibition of this succinate-dependent H_2O_2 generation, which is analogous to the effects observed in isolated brain mitochondria (cf. Refs. [7,8,14,34]). As shown in Figs. 2c and d qualitatively similar effects can be observed with homogenates from whole mouse brain and human parahippocampal gyrus (grey matter). Table 1 summarizes the quantitative data observed in a series of these experiments. These data indicate that between 50 and 56% (in the average 53%) of the H_2O_2 production in the presence of succinate (SUCC-CAT) of digitonin-treated brain homogenates can be attributed to a reverse electron flow-dependent mitochondrial site (SUCC-TTFB), which is very likely located in respiratory chain complex I. The antimycin A-dependent H_2O_2 production (SUCC+ANTI-A-CAT), very likely originating from semiquinone center 'o' of complex III, was observed to be slightly lower (~85% in comparison to the reverse electron flow-dependent H_2O_2 generation).

As shown in Table 2, direct measurements of superoxide production in digitonin-treated brain homogenates using the oxidation of dihydroethidium (cf. Ref. [14]) yielded lower values than the hydrogen peroxide generation rates. This is due to the presence of high SOD2 activities in the mitochondrial compartment. Therefore only superoxide outside the mitochondrial inner membrane is detected, since SOD1 is released from the cytosolic and synaptosomal compartments by the digitonin treatment (the digitonin addition stimulated the superoxide generation rate approximately 1.3-fold, data not shown). Interestingly, in digitonin-treated brain homogenates the superoxide production rates were higher in the endogeneous state, in the uncoupled state and in presence of antimycin, than in presence of succinate alone (Table 2). Since succinate is exclusively metabolised by mitochondria, this indicates a substantial contribution of the semiquinone center 'o' at respiratory chain complex III to the superoxide production rate of digitonin-treated brain homogenates. Since the complex III-dependent mitochondrial superoxide producing site releases superoxide to the extramitochondrial space [13,14] the formed superoxide can be detected by direct measurements. In this context it is noteworthy to mention, that the amount of superoxide which was

Table 1
Generation of hydrogen peroxide by succinate-oxidising digitonin-permeabilized mouse, rat and human brain tissue homogenates

	Mouse (whole brain, $n=5$)	Rat (hippocampus, $n=9$	Human (parahippocampal gyrus, $n=3$)
Endogeneous H_2O_2 generation (DIG-CAT)	480±45	367±48	90±51
Total succinate-dependent H_2O_2 generation (SUCC-CAT)	825±74	594±46	157±17
Uncoupler-sensitive H_2O_2 generation (SUCC-TTFB; presumably by complex I)	429±59	295±30	88±12
Uncoupler-insensitive H_2O_2 generation (SUCC+TTFB-CAT; presumably by complex III and extramitochondrial sources)	352±48	291±22	89±14
Antimycin A-dependent H_2O_2 generation (SUCC+ANTI-A-CAT)	365±67	251±35	88±16

The H_2O_2 generation was measured by Amplex red/HRP-coupled method in presence of SOD excess and is expressed in pmol H_2O_2/min/mg protein (average±SEM). The quantitative data were generated from experiments like presented in Fig. 2 b–d. To calculate total H_2O_2 generation we determined the difference between the slopes after succinate (SUCC — 10 mM) and after catalase (CAT — 13,800 U/ml) additions. To estimate the complex I-dependent part the slope difference between the additions of succinate and the uncoupler TTFB (4,5,6,7-tetrachloro-2-trifluoromethylbezimidazole - 1 μM) was used. The catalase-sensitive slope remaining in presence of TTFB was considered to be caused by complex III and extramitochondrial sources. The Antimycin A-dependent part was determined as difference between additions of 10 mM succinate +0.5 μM antimycin A (SUCC + ANTI-A) and catalase (CAT — 13,800 U/ml).

Table 2
Generation of superoxide by succinate-oxidising digitonin-permeabilized mouse, rat and human brain tissue homogenates

	Mouse (whole brain, $n=3$)	Rat (hippocampus, $n=4$)	Human (parahippocampal gyrus, $n=3$)
Endogeneous superoxide generation (DIG-SOD)	279±47	277±44	89±16
Superoxide production in the presence of succinate (SUCC-SOD)	64±60	124±54	9±5
Antimycin A-dependent superoxide generation (SUCC+ANTI-A-SUCC)	207±84	268±113	n.d.

The superoxide generation of brain tissue homogenates (rat hippocampal homogenate (0.4 mg protein/ml), whole mouse brain homogenate (0.5 mg protein/ml), human parahippocampal gyrus homogenate (0.7 mg protein/ml) was determined in the presence of 100 μg/ml digitonin following the oxidation of dihydroethidium as described in Materials and methods. Additions: SUCC — 10 mM succinate, ANTI-A — 0.5 μM antimycin A, SOD — superoxide dismutase, 176 U/ml. The quantitative values are expressed in pmol H_2O_2 eq./min/mg protein (average±SEM) after calibration of generated superoxide by xanthine/xanthine oxidase. To express the xanthine oxidase generated superoxide in H_2O_2 equivalents, its rate of hydrogen peroxide generation was assessed in the presence of 176 U/ml SOD by the pHPAA-based method (cf. Fig. 1).

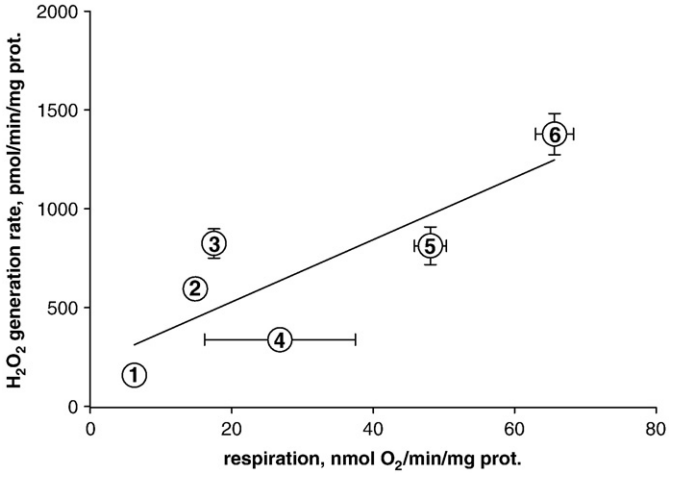

Fig. 3. Dependency of succinate-driven hydrogen peroxide formation on respiration rates. The hydrogen peroxide generation rates of human parahippocampal gyrus homogenates (1), of rat hippocampal homogenates (2), of mouse whole brain homogenates (3), of human (4), rat (5), and mouse (6) brain mitochondria were plotted versus the resting state respiration rates in the presence of succinate. The H_2O_2 generation (in the presence of 10 mM succinate) was measured by pHPAA/HRP-coupled method in isolated mitochondria (4, 5, 6), or by Amplex red/HRP-coupled method in digitonin-treated brain homogenates (1, 2, 3). Number of independent experiments: human parahippocampal gyrus homogenates (1) — 5, rat hippocampal homogenates (2) — 13, mouse whole brain homogenates (3) — 5, human brain mitochondria (4) — 4, rat brain mitochondria (5) — 4, mouse brain mitochondria (6) — 5. The presented data are averages±SEM. The correlation coefficient of the plotted linear regression line is 0.69.

measured in the presence of succinate and antimycin A (Table 2) was found to be comparable to the amount of hydrogen peroxide formed in the presence of succinate, antimycin A and SOD excess (Table 1). The low rates of superoxide production in the presence of succinate alone, i.e. under the conditions of reversed electron flow, are very likely a result of the high redox state of the QH_2/Q redox couple, since not the $QH_2/QH^{\cdot}$, but only the $QH^{\cdot}/Q$ redox couple has a sufficient redox potential to reduce molecular oxygen to superoxide. Therefore, in the presence of succinate alone only extramitochondrial sources appear to contribute to the residual superoxide production (SUCC-SOD in Table 2), which is under the particular conditions very low.

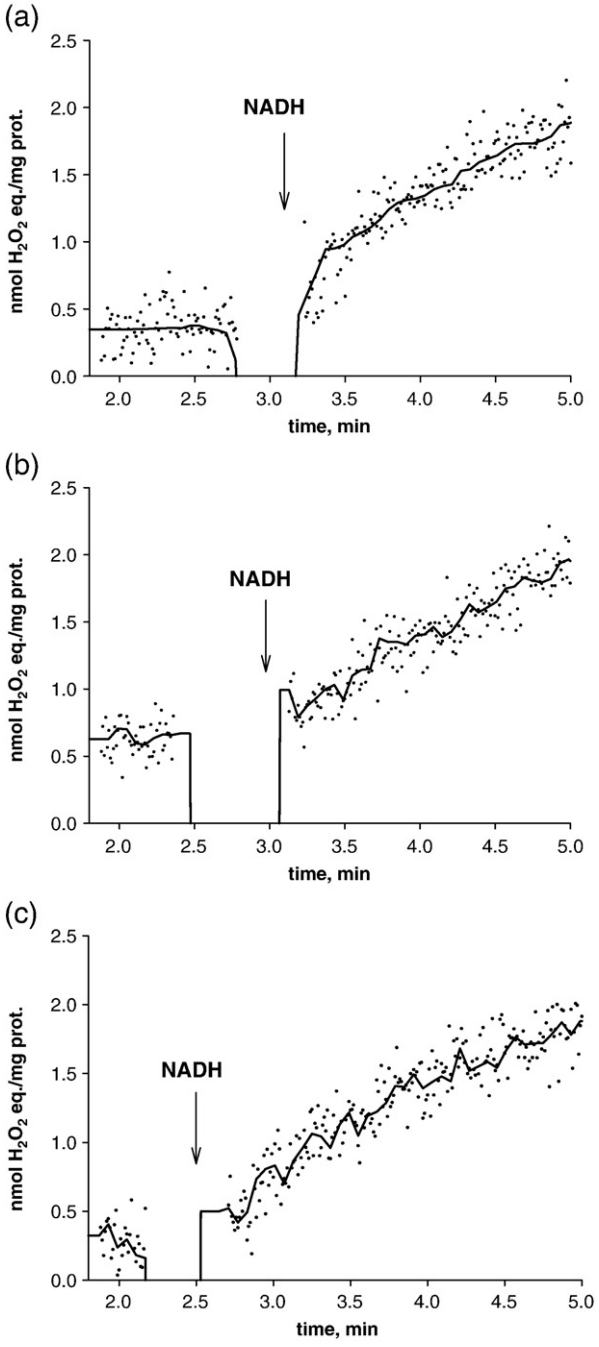

Fig. 4. Experimental traces of superoxide generation by rat brain SMP (0.1 mg protein/ml) in the presence of different mitochondrial inhibitors. The trace shows the reduction of acetylated cytochrome *c* which was obtained as digital difference of the experiment in the absence and in the presence of 200 U/ml SOD. (a) — 6.7 μM rotenone initially present, (b) — 1 μM stigmatellin initially present, (c) — 1 μM myxothiazol initially present. When indicated 200 μM NADH were added.

Table 3
Generation of superoxide in rat brain submitochondrial particles

Substrate/inhibitor	$O_2^{\cdot}$ generation, in pmol H_2O_2 eq./min/mg prot.
NADH	513±174
NADH+ROT	1124±196
NADH+DPI+ROT	286±189
NADH+MYXO	960±260
NADH+STIGMA	908±318
SUCC	898±220
SUCC+ANTI-A	820±127

Superoxide generation of SMP (average±SEM of 3 independent preparations, about 0.1 mg protein/ml) was measured following the reduction of acetylated cytochrome *c*. Superoxide generation is expressed in pmol H_2O_2 eq./min/mg prot. after calibration of generated superoxide by xanthine/xanthine oxidase. The xanthine oxidase generated superoxide expressed in H_2O_2 equivalents, was assessed in the presence of 176 U/ml SOD by the pHPAA-based method. Substrates and inhibitors were used with following concentrations: NADH — 200 μM NADH, ROT — 6.7 μM rotenone, DPI — 10 μM DPI, MYXO — 1 μM myxothiazol, STIGMA — 1 μM stigmatellin, SUCC — 10 mM succinate, ANTI-A — 0.5 μM antimycin A.

3.2. Relationship between respiration and ROS generation of different brain subcellular preparation

To further investigate the potential contribution of the mitochondrial compartment to total ROS generation we compared respiration rates and H_2O_2 generation rates in different succinate-oxidizing subcellular preparations of brain tissue containing mitochondrial inner membranes at different levels of purity.

In Fig. 3 the results of H_2O_2 generation rates with succinate as substrate obtained with rat, mouse and human digitonin-permeabilized homogenates and isolated rat, mouse and human mitochondria are plotted versus the corresponding resting state respiration rates with this particular substrate. We obtained a linear relationship between the succinate oxidation rate and the rate of H_2O_2 generation in the different preparations. From the slope of this line – approximately 100 nmol O/min/mg respiration corresponds to 1 nmol H_2O_2/min/mg ROS production – it is possible to calculate that under the conditions of oxygen saturation and with succinate as respiratory substrate about 1% of the respiratory chain electron flow is redirected to form ROS (Fig. 3).

3.3. Inhibitor studies with rat brain submitochondrial particles

Rat brain SMP, which consist of purified mitochondrial inner membranes, are an appropriate model to re-investigate inhibitor effects on superoxide generation from respiratory chain. This re-investigation is necessary, since work with isolated mitochondria and cells from different tissues – including skeletal muscle [11] and brain [16] – provided conflicting data about the potential superoxide producing site in complex I. In Fig. 4 the results of typical experiments using the SOD-sensitive reduction of acetylated cytochrome *c* are shown. It can be seen, that the rates of superoxide formation from NADH are very similar, if the complex I inhibitors rotenone (Fig. 4a) and piericidin A (data not shown) or the complex III inhibitors myxothiazol (Fig. 4b) or stigmatellin (Fig. 4c) are applied. Only DPI strongly inhibited the rotenone-induced generation of superoxide by complex I (cf. Table 3). These data indicate the presence of a superoxide generating site upstream of the CoQ-reduction site, which is inhibited by rotenone and piericidin A. Differential effects of stigmatellin or myxothiazol, in comparison to rotenone or piericidin A, on superoxide production, reported in studies with isolated rat skeletal mitochondria [11], were not confirmed in these experiments with rat brain submitochondrial particles. The relatively low superoxide generation rates with succinate alone can be explained by the relatively high amount of uncoupled inner membrane particles in this particular SMP preparation, since uncoupler stimulated the succinate-driven respiration only about 1.1-fold (data not shown).

3.4. Hydrogen peroxide generation of rat brain mitochondria depends solely on reduction state of the NAD system

To test if the mitochondrial membrane potential or ΔpH might be a factor influencing the complex I-dependent superoxide generation we compared the H_2O_2 generation rates in isolated mouse brain mitochondria (Fig. 5a) and isolated rat brain mitochondria (Fig. 5b) during forward electron flow (in presence or absence of rotenone) with those of reversed electron flow. Since the NAD redox state has been shown to be an important factor determining complex I-mediated ROS generation [34] we plotted the different rates of H_2O_2 generation versus the NAD redox state. The NAD redox state under conditions of forward electron flow was altered with the use of different substrates (Fig. 5, circles). The NAD redox state under conditions of reversed electron flow was altered by malonate additions (in the presence of succinate, Fig. 5, squares). Since we postulated in accordance with earlier work [8,14] that the FMN moiety of respiratory chain complex I is the major site responsible for superoxide generation we expected independently from the direction of electron flow a unique dependency of superoxide generation rate on the mitochondrial NAD redox state. As shown in Fig. 5, there is within experimental error really a unique dependency of H_2O_2 generation rate on NAD redox state. Since mitochondria have under conditions of forward electron transport (circles) due to rotenone inhibition a very low membrane potential (or low ΔpH), but under conditions of reversed electron flow (squares) a considerable membrane potential (or high ΔpH) the presence of a unique dependency on NAD redox state clearly excludes (at least for brain mitochondria) considerable effects of membrane energization on superoxide production.

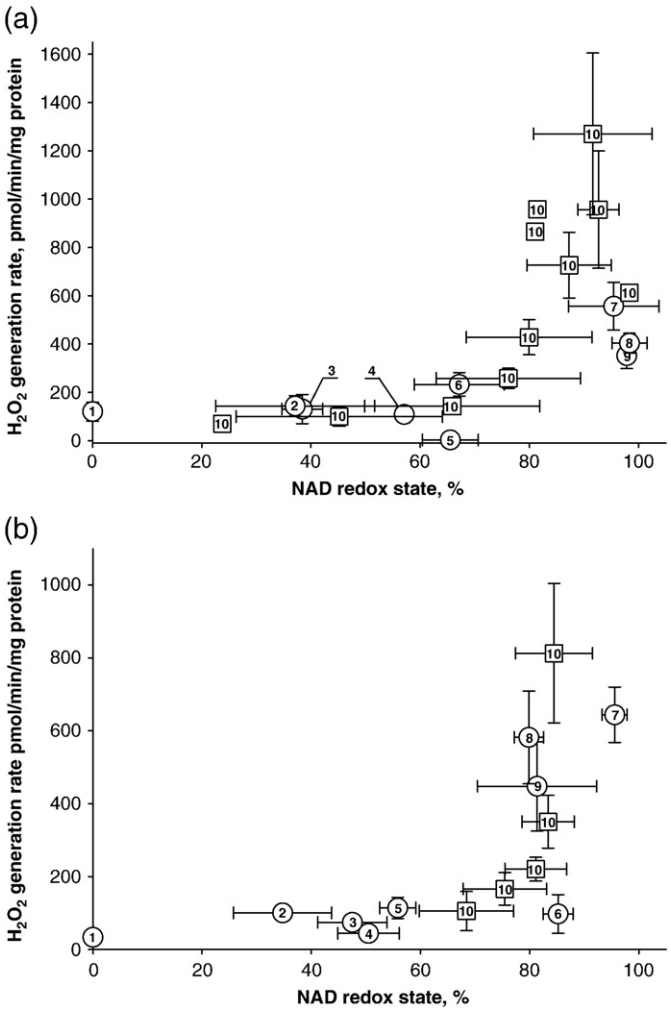

Fig. 5. NAD redox state dependency of H_2O_2 generation by complex I in mouse (a) and rat (b) brain mitochondria. The NAD redox state was determined by NAD(P)H fluorescence measurements as described in Materials and methods. (a) 1 — endogenous state, 2 — 6.7 μM rotenone, 3 — 10 mM glutamate, 4 — 5 mM malate, 5 — 10 mM succinate + 10 mM glutamate + 5 mM malate + 2 mM ATP, 6 — 10 mM glutamate + 5 mM malate, 7 — 10 mM glutamate + 5 mM malate + 6.7 μM rotenone, 8 — 10 mM succinate + 10 mM glutamate + 5 mM malate, 9 — 5 mM malate + 6.7 μM rotenone, 10 — 10 mM succinate + malonate titration (0 mM, 0.07 mM, 0.14 mM, 0.21 mM, 0.28 mM, 0.42 mM, 0.57 mM, 0.71 mM, 0.85 mM, 1.14 mM, 2.04 mM respectively). $n=5$. (b) 1–9 conditions as above, 10 — 10 mM succinate + malonate titration (0 mM, 0.14 mM, 0.28 mM, 0.56 mM, 1.12 mM respectively). $n=6$.

4. Discussion

Even though the predominant mitochondrial origin of *in vivo* ROS has been widely accepted in the literature on the basis of calculations originally made for rat liver and rat or pigeon heart (about 2% of electrons through respiratory chain can give rise to H_2O_2 [23]), some more recent reports questioned the exclusive mitochondrial origin of cellular ROS (cf. discussion in [2] and [24–27]). Due to the lack of experimental data from brain tissue available in the literature we have analyzed digitonin-permeabilized homogenates from rat hippocampus, whole mouse brain and human parahippocampal gyrus for their hydrogen peroxide formation rates. The application of brain tissue homogenates has the following advantages:

(i) The mitochondrial quality in these homogenates is very good (the respiratory control index with pyruvate + malate is ~5, cf. [28]).
(ii) The ROS production of selected brain areas (e.g. hippocampus), which contain well defined cell populations, can be studied quantitatively.
(iii) Other organelles contributing potentially to tissue ROS production (e.g. peroxysomes) are functionally intact.
(iv) The digitonin treatment allows to study the complete mitochondrial population, including mitochondria entrapped in synaptosomes.

But it needs to be mentioned that brain tissue homogenates have also the following serious limitations:

(i) They are a very complex system, containing in addition to intact organelles, also synaptosomes and many membrane fragments.
(ii) Cytosolic conditions are only poorly reflected, since important substrates and enzymes are diluted by the measurement medium. Therefore, the cytosolic contribution to ROS production is in homogenates very likely underestimated.

Despite the mentioned limitations, homogenates obviously provide a useful link between isolated brain mitochondria and intact brain tissue to study the potential mitochondrial contribution to tissue ROS production. We observed that under conditions of oxygen saturation with succinate as substrate relatively high H_2O_2 production rates can be monitored. Taking the rates of respiration of these homogenates into account a maximum of 1% of electron flow through respiratory chain is bypassed to generate superoxide which is readily converted by the SOD2 to H_2O_2. Since the *in vivo* oxygen saturation is much lower, this value is definitely the upper estimate, in line with classical observations [35] that hyperoxia increases to H_2O_2 production by brain *in vivo*. For the following reasons, mitochondria have the potential to contribute to the majority of brain tissue hydrogen peroxide formation. (i) In digitonin-treated brain tissue homogenates the H_2O_2 generation is considerably stimulated by the mitochondrial substrate succinate. (ii) In nonpermeabilized homogenates the rate of H_2O_2 production is sensitive to mitochondrial energetization, since it is diminished by uncoupler additions. (iii) And finally and most importantly, with succinate as substrate brain tissue preparations containing mitochondrial inner membranes at a different purity generate H_2O_2 proportionally to the corresponding rates of oxygen consumption. It has to be mentioned however, that nevertheless a

considerable part of H_2O_2 generation in brain homogenates is due to extramitochondrial sources, which contribution might be underestimated under the present conditions. These include cytoplasmic, plasma membrane and perhaps also peroxisomal sources, which individual contributions remain to be elucidated yet.

To identify the potential ROS producing constituents of mitochondrial inner membranes we isolated rat brain submitochondrial particles and studied the effects of different inhibitors on superoxide generation. We observed that the basal ROS generation in the presence of NADH alone was stimulated by all applied inhibitors of complexes I and III (rotenone, piericidin A, myxothiazol, stigmatellin and antimycin) approximately two-fold, while with succinate without inhibitor a similar rate was obtained like with NADH in presence of inhibitors. DPI was able to inhibit the superoxide production in presence of NADH and the inhibitors. These results clearly indicate that a site upstream of the CoQ-reduction site of complex I is the likely donor of electrons for superoxide generation. The substantial superoxide generation in the presence of NADH alone is in line with previous observations [7,8,14] that the flavin moiety of complex I is this potential superoxide generator. This viewpoint is further supported by the obtained unique dependency of hydrogen peroxide production of rat and mouse brain mitochondria on mitochondrial NAD redox state. Available structural data [36] and investigations of the isolated complex I [37] also corroborate this idea, and suggest that the $FMNH_2$ is perhaps the most relevant species for single electron reduction of molecular oxygen in brain mitochondria.

In summary, we substantiated previous experimental evidence [8] that in brain tissue a potentially most relevant superoxide generating site is the flavin moiety of complex I. It has to be stated, however, that even under the particular conditions of oxygen saturation with succinate as substrate only a maximum of 1% of respiratory chain electrons are redirected to form ROS, which is much less than previously suggested [23].

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