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Biochimica et Biophysica Acta 1229 (1995) 363–372

BBA Biochimica et Biophysica Acta

A metabolite of carcinogenic 2-acetylaminofluorene, 2-nitrosofluorene, induces redox cycling in mitochondria

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Received 31 October 1994; revised 2 January 1995; accepted 25 January 1995

Abstract

The present study was designed to confirm the recent proposal that 2-nitrosofluorene (2-NOF) as well as *N*-hydroxy-2-aminofluorene (N-OH-AF) induce a redox-cycle in rat liver mitochondria as part of the chronic toxic effects of the carcinogen 2-acetylaminofluorene (2-AAF). The formation of O_2^- was demonstrated in submitochondrial particles by the formation of adrenochrome with NADH and succinate as respiratory substrates. 2-NOF was as effective as paraquat, a known redox-cycler, the lowest effective concentration being 0.4 nmol 2-NOF/mg protein. Experiments with isolated mitochondria showed that 2-NOF, in contrast to N-OH-AF, induces cyanide-resistant O_2 consumption only in the presence of respiratory substrates, indicating that the reduction, but not the reoxidation, depends on a continuous flow of electrons through the respiratory chain of the mitochondrial membrane. Lipid peroxidation was estimated by the formation of thiobarbituric-acid-reactive substances. In comparison to the well-known prooxidant *tert*-butylhydroperoxide, 2-NOF was not significantly active. The results support the notion that 2-NOF induces oxidative stress by mitochondrial redox-cycling in vivo. Effects other than lipid peroxidation seem to be important for the chronic toxicity of 2-AAF.

Keywords: Redox cycle; Superoxide anion; Lipid peroxidation; Mitochondrion; Submitochondrial particle; Respiratory chain; (Rat liver)

1. Introduction

2-Acetylaminofluorene (2-AAF) is a complete carcinogen for rat liver, but it is less genotoxic in the liver than other arylamines, that are not complete liver carcinogens [1]. This led to the search for non-genotoxic effects of this chemical which may contribute to its carcinogenicity [2,3].

It is widely accepted that the first activation step of 2-AAF-metabolism is the *N*-hydroxylation to the hydroxamic acid *N*-hydroxy-AAF [4–8]. Although *N*-hydroxylation elevates the carcinogenic potential of the parent compound, it is not considered the ultimate reactive species.

A role of metabolically produced free-radicals was first proposed by Bartsch et al. [9], who demonstrated that one-electron oxidation of *N*-hydroxy-AAF yields a nitroxyl radical which dismutates to *N*-acetoxy-AAF and 2-NOF, two potent carcinogens. Subsequently, this reaction could be catalysed by purified enzymes, but was not observed in rat liver preparations [9,10]. A potential role in vivo became more likely when linoleic acid hydroperoxide was detected as a possible co-substrate for the oxidation with hematin [11]. Another approach to confirm the significance of free-radicals in 2-AAF-carcinogenesis was made possible by the observation that 2-NOF reacted with unsaturated lipids in microsomal membranes and liposomes to form a nitroxyl free radical [12]. However, a role of these reactions in the process of 2-AAF-carcinogenesis has not yet been established.

Studies with rats consuming a diet containing 0.02% AAF revealed a 20% increase of oxygen consumption in perfused liver preparations [2,13]. Mitochondria were therefore considered a possible target for 2-AAF toxicity [2]. 2-NOF, which in vivo may be formed in microsomes by deacylation of N-OH-AAF and subsequent oxidation [12], induced a cyanide-resistant O_2 consumption in isolated rat liver mitochondria. To account for the elevated O_2 consumption, a redox cycle was proposed, according to which 2-NOF is reduced by components of the respiratory

Abbreviations: FCCP, carbonylcyanide-*p*-(trifluoromethoxy)-phenylhydrazone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBARS, thiobarbituric-acid-reactive substances; SOD, superoxide dismutase; t-BHP, *tert*-butylhydroperoxide; 2-NOF, 2-nitrosofluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene; Glu/Mal, glutamate/malate; Succ, succinate; KCN, potassium cyanide; Asc, ascorbate.

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SSDI 0005-2728(95)00019-4

chain to *N*-hydroxy-2-aminofluorene (N-OH-AF) or the nitroxyl free radical, both of which are able to reduce molecular oxygen to superoxide anions [2].

We now provide in vitro data supporting the generation of superoxide anions ($O_2^{\cdot-}$) and the proposed redox-cycling mechanism.

2. Materials and methods

2-Nitrofluorene and potassium cyanide (KCN) were purchased from Aldrich (Steinheim, Germany); FCCP, glutamate (Glu), malate (Mal), succinate (Succ), *t*-butyl hydroperoxide (t-BHP), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), sulfonazo III, paraquat, epinephrine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (essentially fatty-acid free) and superoxide dismutase (SOD) were purchased from Sigma (Deisenhofen, Germany).

N-OH-AF and 2-NOF were synthesized from 2-nitrofluorene according to [14]. The purity of 2-NOF was checked by reverse-phase HPLC on a Zorbax ODS C-8 column (Bischoff, Leonberg, Germany) and was greater than 98%. In accordance with the data from literature the absorption maxima of 2-NOF were 361 nm ($\epsilon = 21\,400$) and 244 nm in 95% ethanol. N-OH-AF gave UV-visible absorption maxima at 285 nm ($\epsilon = 19\,200$) and a shoulder at 315 nm. In aqueous media, the absorption maxima shifted to longer wavelengths. Both chemicals were stored under N_2 at $-20°$ C. For in vitro experiments with isolated mitochondria N-OH-AF and 2-NOF were dissolved in DMSO immediately prior to each experiment. The total concentration of DMSO in incubation media did not exceed 0.2%. For spectrophotometric determinations stock solutions of N-OH-AF and 2-NOF were prepared in acetone.

Male Wistar rats (200–260 g) were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) and had free access to standard diet (Altromin 1324, Altrogge, Lage/Lippe, Germany) and water. They were kept under controlled conditions of temperature and humidity on a 12 h light, 12 h dark cycle.

Rat liver mitochondria were isolated by differential centrifugation as described in [15] with some modifications. Animals were anaesthetized with ether and the liver was perfused with saline. All subsequent procedures were performed at 0–4° C. The liver was immediately minced with a scalpel into 30 ml of fresh ice-cold isolation buffer containing 0.21 M D-mannitol, 0.07 M sucrose, 10 mM Hepes, 1 mM EGTA and 0.1% (w/v) fatty acid-free bovine serum albumin (pH 7.4) and homogenized by hand in a Potter-Elvehjem glass vessel fitted with a Teflon pestle. The homogenate was initially centrifuged at $450 \times g$ for 5 min at 4° C. The supernatant was kept on ice in a precooled centrifugation tube, while the pellet was resuspended and again centrifuged at $450 \times g$ for 5 min at 4° C. The combined supernatant fractions were centrifuged at $7000 \times g$ for 5 min at 4° C. The resulting mitochondrial pellet was washed twice by gentle resuspension and recentrifugation. The final mitochondrial pellet was gently resuspended in isolation buffer at a final concentration of approx. 20 mg of mitochondrial protein per ml. Protein concentration was determined by the method of Lowry et al. [16], with bovine serum albumin as standard.

SOD-depleted particles were prepared by sonicating thawed mitochondria for 3×1 min in a medium containing 0.25 M sucrose and 2 mM EDTA (pH 7.4). The sonicate was diluted with an equal volume of 0.25 M sucrose, centrifuged for 5 min at 9000 rpm to pellet intact mitochondria. The supernatant was centrifuged for 40 min at $105\,000 \times g$. The pelleted particles were washed in 10 vols. 0.25 M sucrose, sedimented by centrifugation and stored frozen.

The formation of $O_2^{\cdot-}$ was determined by the oxidation of epinephrine according to [17] except that KCN was used as inhibitor instead of antimycin A. The reaction volume was reduced to 1 ml instead of 2 ml and incubations were followed for 60 min at 37° C. For each group, one preparation lacking the ultimate test substance was run as a blank.

The rate of oxygen consumption was measured polarographically with the aid of a Clark oxygen electrode DW1 (Bachofer, Reutlingen, Germany) equipped with an ultrathin Teflon membrane. Oxygen consumption rates were determined at 25° C in respiration buffer, containing 0.3 M sucrose, 5 mM Mops, 1 mM EGTA, 5 mM KH_2PO_4, 5 mM $MgSO_4$ and 0.1% (w/v) fatty acid-free bovine serum albumin (pH 7.4). Cyanide-insensitive respiration was determined by the addition of mitochondria (2.5 mg/ml) to an incubation mixture containing 1 mM KCN and 5 mM respiratory substrates. FCCP (1 μM) was added to each reaction to deplete endogenous substrates and to allow maximal oxidation of substrates [18]. Oxygen consumption calculations are based on an oxygen solubility in air-saturated respiration buffer at 25° C of 260 nmol O_2/ml. Oxygen consumption rates are expressed as nmol O_2 per min per mg mitochondrial protein.

The degree of lipid peroxidation was estimated by measuring the concentration of TBARS according to [19] with slight modifications. Mitochondria (2.5 mg/ml) in Tris-KCl buffer (10 mM Tris, 100 mM KCl), pH 7.4, were incubated at 37° C under continuous stirring with different amounts of prooxidants in the presence of 10 mM Succ. After 5 min the reaction was stopped by adding 1 ml of the mitochondrial suspension to 2 ml of 0.6% (w/v) thiobarbituric acid containing 1% trichloroacetic acid (pH 1.0). The reaction mixture was heated at 95° C for 30 min. The thiobarbituric acid-malondialdehyde-complex formed was extracted with n-butanol and the concentration of TBARS was determined fluorometrically according to [20], with an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Standard malondialdehyde was prepared

by acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) and the level of lipid peroxides was expressed as pmol malondialdehyde equivalents per mg mitochondrial protein.

UV-visible absorbance measurements were carried out with a Kontron Uvikon 860 spectrophotometer (Kontron Instruments, Neufahrn, Germany) at 25° C. The reaction was started by the addition of either N-OH-AF or 2-NOF plus a reductant. For measuring the change of spectrophotometric properties during reduction of 2-NOF, DTT was used as reducing agent, because it does not absorb in the studied λ-range.

The rate of superoxide formation during autoxidation of N-OH-AF was measured by the reduction of the tetrazolium salt MTT at 560 nm under aerobic conditions [21,22].

3. Results

3.1. Superoxide anion formation during autoxidation of N-OH-AF

The hydroxylamine, N-OH-AF is not stable in 20 mM buffer and is converted to 2-NOF by autoxidation (Fig. 1). The two sharp isosbestic wavelengths at 253 nm and 320 nm are characteristic for the conversion of N-OH-AF to 2-NOF.

By addition of DTT in Hepes (pH 7.4), 2-NOF, on the other hand, is rapidly reduced to N-OH-AF (Fig. 2). The formation of N-OH-AF is succeeded by autoxidation of the hydroxylamine as shown by the reappearance of the 2-NOF-spectrum. The rate of reoxidation of N-OH-AF was approximately half as fast as the reduction of 2-NOF under the applied conditions (Fig. 2). Reduction of 2-NOF to N-OH-AF was almost complete (98%) at a concentration of 500 μM DTT (data not shown); reoxidation yielded 88% of the initial concentration of 2-NOF, indicating that only a small amount of byproducts is being formed.

To demonstrate the generation of $O_2^{\cdot-}$, the reduction of MTT was measured. The addition of MTT to a mixture of 2-NOF and the reducing agent Asc in Hepes (pH 7.4) induced an increase in absorbance at 560 nm due to the accumulation of the blue formazan derivative (Fig. 3). The initial rate of Asc-dependent increase of absorbance (0.014/min) was enhanced more than 4-fold by the addition of 2-NOF (0.064/min). 2-NOF alone did not induce the reduction of MTT (data not shown). The reduction of MTT was inhibited by SOD, thus indicating the participa-

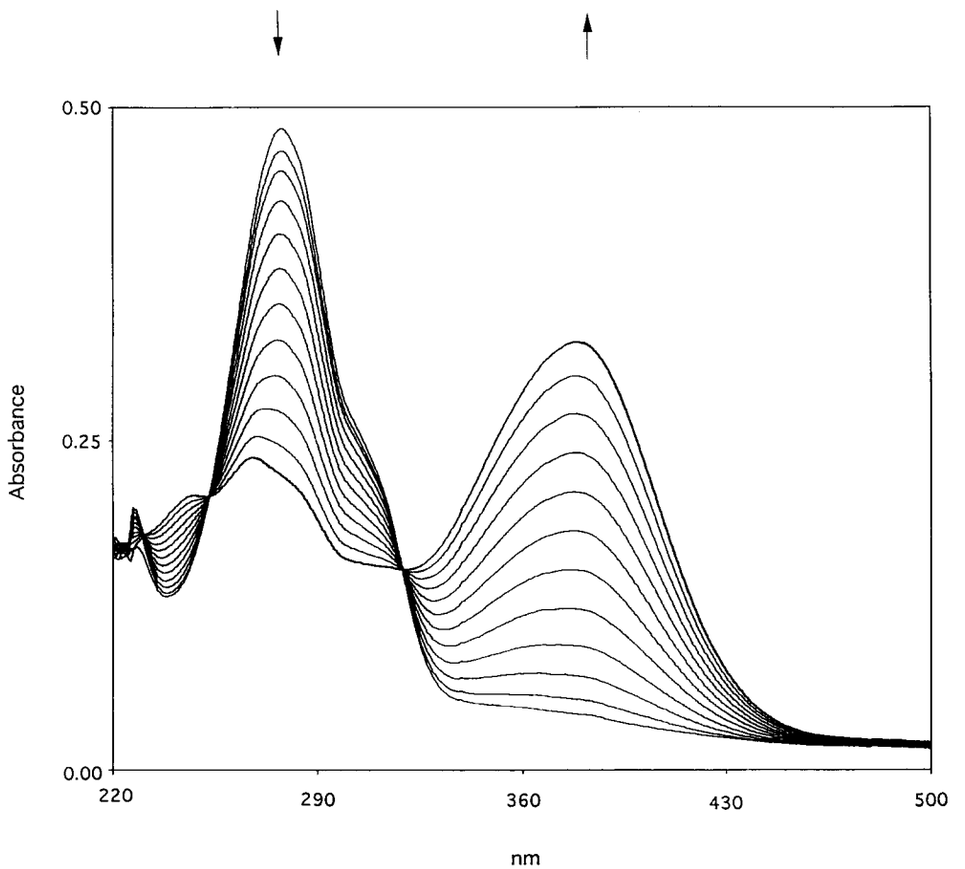

Fig. 1. The spectrophotometric determination of the autoxidation of N-OH-AF. N-OH-AF (25 μM) was added to Hepes buffer (pH 7.4), in a 3 ml quartz cuvette. Spectra were recorded at 1 min intervals.

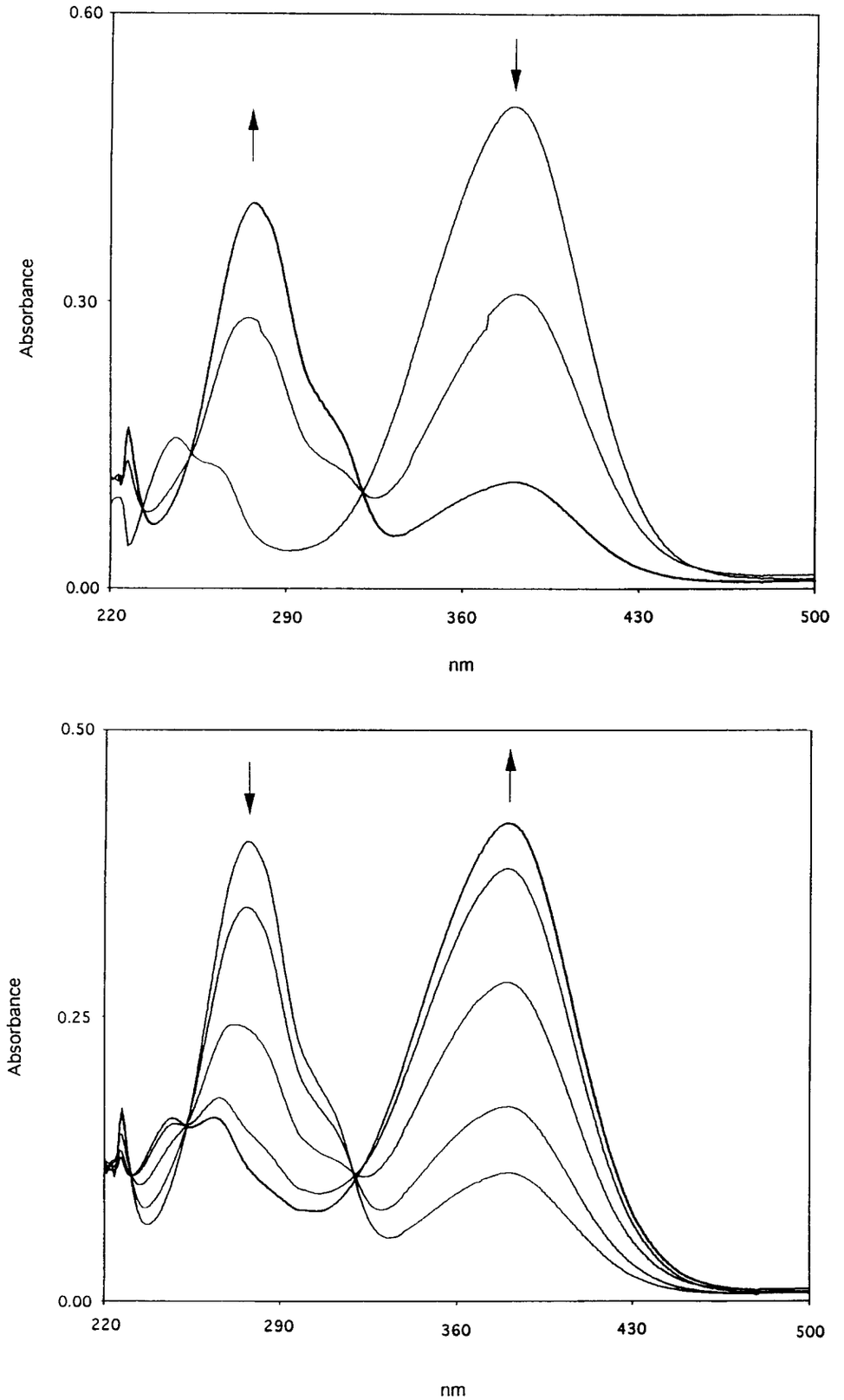

Fig. 2. The reduction of 2-NOF to N-OH-AF is succeeded by autoxidation of N-OH-AF. In panel (A) (upper panel), 2-NOF (25 μM) in Hepes (pH 7.4), was reduced with DTT (50 μM) to N-OH-AF. Spectra were recorded in 2 min intervals. After 6 min (panel B; lower panel) the reductant was exhausted – and N-OH-AF reoxidized as shown by the reappearance of the 2-NOF-spectrum.

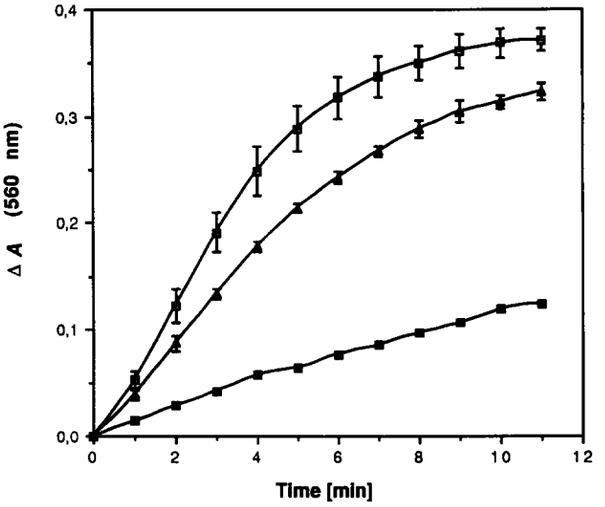

Fig. 3. The generation of $O_2^{\cdot-}$ during autoxidation of N-OH-AF and the effect of superoxide dismutase as determined by the reduction of MTT (increase of absorption at 560 nm). 2-NOF (25 μM) was reduced with Asc (500 μM) in 20 mM Tris buffer (pH 7.4), containing 50 μM MTT (□). $O_2^{\cdot-}$ is formed by reoxidation of N-OH-AF and reduces MTT to a formazan-derivative, whose UV-absorption is measured. In parallel vessels, 10 U SOD were added to the reaction mixture (▲). (■) represents control experiments containing MTT and Asc, but without addition of 2-NOF. The data represent mean values ± S.D. of four separate experiments.

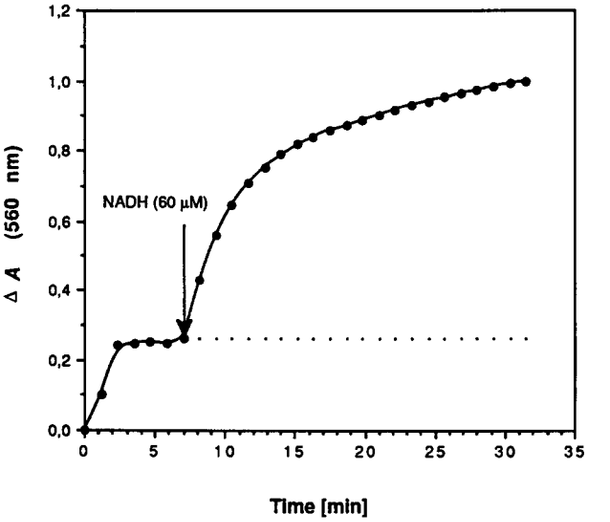

Fig. 4. Redox cycling of the redox couple N-OH-AF/2-NOF in the presence of NADH. Autoxidation of N-OH-AF (60 μM) in Hepes buffer (pH 7.4), containing MTT (50 μM) yields only a limited amount of $O_2^{\cdot-}$, as indicated by MTT-reduction. After the addition of a reducing agent (NADH) a redox-cycle is initiated.

tion of $O_2^{\cdot-}$ in the autoxidation of the hydroxylamine. The free-radical scavenger α-tocopherol and the antioxidant BHT also inhibited the reduction of MTT (data not shown).

The rate of autoxidation depends on the pH of the buffer (data not shown): the higher the pH, the less stable the hydroxylamine. The initial rate of autoxidation of N-OH-AF (60 μM) was 15-times faster at pH 9.0 as compared to the rate of autoxidation at pH 6.5.

In the absence of a reducing agent only a limited amount of $O_2^{\cdot-}$ was generated and the stable nitroso-derivative was formed. But adding a reducing agent to the reaction mixture initiated a redox cycle, resulting in the superstoichiometric formation of $O_2^{\cdot-}$ as indicated by the reduction of MTT (Fig. 4).

3.2. Superoxide anion formation in submitochondrial particles

The oxidation of epinephrine was used to demonstrate the generation of $O_2^{\cdot-}$. 2-NOF, like the two known redox cyclers paraquat and sulfonazo III gave positive reactions and the efficiency was comparable to that of paraquat (Fig. 5). The values of the positive controls correspond to those previously reported [17]. The reaction depends on a functioning respiratory chain. In this case NADH was used as a substrate. If succinate was used, paraquat and sulfonazo III are negative, but 2-NOF remains positive (Fig. 6), indicating that it receives its electrons at a different site downstream of complex I. Conversely, using antimycin A as an inhibitor, 2-NOF-dependent adrenochrome formation was inhibited, but not that of paraquat and sulfonazo III (data not shown).

The lowest effective concentration of 2-NOF is 100 nM, which corresponds to a dose of 0.4 nmole/mg protein (data not shown). Dose-dependent effects are also seen with higher concentrations.

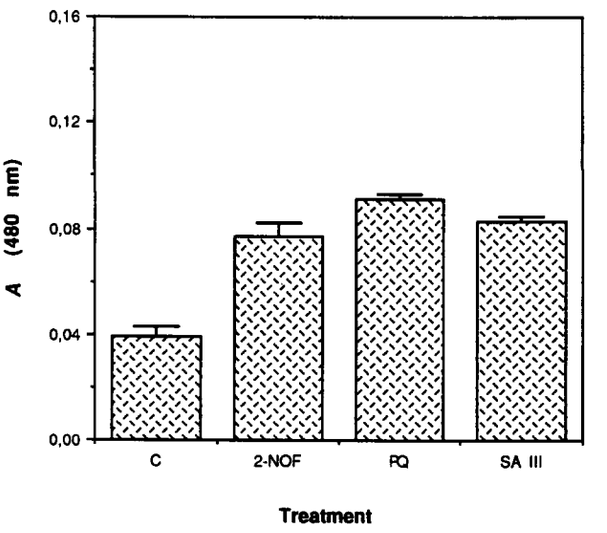

Fig. 5. Effect of 2-NOF (100 μM), paraquat (PQ; 390 μM), sulfonazo III (SA III; 1.45 μM) and control (C) on NADH (125 μM)-dependent superoxide generation in SMP as measured by the absorption of adrenochrome at 480 nm. The incubation mixture contained 1 ml of 50 mM Hepes buffer (pH 7.2), epinephrine (1.5 mM), 0.5 mg SMP and KCN (100 μM). The mixtures were incubated for 60 min at 37° C. Mean values ± S.E. are presented.

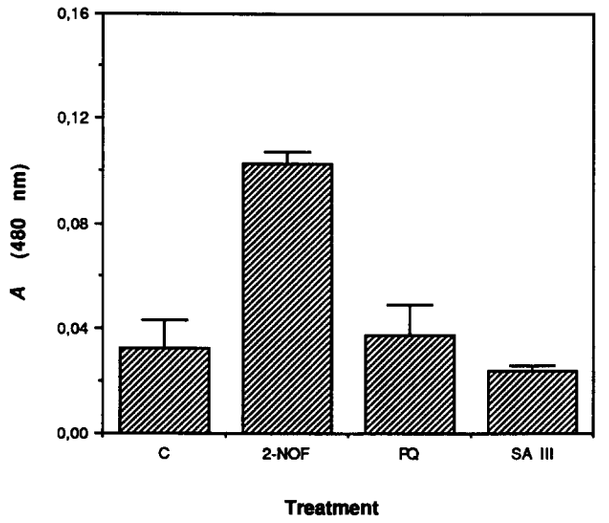

Fig. 6. Effects of different redox cyclers on superoxide generation in SMP. Concentrations and conditions as in Fig. 5, except that NADH was exchanged for succinate (5 mM).

3.3. Effects of 2-NOF and N-OH-AF on respiration in isolated rat liver mitochondria

2-NOF induces cyanide-resistant O_2 consumption in isolated mitochondria, which depends on the presence of respiratory substrates (Fig. 7). 2-NOF did not induce cyanide-resistant O_2 consumption in the absence of Glu/Mal in mitochondria pretreated with FCCP, to deplete endogenous substrates and with KCN, to inhibit state-4-respiration (Fig. 7, panel B). But as soon as Glu/Mal was added to the reaction mixture, O_2 consumption increased. The same results were obtained with Succ as respiratory substrate (data not shown).

In contrast to 2-NOF, N-OH-AF stimulates O_2 consumption in the absence of respiratory substrates (Fig. 8, panels A and B). When N-OH-AF was added to mitochondria, treated with FCCP and KCN but without respiratory substrates, O_2 was rapidly consumed, but this O_2 consumption ceased after 1 min. When either Glu/Mal or Succ was added thereafter, cyanide-insensitive O_2 consumption resumed. Since total O_2 consumption could not be determined in this experiment (Fig. 8), it was measured separately under the same conditions and compared with the O_2 consumption by autoxidation of N-OH-AF to support the occurrence of a redox cycle. Glu/Mal-stimulated reduction with concurrent autoxidation consumed twice as much O_2 as autoxidation alone (data not shown). This result proves a cyclic reaction of a redox couple.

The succinate-dependent O_2 consumption was inhibitable with antimycin A (Fig. 8B).

3.4. Effect of albumin on 2-NOF and N-OH-AF-dependent O_2 consumption in isolated rat liver mitochondria

Neither N-OH-AF nor 2-NOF plus Glu/Mal was effective in albumin-free respiration buffer (pH 7.4). When albumin was added to the reaction mixture to a final concentration of 0.1%, O_2 consumption started immediately and showed the same features as shown before. For instance, the N-OH-AF-induced O_2 consumption could be restimulated by addition of Glu/Mal (Fig. 9).

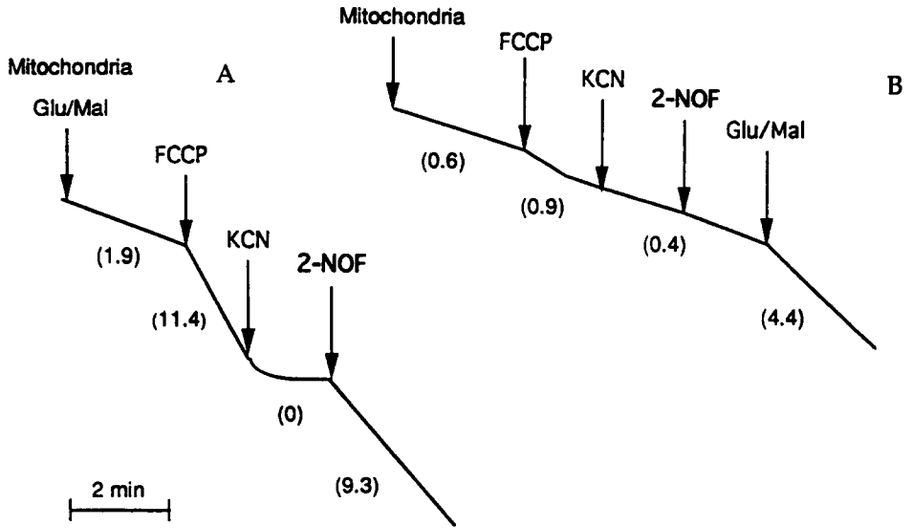

Fig. 7. Effect of 2-NOF on the oxygen consumption of isolated rat liver mitochondria in the presence (A) and absence (B) of respiratory substrates. Mitochondria (2.5 mg/ml) in respiration buffer (pH 7.4), in the presence (A) and absence (B) of 5 mM Glu/Mal were incubated at 25° C with FCCP (1 μM), KCN (1 mM) and 2-NOF (30 nmol/mg), successively. The respiratory rates are given in nmol/min per mg.

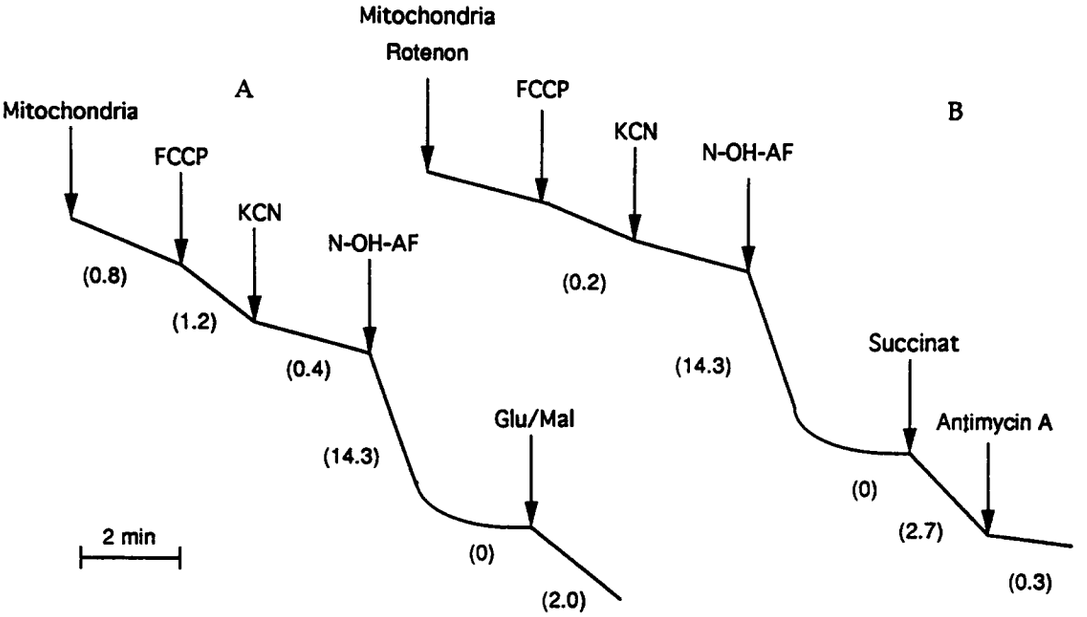

Fig. 8. Effect of N-OH-AF on the O_2 consumption of isolated rat liver mitochondria and the restimulation of a cyanide-insensitive O_2 consumption with the respiratory substrates Glu/Mal (A) and Succ (B). (A) Mitochondria (2.5 mg/ml) in respiration buffer (pH 7.4) were incubated at 25° C with FCCP (1 μM) and KCN (1 mM) prior to the addition of N-OH-AF (20 nmol/mg). After the N-OH-AF-induced O_2 consumption stopped, 5 mM Glu/Mal were added to the mitochondrial suspension. The respiratory rates are given in nmol/min per mg. (B) Mitochondria (2.5 mg/ml) in respiration buffer (pH 7.4), in the presence of rotenone (5 mM) were incubated at 25° C with FCCP (1 μM) and KCN (1 mM) prior to the addition of N-OH-AF (20 nmol/mg). After the N-OH-AF-induced O_2 consumption stopped, 5 mM Succ was added to the mitochondrial suspension. The restimulation of the cyanide-resistant O_2 consumption was inhibited by addition of antimycin A (4 μg/ml). The respiratory rates are given in nmol/min per mg.

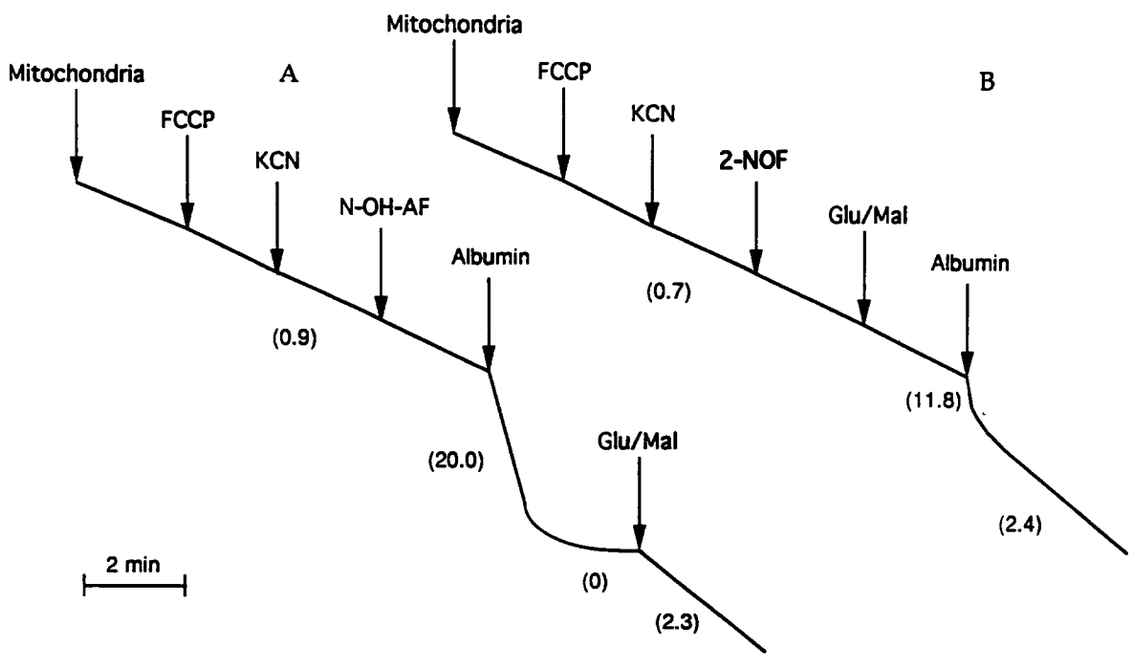

Fig. 9. Effect of albumin on the cyanide-insensitive O_2-consumption of N-OH-AF and 2-NOF in isolated rat liver mitochondria. Mitochondria (2.5 mg/ml) in respiration buffer (pH 7.4) without albumin were treated according to the described procedure of Fig. 8. After the addition of N-OH-AF (A) and 2-NOF plus Glu/Mal (B), respectively, albumin (fatty-acid-free) was added to the mitochondrial suspension to a final concentration of 0.1%. The respiratory rates are given in nmol/min per mg.

To understand the effect of albumin, N-OH-AF-induced autoxidation was measured polarographically in imidazole buffer (pH 7.4), in the absence and presence of albumin. The rate of O_2 consumption induced by 50 μM N-OH-AF (0.65 nmol/min) increased more than 20-fold (15.6 nmol/mg) after addition of albumin (data not shown). This observation suggests that albumin facilitates the autoxidation of the hydroxylamine. A chemical reaction of albumin with N-OH-AF in imidazole buffer is not expected, since O_2 consumption was restimulated by the reducing agent NADH, indicating that 2-NOF was formed during the O_2 consumption burst.

Autoxidation is also enhanced by albumin in mitochondrial suspensions. However, when N-OH-AF was added to such suspensions containing albumin (0.1%), 3-times as much O_2 was consumed as compared with the autoxidation in mitochondria-free buffer containing the same concentration of albumin. This indicates that, in addition to albumin, mitochondria also facilitate autoxidation.

3.5. Effect of 2-NOF-stimulated redox cycling on lipid peroxidation in isolated rat liver mitochondria

Damage of the mitochondrial membrane as a result of lipid peroxidation was tested by measuring TBARS-production of respiring mitochondria. TBARS-production was slightly but significantly increased with a maximal value at 40 nmol/mg 2-NOF. The addition of albumin to the incubation medium did not increase the formation of TBARS (data not shown). Compared to the TBARS-production of the known prooxidant t-BHP, the 2-NOF-induced lipid peroxidation seems to be irrelevant (Fig. 10).

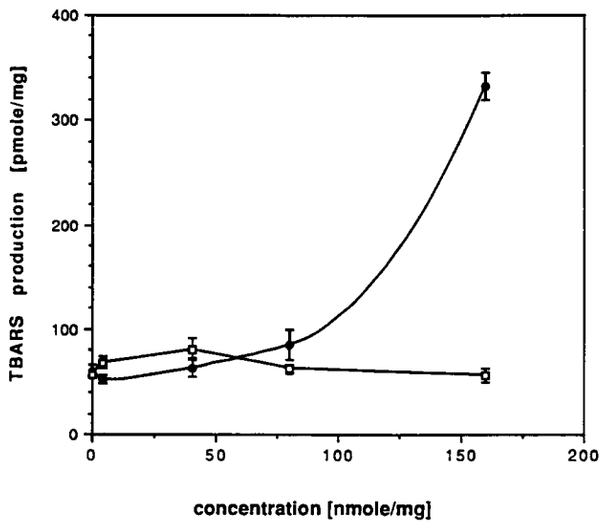

Fig. 10. Effect of the prooxidants 2-NOF and t-BHP on lipid peroxidation in isolated rat liver mitochondria. Mitochondria (2.5 mg/ml) in KCl-Tris-buffer (pH 7.4) were incubated under continuous stirring at 37° C with different amounts of the prooxidants 2-NOF (□) and t-BHP (●). After 5 min the reaction was stopped by heating 1 ml of the mitochondrial suspension in 2 ml TBA-TCA-solution at 95° C for 30 min. The formed TBA-MDA-complex was extracted with n-butanol and determined fluorimetrically. The data represent mean values ± S.D. of four separate experiments.

4. Discussion

It has been previously proposed that N-OH-AF and 2-NOF, metabolites of the carcinogen 2-AAF, may constitute the members of a redox cycle which generates reactive oxygen in mitochondria [2,13]. We now confirm this proposal by demonstrating the generation of $O_2^{\cdot-}$ and we present a more detailed mechanism of redox-cycling in intact mitochondria.

The chemistry of activation of 2-NOF is poorly understood. Subcutaneous injection of 2-NOF produces tumors at the site of injection as well as tumors of the mammary gland in female rats [23].

A role for free radical processes in 2-NOF-metabolism was proposed by Floyd et al. [12], who showed that 2-NOF adds to double bonds of unsaturated fatty acids of rat liver microsomal membranes to form a secondary hydroxylamine, which may autoxidize to a nitroxyl free radical, by using electron spin resonance spectroscopy. This radical may form a redox-couple with the corresponding *N*-hydroxylamine. The relevance of this reaction in vivo, however, may be considered limited because only a small fraction of 2-NOF, i.e., 1%, reacts with lipid membranes. We now demonstrate that 2-NOF is able to initiate an efficient redox cycle by accepting electrons from the mitochondrial respiratory chain.

2-NOF, in contrast to N-OH-AF induces cyanide-resistant O_2 consumption only in the presence of respiratory substrates (Figs. 7 and 8), indicating that the reduction, but not the reoxidation depends on a continuous flow of electrons through the respiratory chain of the mitochondrial membrane. After inhibition of the electron flow with antimycin A, an inhibitor of complex III of the respiratory chain, the O_2 consumption stopped immediately (Fig. 8B).

The reduction of 2-NOF by electrons of the respiratory chain yields either the nitroxyl free radical or N-OH-AF which are reoxidized by oxygen (Fig. 11). The associated generation of short-lived $O_2^{\cdot-}$ was demonstrated by sensitive chemical methods based on trapping the radical with suitable scavengers. The oxidative properties of $O_2^{\cdot-}$ were used by measuring the formation of adrenochrome from epinephrine [24,25], the reductive properties by the reduction of MTT, a tetrazolium salt, to a blue formazan derivative [26,27]. Although neither method is specific, the inhibiting effects of SOD favor the given interpretation of these results.

Thus, the 2-NOF-dependent generation of $O_2^{\cdot-}$ supports the existence of a redox cycle in mitochondrial membranes (Figs. 5 and 6). It may also be emphasized that 2-NOF was as efficient in forming adrenochrome as the established redox cycler paraquat and that it receives the electrons

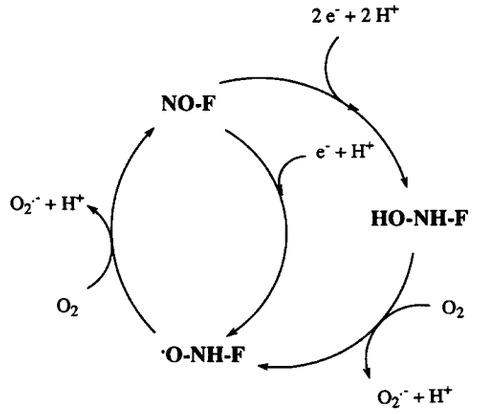

Fig. 11. Proposed mechanism of redox cycling.

from a different site than paraquat, i.e., most likely from complex III or beyond.

The in vitro experiments without mitochondria clearly show that N-OH-AF is readily oxidized by oxygen and that redox-cycling, i.e., the superstoichiometric consumption of O_2, requires the presence of a reducing agent. In mitochondria the functioning respiratory chain serves this purpose. The proposed redox cycle is summarized in Fig. 11.

The question remains to be answered whether one- or two-electron reduction of 2-NOF prevails in mitochondria. The flavin nucleotides, $FMNH_2$ and $FADH_2$, as well as coenzyme Q are able to catalyze both kinds, whereas cytochromes and iron-sulfur centers catalyze only one-electron reductions. If the site of 2-NOF reduction lies downstream of coenzyme Q (complex III, cytochrome *c* or complex IV), the nitroxyl free radical should be the predominant reduction product. Studies are in progress to locate more precisely the reaction site. However, arylnitroxides are also known to disproportionate readily to hydroxylamines and nitroso-derivatives [28], and it is possible that N-OH-AF is formed after one-electron reduction of 2-NOF in this way.

Surprisingly, neither N-OH-AF nor 2-NOF increased cyanide-resistant O_2 consumption in albumin-free buffered mitochondrial suspensions in the presence of respiratory substrates. The reactions could be started immediately, however, if albumin was added. Typically, albumin acts as a solubilizer and we assume that neither N-OH-AF nor 2-NOF reaches the mitochondrial membranes in aqueous suspensions. In addition, albumin facilitates the autoxidation of N-OH-AF in aqueous solutions.

Additional effects may influence O_2 consumption. Autoxidation of N-OH-AF in the presence of albumin was 3-times higher in mitochondrial suspensions than in aqueous buffer. Since heme compounds facilitate the oxidation of hydroxylamines [29], cytochromes and also non-heme iron-sulfur centers may catalyze the autoxidation of N-OH-AF in mitochondrial membranes.

The contribution of lipid peroxidation to acute 2-AAF-toxicity has been previously addressed in livers of rats with cannulated bile ducts treated with 1 mmol 2-AAF/kg body weight and in isolated perfused livers with concentrations up to 50 μM 2-AAF [3]. Measurable lipid peroxidation could not be demonstrated in both cases. We now determined 2-NOF-induced TBARS-production over a range of concentrations in isolated mitochondria. The same concentrations of t-BHP were used as positive controls. A small but significant dose-dependent effect was seen with 2-NOF with concentrations up to 40 nmol/mg, which decreased with higher concentrations. Lipid peroxidation was clearly induced with t-BHP, and the values were more than 6-fold higher with t-BHP than with 2-NOF at a concentration of 160 nmol/mg.

The biphasic effect of 2-NOF on lipid peroxidation indicates that a second process may antagonize the formation of radicals at higher concentrations. Preliminary results obtained by measuring DNP-uncoupled respiration show that 2-NOF indeed inhibits mitochondrial respiration at these higher concentrations (Klöhn and Neumann, unpublished data).

$O_2^{\cdot-}$ causes lipid peroxidation in reaction mixtures containing iron complexes like Fe^{3+}/EDTA [30] or Fe^{2+}/Asc [31]. But in these systems the disproportionation of H_2O_2 to the reactive $OH^{\cdot}$ and OH^- according to the Fenton reaction is promoted by the presence of free iron. Although mitochondria contain iron proteins, the Fenton reaction does not take place, unless iron is released from the protein [32].

An important question still remains to be answered: how relevant is the observed mitochondrial redox-cycling for 2-AAF-toxicity?

Although the proportion of non-enzymic reduction of 2-NOF by cellular reductants (NAD(P)H, Asc, glutathione) needs to be elucidated, the in vitro results clearly demonstrate the participation of the mitochondrial respiratory chain by the facts that 2-NOF-induced redox-cycling does not occur in the absence of respiratory substrates and that Succ- as well as Glu/Mal-supported O_2 consumption is almost completely inhibited by AA, an inhibitor of the respiratory chain.

In the in vivo situation redox-cycling could be favored, because 2-NOF is highly lipophilic and may be more readily available to the respiratory chain by sequestration into the mitochondrial membrane.

The formation of reactive oxygen radicals may not necessarily produce mitochondrial toxicity. Mitochondria possess an efficient enzyme system – including SOD, glutathione peroxidase and glutathione reductase – to detoxify $O_2^{\cdot-}$, which is also formed under physiological conditions as a result of incomplete reduction of O_2 [33–37].

However, the prooxidant may be formed at a rate sufficient to produce oxidative stress, since it has already been shown that oxidized glutathione accumulates in mito-

chondria during 2-AAF-feeding (0.02%). Ca^{2+} release and mitochondrial swelling have been observed in vitro [2]. Lipid peroxidation, on the other hand, does not seem to be a relevant consequence of redox-cycling.

Work in progress in this laboratory addresses the question whether oxidative phosphorylation is influenced under these conditions.

Acknowledgements

This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 172). We acknowledge the skilful technical assistance of Mrs. Corinna Kopp.

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