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Abstract

Electron paramagnetic resonance (EPR) spectroscopy is a powerful and widely used technique for studying structure and dynamics of biomolecules under bio-orthogonal conditions. In-cell EPR is an emerging area in this field; however, it is hampered by the reducing environment present in cells, which reduces most nitroxide spin labels to their corresponding diamagnetic N-hydroxyl derivatives. To determine which radicals are best suited for in-cell EPR studies, we systematically studied the effects of substitution on radical stability using five different classes of radicals, specifically piperidine-, imidazolidine-, pyrrolidine-, and isoindoline-based nitroxides as well as the Finland trityl radical. Thermodynamic parameters of nitroxide reduction were determined by cyclic voltammetry and the rate of reduction in the presence of ascorbate, cellular extracts and after injection into oocytes was measured by continuous-wave (CW) EPR spectroscopy. Our study revealed that tetraethyl-substituted nitroxides are good candidates for in-cell EPR studies, in particular pyrrolidine derivatives, which are slightly more stable than the trityl radical.

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Sterically shielded spin labels for in-cell EPR spectroscopy: Analysis of stability in reducing environment

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Short title: Reductive stability of radicals

Abstract

Electron paramagnetic resonance (EPR) spectroscopy is a powerful and widely used technique for studying structure and dynamics of biomolecules under bio-orthogonal conditions. In-cell EPR is an emerging area in this field; however, it is hampered by the reducing environment present in cells, which reduces most nitroxide spin labels to their corresponding diamagnetic *N*-hydroxyl derivatives. To determine which radicals are best suited for in-cell EPR studies, we systematically studied the effects of substitution on radical stability using five different classes of radicals, specifically piperidine-, imidazolidine-, pyrrolidine-, and isoindoline-based nitroxide as well as the Finland trityl radical. Thermodynamic parameters of nitroxide reduction were determined by cyclic voltammetry and the rate of reduction in the presence of ascorbate, cellular extracts and after injection into oocytes was measured by continuous-wave (CW) EPR spectroscopy. Our study revealed that tetraethyl-substituted nitroxides are good candidates for in-cell EPR studies, in particular pyrrolidine derivatives, which are slightly more stable than the trityl radical.

Keywords: nitroxide reduction, aminoxyl radical, radical stability, spin labeling, trityl radical

1. Introduction

RNA molecules have a central role in cellular processes and gene regulation. Their three-dimensional structures and conformational dynamics are essential for their functions as biological catalysts, structural scaffolds and regulators of gene expression [1]. Thus, information about structure and motion can give insights into RNA function and how it might be modulated. Besides X-ray crystallography [2], NMR [3] and fluorescence spectroscopy [4], pulsed electron-electron double resonance (PELDOR or DEER) spectroscopy [5] has over the past few years demonstrated its applicability to map the global structure of nucleic acids [6,7] and other macromolecules [8,9] through distance measurements in the range of 1.5-8 nm, utilizing distance-dependent magnetic dipole-dipole interaction between two covalently attached aminoxyl (nitroxide) radicals. EPR spectroscopy also holds promise for in-cell measurements because there is no background interference, as can be the case with fluorescence spectroscopy. In fact, PELDOR was recently used to study structural aspects of nucleic acids [10,11] and proteins [12] inside intact *Xenopus laevis* oocytes. However, the short lifetimes of nitroxide spin labels under cellular conditions is a severe limitation to the general applicability of PELDOR for in-cell measurements [13].

In addition to the use as labels for EPR spectroscopy, cyclic nitroxide radicals are an important class of compounds for biological and medical applications [14-16], such as contrast agents for magnetic resonance imaging (MRI) [17], as antioxidants and as superoxide dismutase mimics [18], where the nitroxide is involved in redox reactions by a one-electron exchange

between its reduced and oxidized state [19]. Additionally, nitroxide radicals have been used to determine the partial pressure of oxygen [20] and pH [21] values in living tissues via EPR spectroscopy. The aforementioned applications of nitroxides in biological fluids are adversely affected when the paramagnetic center is readily reduced to the EPR-silent hydroxylamine [22]. Since the application of nitroxides inside living cells is of growing interest, extensive efforts have been taken in the design and synthesis of nitroxide radicals that are more resistant towards reduction [23,24]. Several factors affect the stability of nitroxides, such as ring size (piperidine, pyrrolidine, isoindoline), the presence of heteroatoms within the ring (imidazolidine), substituents (neutral or charged) and the identity of the alkyl substituents in the positions adjacent to the nitroxide functional group. Although the stability of a variety of nitroxides in the presence of ascorbate has been reported [22,25-36], the biostability of radicals has invariably been investigated under different conditions [26-30,37-41]. Furthermore, each of these biostability studies has focused on a small number of radicals. The study of Kinoshita et al. is similar to the work described here in terms of the techniques and conditions used for evaluation of radical stability. They also compared tetramethyl and tetraethyl piperidine nitroxides; however, our work additionally includes imidazolidine and isoindoline nitroxides as well as a triphenylmethyl (trityl) radical [37].

In this paper, we compare redox properties of a series of nitroxides varying with ring type, substituents and charge, using several approaches with the aim to identify radicals that are suitable for in-cell studies. A trityl radical, which has been shown to be relatively stable under reductive conditions [42],

was chosen for comparison. We also determined the hyperfine coupling constants (A_{iso}) of all the nitroxides, that are sensitive to the polarity around the nitroxide moiety, by continuous wave (CW) EPR. With regards to the nitroxides, we focused on tetraethyl-substituted radicals with pyrrolidine-, piperidine-, isoindoline- and imidazolidine-based structures and compared them with the more reactive tetramethyl-substituted analogues. The effect of electronegative and charged substituents on the nitroxide rings was also evaluated. In addition to determination of the kinetic and thermodynamic stability of the radicals in the presence of ascorbic acid, their stability was tested in a cytosolic extract from *Xenopus leavis* oocytes and inside living oocyte cells.

2. Material and methods

2.1. Preparation of radicals

Chemicals were purchased primarily from Sigma-Aldrich Chemical Company and Acros- Belgium, and were used without further purification. Thin layer chromatography (TLC) was performed on glass backed TLC plates with extra hard layer (Kieselgel 60 F_{254} , 250 µm, Silicycle) and compounds were visualized by UV light at 254 nm. Silica gel (230-400 mesh, 60 Å) was purchased from Silicycle, and used for flash chromatography. Molecular mass of organic compounds were determined by HR-ESI-MS (Bruker, MicroTof-Q).

Tetramethyl-derived spin labels 1, 2, 3 and pyrrolidine spin label 12 were purchased, while tetraethyl piperidine derivative 4 was prepared according to the reported procedure [37] and used for the synthesis of compounds 5 and 6

[37,43]. Compounds **11** and **13-15** were synthesized as previously described [44-47].

Compounds **8** and **10** were prepared using a modification of previously reported method [48]. In the reported procedure for the synthesis of **8**, compound **16a** was oxidized and then nitrated to obtain **18a**. However, nitration gave multiple spots in our hands and, therefore, compound **16a** was first nitrated to obtain compound **17a** (Scheme 1) [49]. Subsequent oxidation with m-CPBA yielded compound **18a** in moderate yields, which was converted to **8** using the previously published protocol [48]. This modified procedure was also used for the synthesis of the corresponding tetraethyl derivative **10**. Preparation of **8** using this strategy has recently been reported by Mileo *et al.* [50].

1,1,3,3-Tetramethyl-5-nitroisoindoline 18a

To a solution of compound **17a** (0.9 g, 4 mmol) in CH₂Cl₂ (5 mL) at 0 °C was added a solution of *m*-CPBA (1.4 g, 8.17 mmol) in CH₂Cl₂ (6 mL). The resulting solution was stirred at 0 °C for 1 h and then at 24 °C for 2h. The reaction mixture was diluted with CH₂Cl₂ (10 mL), the organic layer washed successively with an aqueous NaOH solution (2.5 N, 2 x 10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and then concentrated *in vacuo*. The crude reaction mixture was purified by column chromatography (5% MeOH in CH₂Cl₂) to yield **18a** as a yellow solid (0.9 g, 94% yield). HR-ESI-MS: 258.0975 (M+Na), calcd. 235.0953 for C₁₂H₁₅N₂O₃.

Tetraethyl-5-nitroisoindoline 18b

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To a solution of compound **17b** (0.120 g, 0.43 mmol) in CH₂Cl₂ (2 mL) at 0 °C was added a solution of *m*-CPBA (0.089 g, 0.5 mmol) in CH₂Cl₂ (1 mL). The resulting solution was stirred at 0 °C for 1 h and then at 24 °C for 3h. The reaction mixture was diluted with CH₂Cl₂ (5 mL), the organic layer washed successively with an aqueous NaOH solution (2.5 N, 2 x 10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and then concentrated *in vacuo*. The crude reaction mixture was purified by column chromatography (30 % EtOAc in petroleum ether) to yield **18b** as a yellow solid (0.1 g, 79% yield). HR-ESI-MS: 314.1602 (M+Na), calcd.

291.1601 for C₁₆H₂₃N₂O₃

2.2. Cyclic voltammetry

Cyclic voltammetry experiments were performed in a situ 4 mM phosphate buffer (PBS) solution (pH 7.2) containing 1 mM radical concentration. A three-electrode cell arrangement was used encompassing glassy carbon working electrode, which was polished before each measurements, a reference electrode Ag/AgCl/KCl(3M), and a double-wire platinum counterelectrode. The following parameters were used: 0.1 V/s scan rate, 0.0 V starting potential, 1.0 V upper vertex potential, -1.5 lower vertex potential, -0.00244 V cathodic step potential, 0.00244 V anodic step potential. In case of **6**, the starting potential was -0.2 V, because of similar anodic and cathodic peak potentials.

2.3. Monitoring reduction of radicals in ascorbic acid and in cell extract using CW-EPR

Preparation of the crude cytoplasmic extracts from *Xenopus laevis* oocytes was as previously described [10]. 19 μ L of 5 mM ascorbic acid solution in 4

mM PBS (pH = 7.4), or 19 μ L of cell extract were mixed with 1 μ L of a 4 mM radical solution (final radical concentration 200 μ M). The solution was transferred into an EPR tube (1 mm inner diameter, Wilmad, USA) and the EPR signal intensity was measured as a function of time using a Bruker E500 CW-EPR spectrometer at X-band (9.43 GHz), using the following settings: 9.43 GHz modulation frequency, 40.96 ms time constant, 1.0 mW microwave power, 40.96 ms conversion time, 1.0 G modulation amplitude, 1024 number of points, 70 G field sweep and 160-170 sweeps.

2.4. Monitoring reduction of radicals inside cells by CW-EPR

Oocytes from *Xenopus laevis* trapped in stage VI, characterized with ca.1 mm diameter and ca.1 μ L volume were used [10]. Samples were prepared by microinjection of ca. 40 nL of 4 mM spin label stock solution into 50 oocytes and subsequent incubation at room temperature for a variable time (0, 15, 30, 60, 120 min) before freezing the cells in liquid nitrogen, until the radical concentration was determined by CW-EPR: microwave power 0.2 mW, modulation amplitude 2.0 G, number of scans 10, temperature 125 K.

3. Results and discussion

3.1. Selection of radicals

Five classes of spin labels were investigated, each of which was based on a specific cyclic nitroxide, i.e. piperidine- (Figure 1A), isoindoline- (Figure 1B), imidazolidine- (Figure 1C), pyrrolidine-derivatives (Figure 1C) or a trityl radical (Figure 1C). The piperidine series served to investigate the effects of the substituents on the ring, all of which were electron withdrawing, except for the alkyl groups flanking the nitroxide. The isoindolines (Figure 1B) had ionizable groups to facilitate solubility in

aqueous solutions, a sulfonate in compounds **7** [51,52] and **9** [51,52], and a tetraalkylammonium ion in compounds **8** and **10**. Each type of nitroxide was prepared as tetramethyl- and tetraethyl-derivatives. The trityl radical **15** was the only carbon-based radical in this study.

3.2. Thermodynamic parameters for reduction in the presence of ascorbic acid

Ascorbic acid, a reducing agent present to some extent in biological systems [53-55], is commonly used for evaluation of radical stability [29] and was used to investigate the thermodynamic parameters of the radicals. To determine the Gibbs free energy (ΔG) and the reaction equilibrium constant (*K*) for the reduction, the redox potential of each radical was measured by cyclic voltammetry. The half-wave potentials correspond to standard redox potentials as long as the diffusion coefficients of the reduced and oxidized state are equal, which is generally the case.

A typical voltammogram of a nitroxide, recorded by sweeping the potential of a glassy carbon electrode between -1.5 and 1 V (vs Ag/AgCl/KCl) in both the anodic and the cathodic direction, respectively, contains two peak couples (Figure S4A). The first peak couple appears at the anodic potential of the voltammogram and originates from the reversible oxidation of the nitroxide to an oxoammonium cation (Figure S4B). The peak separation is close to the theoretical value of 59 mV for a one electron transfer. This reaction is not important in a biological context, where nitroxide radicals are reduced. The second peak-couple represents the reduction of the nitroxide (Figure S4C) and the corresponding oxidation of hydroxylamine. In this case, the electron transfer to the nitroxide is coupled to a chemical reaction,

Free Radic Res Downloaded from informahealthcare.com by Dicle Univ. on 11/04/14 For personal use only. making the peak separation larger. Since the redox behavior of trityl radicals differs significantly from nitroxides, **15** was not studied by cyclic voltammetry.

The half-wave potentials $(E_{1/2})$ were determined from the cyclic voltammograms as the midpoint between the oxidation and the reduction peak potentials for the second peak-couple: $E_{1/2} = (E_p^{\text{ox}} + E_p^{\text{red}})/2$ (Table 1). The tetramethyl-substituted piperidines had the highest $E_{1/2}$ value (*ca.* -0.07 V), while the tetraethyl-substituted isoindoline- and pyrrolidine-derived nitroxides displayed the lowest redox potential (-0.32 V), making them the most resistant to reduction. The difference between the standard redox potential of ascorbic acid ($E_0 = -0.15$ V vs Ag/AgCl/KCl) and the measured $E_{1/2}$ of each nitroxide, were used to determine ΔG and K for the reduction of the nitroxide with ascorbate [56] (Table 1). The ΔG values for the reduction were negative for the tetramethyl-derived piperidines, thus making the nitroxide reduction energetically favorable. In contrast, the tetraethylsubstituted nitroxides yielded ΔG values of about 6-16 kJ/mol and small equilibrium constants, showing that their equilibrium lies towards the educts.

Using the equilibrium constants determined from the cyclic voltammetry measurements and known starting concentrations of educts, the equilibrium concentrations of the nitroxide radicals were calculated, normalized to the starting concentration ($[N]_{eq}/[N]_0$) and expressed in Table 1 as percentages. The equilibrium concentrations of the nitroxides in reaction with ascorbic acid were also determined experimentally by following the intensity of the low-field nitroxide line of the EPR spectra as a function of time (Figure 2A). The decaying EPR signal was fitted with the pseudo-first order reaction

kinetic implicitly including the equilibrium signal as a fit parameter (see 3.3). This signal originates from radicals present at equilibrium and was compared with the equilibrium concentration of nitroxides that were calculated from the redox potentials. Comparison of the last two columns in Table 1 shows an excellent agreement between the equilibrium concentrations of the nitroxide radicals as measured by EPR spectroscopy and those calculated from the thermodynamic parameters. Thus, knowledge of the redox properties of radicals can be used to predict with high precision the equilibrium concentration for their reduction with ascorbate anion.

3.3. Kinetics of the reduction of radicals in ascorbic acid solution

The large excess of ascorbate that was used for reduction of radicals ensured (pseudo) first-order kinetics and allowed fitting of the decay curve with a first order exponential function: $[N] = [N]_{eq} \div [N]_{0} \cdot e^{-k' \cdot q}$, where [N] is the concentration of the radical at time t, $[N]_{0}$ is the initial concentration and k' is the pseudo first order rate constant. $[N]_{eq}$ is a constant offset given by the thermodynamic equilibrium of nitroxide radicals under steady state conditions. Dividing k' by the concentration of ascorbic acid yielded the bimolecular rate constants (Table 2).

The calculated bimolecular rate constants confirmed that the rate of reduction depends on several factors: the size of the nitroxide ring system, the nature of the substituents and shielding of the nitroxide moiety (Figure 2A, Figure S1). Replacing the methyl groups adjacent to the nitroxides with ethyl groups had the largest impact on radical stability, presumably by sterically restricting access of reductants to the nitroxide [22,25]. The hyperfine coupling constants (A_{iso}) of the radicals were determined from their CW-EPR spectra

(Table S1) and confirmed that the ethyl-derivatized nitroxides had a lower A_{iso} value than the corresponding methyl derivatives, as expected for a more hydrophobic environment. The steric effect was most pronounced in the case of the piperidine derivatives, where the tetraethyl-substituted nitroxides **4**, **5** and **6** were found to be ca. 100 times more stable against reduction than the tetramethyl-derivatives **1**, **2** and **3**. This data is in agreement with the recently published study of Kajer *et al* [57]. It has been shown that cyclohexyl or pyran groups do not further increase radical stability [22,58], making tetraethyl-derived nitroxide radicals good candidates for *in vivo* EPR.

Other substituents also affected stability of the radicals, although to a lesser extent than the alkyl groups adjacent to the nitroxides. Positively charged substituents decreased the stability of the radicals, presumably by attracting the ascorbate anion. For example, **3**, where the amino group is protonated at pH 7, is three-fold more reactive than **2**. The imidazolidine **13** was considerably less stable than the other tetraethyl derivatives, presumably due to protonation [44]. For the isoindoline derivatives, the effect of charge was not as pronounced, presumably because the charge is farther from the nitroxide functional group; the positively charged **8** is less than two-fold more reactive than the negatively charged **7**, while no difference was observed for the tetraethyl-substituted compounds **9** and **10**.

The largest effect on the nitroxide stability of the tetramethyl-substituted nitroxides was caused by the structure of the nitroxide-bearing ring. The five-membered pyrrolidine, imidazolidine and isoindoline-derivatives were three-to ten-fold more stable towards reduction than the six-membered piperidine derivatives [32]. Similar rates of reduction for 7 and 12 indicate that the

pyrrolidine- and isoindoline-rings have similar stability. The slowest reduction rate was found to be that for the negatively charged tetraethyl pyrrolidine derivative **14**.

3.4. Radical stability in cell extract

The rate of radical reduction was also investigated in cytoplasmic extracts of oocytes and as in the case of ascorbate reduction, all tetramethyl substituted radicals were rapidly reduced in the cellular extract (Figure 2B, Figure S2). The EPR time traces (Figure 2B) did not follow a single exponential decay, presumably due to the presence of various reducing agents (ascorbate, glutathione, cysteine, NADH, NADPH) and a significant amount of dissolved oxygen in the cell extract. Therefore, signal intensities after 2 h of reaction time were used for comparing the stability of the radicals in the cytosolic fluid. As seen from Figure 2B and Table 2, the most resistant radical is the pyrrolidine-derivative 14, which was 95% intact after 2 h, followed by the tetraethyl-piperidines 4 and 5 (75%), tetraethylimidazolidine and tetraethyl-piperidine 6 (65%), trityl (60%) and tetraethylisoindoline (50%). The different relative stability of isoindoline vs. piperidine nitroxides in the cell extract, compared to ascorbate reduction, is presumably due the presence of different reducing agents in the cell. In addition, different rates of re-oxidation of the corresponding hydroxylamines to nitroxides by oxidants in the cell extract, including molecular oxygen, could also be a contributing factor [59].

3.5. Radical stability inside cells

As stated above, the primary aim of this study was to compare, under identical experimental conditions, the stability of potential spin labels for

investigation of biomolecules in living cells by pulsed EPR spectroscopy. Therefore, the ultimate test was their persistence in oocyte cells. Charged radicals **6**, **9**, **10**, **14** and **15** gave reduction profiles that are very similar to those recorded in cell extracts (Table 2), presumably because they were distributed in the cytosol which should yield similar reduction kinetics to cell extracts (see Figure S2 and S3 for decay curves for all radicals in cytosolic extract and inside cells). In contrast, the reduction kinetics for non-charged radicals **4**, **5** and **13** inside cells were much faster than in the cell extracts, *e.g.* only about 15% of **5** remained after 2 h. Radical **6**, containing the same ring size but carrying a protonated amino group, retained 65% of the signal. A possible explanation for this discrepancy is that the non-charged radicals could have entered intracellular membranes and been reduced rapidly by the electron transport chain components in the mitochondrial membrane [60]. Pyrrolidine **14** was the most persistent radical, retaining more than 85% of the signal, with the trityl radical **15** following closely behind (70%).

4. Conclusions

In conclusion, introduction of bulky ethyl groups next to a nitroxide group leads to significant stabilization against reduction by both ascorbic acid and the reductants present in living cells, yielding radicals that are more stable in cells than trityl radical **15**. The tetraethyl-substituted pyrrolidine-based nitroxide carrying a carboxylic group (**14**) demonstrated superior stability against reduction due to combination of sterical shielding, ring size and charge, all of which are factors that should be taken into account for the design of spin labels for *in vivo* studies. Charged and neutral radicals showed different relative stabilities in cell extracts than in cells, indicating that conjugation to biomolecules could have a large effect on the stability of these radicals. The trityl radical exhibited considerable stability towards reduction, especially in cells.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Free Radic Res Downloaded from informahealthcare.com by Dicle Univ. on 11/04/14 For personal use only. in blood in vitro and in vivo. Gen Physiol Biophys 2009;28(4):356-362.

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Table 1. Half-wave potentials $(E_{1/2})$ of nitroxide radicals, Gibbs free energy (ΔG) and equilibrium constants (*K*) for reduction of nitroxides with ascorbic acid, and equilibrium concentrations of the nitroxides (calculated and measured).

Dad *	$E^{\$}_{1/2}$	ΔG^{\dagger}	$\mathbf{V} = e^{-(\Delta G/RT)}$	$[N]_{\rm eq}/[N]_0$ (%)	
Kau.	[V]	[kJ/mol]	$\mathbf{X} = \mathbf{e}^{\mathbf{X}}$	calculated	measured
1	-0.08	-7 ± 2	16 ± 5	1.4 ± 0.5	1.2 ± 0.1
4	-0.27	12 ± 1	0.009 ± 0.003	59 ± 6	53 ± 3
2	-0.08	-7 ± 2	16 ± 5	1.4 ± 0.5	1.4 ± 0.1
5	-0.27	12 ± 1	0.009 ± 0.003	59 ± 6	55 ± 2
3	-0.07	-7 ± 2	16 ± 5	0.9 ± 0.5	1.5 ± 0.3
6	-0.23	8 ± 1	0.04 ± 0.02	33 ± 6	31 ± 2
7	-0.16	1 ± 1	0.7 ± 0.3	4.5 ± 2.0	5.0 ± 0.5
9	-0.32	16 ± 1	0.0012 ± 0.0005	82 ± 4	84 ± 2
8	-0.16	0.5 ± 1	0.8 ± 0.3	3.8 ± 1.2	4.8 ± 0.5
10	-0.32	16 ± 1	0.0012 ± 0.0005	82 ± 4	76 ± 2
11	-0.16	1 ± 1	0.7 ± 0.3	4.5 ± 2.0	5 ± 2
13	-0.21	6 ± 1	0.09 ± 0.04	22 ± 5	16 ± 1
12	-0.18	3 ± 1	0.3 ± 0.1	9 ± 3	11 ± 1
14	-0.32	16 ± 1	0.0012 ± 0.0005	86 ± 4	91± 7

*Radicals; [§]For the half-wave potential ($E_{1/2}$) the error

is ±0.01;

 $\Delta \mathcal{G} = -n \mathcal{F} \Delta \mathcal{G}^{\mathbf{v}} = -\mathcal{S} \mathcal{T} \ln(\mathcal{S}).$

Table 2. Half-life of the radicals and their corresponding second order reaction rate constant for reduction with ascorbic acid, EPR signal intensities after incubation (2 h) in ascorbic acid, in the cell extracts and inside oocyte cells.

		2 nd Order	Extent of reduction ^{\dagger}		
Rad. [*]	Lifetime [§] [s]	rate const. [(M·s) ⁻¹]	Asc.¶	Cell extract	Oocyte cells
1	158 ± 1	5.75 ± 0.04	1	0	-
4	2600 ± 50	0.066 ± 0.001	57	75	34
2	165 ± 2	5.51 ± 0.005	1	0	-
5	2640 ± 60	0.058 ± 0.002	58	78	15
3	54 ± 2	16.8 ± 0.7	1	0	-
6	1480 ± 40	0.115 ± 0.003	31	65	66
7	365 ± 5	0.44 ± 0.01	5	0	-
9	1980 ± 50	0.079 ± 0.003	84	45	51
8	220 ± 3	0.74 ± 0.01	5	0	-
10	2350 ± 60	0.081 ± 0.001	78	52	55
11	100 ± 2	1.62 ± 0.04	5	0	
13	1940 ± 30	0.084 ± 0.002	19	66	25
12	2160 ± 20	0.079 ± 0.001	14	38	-
14	85000 ± 30000	0.002 ± 0.001	94	94	88
15	10500 ± 1000	0.016 ± 0.002	74	60	70

*Radicals. The starting concentration of radicals was 200 mM and concentration of ascorbic acid 5 mM, except for spin label **1-3** where it was 1 mM; [§]calculated as $t_1 = 1/k'$; [†]listed as a percentage, after 2 h of reaction time; [†]ascorbic acid.

Figure legends

Figure 1. Radicals for evaluation of reductive stability. (A) Piperidine-based

nitroxides. (B) Isoindoline-based nitroxides. (C) Imidazolidine- and

pyrrolidine-based nitroxides and a trityl radical.



Figure 2. (A) Reduction of selected radicals with ascorbic acid (200 μ M conc. of radical and 5 mM ascorbic acid in PBS buffer, pH 7.2, except for compounds 1-3, when ascorbate conc. was 1 mM). The EPR signal intensity is plotted as a function of time. (B) Decay curves for the radicals in a cytosolic extract.





