Current Topics in Biophysics 2005, 29(1-2), 123-127

25 YEARS EXPERIENCE ON THE DETECTION OF RADICALS IN BIOLOGICAL SYSTEMS

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We have 25 years experience in the application of EPR technique for the detection of radicals. We found that mitochondria from senescent rats produce more reactive oxygen species (ROS) than the young controls. We used spin labeling for the determination of fluidity changes in the inner mitochondrial membrane. We were unable to confirm the formation of ROS using TIRON. However, anthracycline antibiotics like adriamycin were found to produce ROS. Furthermore we described the existence of a mitochondrial nitrite reductase which may be important by patients using NO-donors. In that case bioenergetic parameters such as ATP production might be critical. We also found that ubisemiquinone replaces ferrous iron in the Fenton reaction. On the other hand we found that ubiquinol in biomembranes which do not recycle or stabilize ubisemiquinones resulting from lipidperoxidation give rise to the formation of prooxidants.

INTRODUCTION

Since the observation that radicals play a role in aerobics, various methods have been applied to detect the various radical species. Spectrophotometric methods were used in the early seventies. However, these methods were susceptible partially producing artefactual radical species. For instance, the use of cytochrome c for the detection of superoxide radicals ($O_2^{\cdot -}$) was uncertain since $O_2^{\cdot -}$ rapidly dismutate to H_2O_2 due to the presence of a matrix SOD. Besides $O_2^{\cdot -}$ and H_2O_2 also hydroxyl radicals (OH) are formed which due to their extremely high oxidation potential destroy biomembranes, proteins and DNA. Consequently oxidation products of these bioorganic compounds were taken as evidence that reactive oxygen species (ROS) occur in aerobics. The introduction of EPR technique in combination with spin trapping was a progress in the detection of free radicals. EPR technique was also applied to measure fluidity changes as a consequence of radical attack to biomembranes.

METHODS

Rat heart mitochondria were prepared according to (Szarkowska & Klingenberg, 1963). Submitochondrial particles were gained according to (Rice & Lindsay, 1997). Order parameters were calculated from molecular motion data of the spin label 2-(3-carboxypropyl)-4-4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (Nohl, Breuninger & Hegner, 1978).

Power saturation response of the ubisemiquinone EPR signal was determined according to (Nohl, Gille & Kozlov, 1996) CPH - EPR measurements were performed as in (Kozlov, Szalay, Umar, Fink, Kropik, Nohl, Redl & Bahrami, 2003). Nitrite reductase activity in mitochondria was measured according to (Kozlov, Staniek & Nohl 1999) Organic Fenton-type reaction was followed as in (Nohl & Jordan, 1981; Nohl, Jordan & Hegner, 1982; Nohl & Jordan, 1984; Nohl & Jordan, 1987). Adriamycin activation and aglycon formation was determined as in (Nohl, 1983; Nohl & Jordan, 1983; Nohl & Jordan, 1984; Nohl, 1988a, b).

RESULTS

In 1974 B. Chance and colleagues reported on the formation of ROS in isolated mitochondria. At that time I was engaged in bioenergetics of mitochondria. Therefore I was interested very much in this new research field. Four years later I published my first paper on this topic. The paper appeared in *Eur. J. of Biochem.* (Nohl *et al.*, 1978). In this paper I tried to confirm the free Radical Theory of Aging set up by D. Harman.

I compared rat heart mitochondria (RHM) from senescent animals with the young controls. At that time I used spectrophotometric methods. Our results clearly demonstrated that senescent rat heart mitochondria generated considerably more ROS

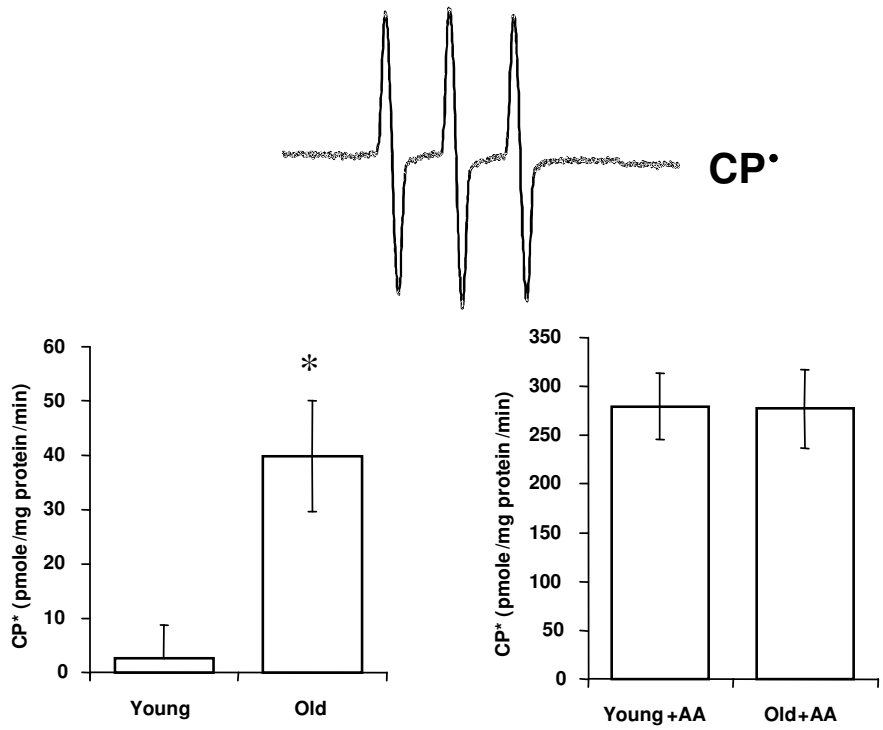

Fig 1 The rate of RONS generation in heart mitochondria isolated from young and old rats. Mitochondria were incubated in the presence of 250 μM CPH for 20 min at 22±1.5 °C. Antimycin A (AA) was added prior to CPH at the concentration of 2 μg/ml. Inset: a typical electron spin resonance spectrum of $CP^{\bullet}$.

than the young controls. Recently we confirmed our earlier results this time using EPR technique.

We used a pyrrolidine derivative (CPH) which is EPR silent unless it becomes oxidized by $O_2^{\cdot-}$ or $ONOO^-$. The resulting 3-carboxy-peroxyl radical gives a triplet (Fig 1). Although $ONOO^-$ formation was not expected we added ebselen to prevent any $ONOO^-$ derived triplet. The method was checked by the addition of antimycin A (AA) to mitochondria which is known to trigger $O_2^{\cdot-}$ release.

The comparison with young adult RHM clearly revealed a significant increase in ROS production of senescent rats (Kozlov, Szalay, Umar, Kropik, Staniek, Niedermüller, Bahrami & Nohl, 2005).

Simultaneously we observed a fluidity change of the inner mitochondrial membrane using spin labelling technique (Nohl *et al.*, 1978). We determined order parameters from the insertion of a spin label and plotted them versus the temperature. We observed a clear shift of the phase transition in senescent lipids from the inner mitochondrial membrane indicating a decrease of the fluidity. This was also confirmed by GC analyses of phospholipids which indicated a decrease of the PUFA/SAFA ratio (not shown).

Some of our colleagues used TIRON to demonstrate mitochondrial $O_2^{\cdot-}$ formation. TIRON is EPR silent unless an e^- is removed. This can be done by a $O_2^{\cdot-}$ radical or components of the respiratory chain. Also transition metals can withdraw a single electron, for instance, using phosphate buffer which are contaminated by adventitious iron.

When an e^- is removed TIRON becomes a radical exhibiting a quartet. The quartet can disappear by reduction via e^- carriers or transition metals. We checked TIRON with respect to mitochondrial $O_2^{\cdot-}$ formation.

Only in the presence of antimycin A (AA) we saw the quartet, while reversed e^- flow failed to demonstrate $O_2^{\cdot-}$ radical formation. Hence higher SOD concentrations used to demonstrate mitochondrial $O_2^{\cdot-}$ formation gave also a quartet. We observed that instead of quenching the TIRON derived EPR quartet SOD itself transformed TIRON to a quartet simulating $O_2^{\cdot-}$ radical formation.

When mitochondrial respiration was blocked upon the addition of AA we observed in the presence of DMPO a quartet indicative of the formation of $^{\cdot}OH$ radicals.

The presence of methanol supports our assumption because a α-hydroxymethyl radical was formed. Bioenergetic data supposed that $UQ^{\cdot-}$ are involved in the reductive homolytic cleavage of H_2O_2. Therefore we investigated various biological

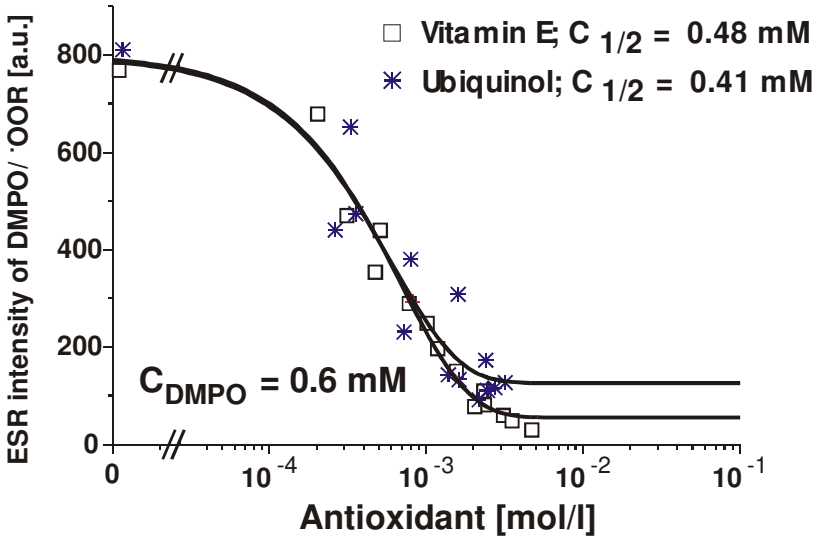

Fig 2 Radical scavenging efficiency of vitamin E (α-tocopherol) and ubiquinol in homogenous solution. The antioxidants were compared for their potency to intercept peroxyl radicals and thereby decreasing the intensity of the DMPO/•OOR spin adduct. Concentrations required for half maximal quenching ($C_{1/2}$) were calculated from the exponential decay of the ESR signal intensity (DMPO/•OOR in acetonitrile: g = 2.006, a_N = 13.0 G, a_H = 9.8 G, Σa = 35.8 G) versus antioxidant concentration.

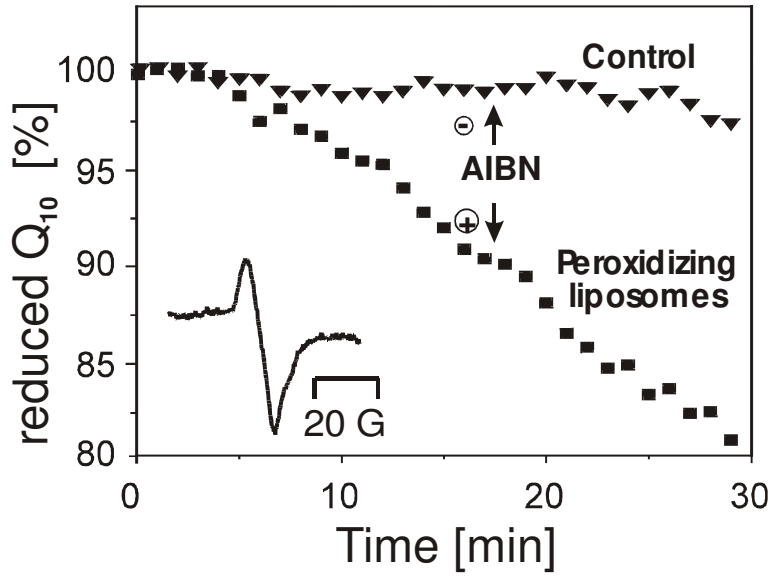

Fig 3 Reoxidation of ubiquinol in liposomes during LPO initiated by UV irradiation of AIBN in presence of air. Control samples were irradiated in absence of AIBN. Liposomes contained a DOPC/$UQ_{10}H_2$ mixture (molar ratio 1:40) and $UQ_{10}H_2$ was quantified photometrically from the absorption difference between 280 and 289 nm (ε = 8800 l/mol*cm). ESR signal of ubisemiquinones ($UQ^{\bullet -}$: g = 2.005, ΔH_{pp} = 8.9 G) in liposomes appearing during LPO due to the antioxidant activity of $UQ_{10}H_2$. The liposomes containing $UQ_{10}H_2$ were UV irradiated in presence of a radical initiator at 77 K and ESR spectra of samples were measured at 200 K.

quinones which were transferred to semiquinones by XOD in the absence of oxygen.

The addition of H_2O_2 resulted in the direct or indirect formation of ·OH derived radicals plus $^-$OH. With the exception of Q_9 all quinones generated ·OH radicals. This was the first demonstration of an organic Fenton reaction (Nohl & Jordan, 1987; Nohl & Jordan, 1981; Nohl *et al.*, 1982; Nohl & Jordan, 1984).

Adriamycin (AQ)-dependent cardiotoxicity was another field of interest (Nohl *et al.*, 1982; Nohl, 1983; Nohl & Jordan, 1984; Nohl, 1988a, b). We described an external NADH-DH responsible for cardiotoxicity (Nohl & Jordan, 1984; Nohl, 1987). In contrast to microsomes which generate $O_2^{\cdot -}$

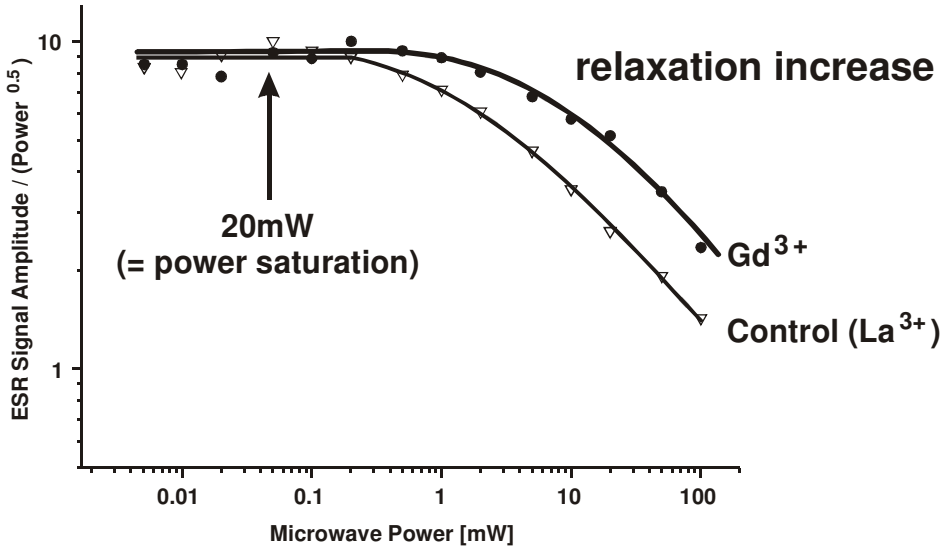

Fig 4 Saturation behavior of the $SQ^{\bullet}$ ESR signals generated during LPO in liposomes from an interaction of lipid radicals with incorporated $Q_{10}H_2$. In the presence of Gd^{3+} the relaxation of $SQ^{\bullet}$ was enhanced resulting in a higher ESR signal intensity as compared with control samples which contained a diamagnetic La^{3+} complex under saturation conditions (n = 3, error bars correspond to s.d.).

radicals in the presence of AQ heart mitochondria transformed AQ to a stable $AQ^{\cdot}$. The semiquinone disappeared in microsomes suggesting autoxidation, while $AQ^{\cdot}$ formation in RHM gave rise to the formation of $^{\cdot}OH$ radicals.

We also investigated the mechanism of OH radical generation (Nohl & Jordan, 1983). We found that AQ after being reduced to $AQ^{\cdot}$ undergoes autoxidation generating $O_2^{\cdot-}$ derived H_2O_2, followed by an intramolelcular derangement which results in the cleavage of the glycolytic bond. The aglycone accumulates in the inner membrane where it takes part in e^- transfer. The $AQ^{\cdot}$ aglycone provides an e^- for reductive homolytic cleavage of H_2O_2 giving rise to the formation of $^{\cdot}OH$ radicals.

We recently described a mitochondrial NO_2^- reductase (Kozlov *et al.*, 1999). NO_2^- is the first oxidation product of NO. NO formation from NO_2^- was followed by 5 coordinated ferrous NO-complex with deoxy-hemoglobine. This complex is EPR detectable by its typical triplet. A prerequisite of NO recycling is the activity of the respiratory chain.

The site where $NO_2^{\cdot-}$ is reduced was determined by the use of selective inhibitors of the respiratory chain, which upregulate the reduction state upstream and downregulate it downstream. Myxothiazole totally suppressed NO recycling indicating the involvement of $UO^{\bullet}_{out}$.

UQH_2 is generally considered as an antioxidant. We will show that in some biomembranes it can become a prooxidant (Nohl, Gille, Schönheit & Liu, 1996; Kagan, Quinn & Nohl, 1996; Nohl, Gille & Kozlov, 1998; Nohl, Gille & Kozlov, 1999; Nohl, Gille, Kozlov & Staniek, 2000). This is prevented in biomembranes like mitochondria where the reaction product ubisemiquinone ($UQ^{\cdot-}$) emerging from LPO is stabilised or recycled. Although the rate constant for UQH_2 is one order of magnitude lower than for α-tocopherol UQH_2 is three times higher concentrated thus chromanoxyl radical formation and $UQ^{\cdot-}$ are more or less equal. In homogeneous solutions α-tocopherol is as effective as UQH_2 in scavenging peroxyl radicals (Fig 2).

In peroxidizing liposomes UQH_2 undergoes two one e^- oxidation steps (Fig 3). The intermediate was a $UQ^{\cdot-}$ which is supposed due to its negative charge to accumulate in the polar head group phase.

We assume that the non-protonated ubisemiquinone interacts with the paramagnetic Gd^{3+} salt which is dissolved in the aqueous phase (Nohl *et al.*, 1999).

Spin-spin interaction under power saturation resulted in a relaxation increase which reveals that $UQ^{\cdot-}$ is close to the aqueous phase where it is not stabilized therefore undergoes autoxidation (Fig 4).

This was documented when measuring $O_2^{\cdot-}$ derived H_2O_2 formation from peroxidizing liposomes where UQH_2 was inserted. Also control liposomes exhibited H_2O_2 formation probably derived from traces of peroxidized lipids due to sonication.

$UQ^{\cdot-}$ derived from xanthin oxidase (XOD) in the absence of O_2 generated $^{\cdot}OH$-radicals trapped by DMPO. Organic peroxides like cumol hydroperoxide (CuOOH) and linoleic-peroxide form degrada-

tion products of the alkoxy radical $(RO)^{\cdot}$, the stable alkyl- and acyl radical DMPO-adduct (carbon centred radicals). Hyperfine splitting data helped us to identify these carbon centred split products of $RO^{\cdot}$.

We conclude based on our experiments that prooxidants are formed during lipid-peroxidation. Peroxyl radicals as well as chromanoxyl radicals are reduced to the non-radical form at the expense of ubiquinole. The resulting ubisemiquinone gives rise to various prooxidants. First of all it oxidizes the antioxidant form of ubiquinol to ubiquinone. Autoxidation leads to $O_2^{\cdot-}$ radicals which decompose to H_2O_2 undergoing homolytic cleavage resulting catalyzed by ubisemiquinone resulting in the formation of $^{\cdot}OH$ radicals.

CONCLUSION

EPR technique allows to detect radicals, to determine fluidity changes of biomembranes and phase transition shifts. This technique is superior to spectrophotometric methods earlier used.

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