REACTIVITY OF FREE RADICALS GENERATED FROM NEUROTRANSMITTERS STUDIED BY ELECTRON SPIN RESONANCE SPECTROSCOPY

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Received December 31, 2003
Accepted May 18, 2004

Free phenoxy radicals derived from catecholamine-type neurotransmitters (dopamine, noradrenaline, adrenaline) σ-coordinated to Co(III) chelates were generated by the reaction of π-coordinated *tert*-butylperoxy radicals with the neurotransmitters in non-polar solvent at ambient temperature. The ESR signals of the formed complexes are split into the basic octet line resulting from the interaction of the unpaired electron of the phenoxy radical with the ^{59}Co nucleus (I = 7/2). Increasing the polarity of the solution starts the decomplexation and the liberated phenoxy radicals of the neurotransmitters disappear by recombination or by H-abstraction from the added antioxidant. When vitamin E is added to the system, only the ESR signal of the stable α-tocopheroxy radicals is detectable. Similarly, in the presence of the antiarrhytmic drug Stobadine, only the signal of the corresponding nitrogen-centred radical is seen. In the presence of both antioxidants, rapid H-transfer occurs from vitamin E to the Stobadine radicals.

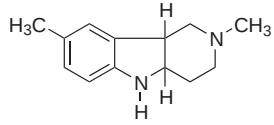
Keywords: Neurotransmitters; Free radicals; ESR spectroscopy; Dopamine; Antioxidants; Vitamin E; Stobadine.

The goal of this paper is to clarify the initial step of free radical generation from three neurotransmitters with basic catecholamine structure, viz. dopamine, noradrenaline and adrenaline, after the attack of one of the two OH groups on the aromatic ring with peroxy radicals in non-polar solvents at ambient temperature.

All three studied neurotransmitters are formed in the brain of mammals in consecutive steps from the amino acid tyrosine.

The two sterically unhindered OH groups on the aromatic ring are highly prone to H-abstraction in contact with reactive peroxy or alkoxy radicals. For the inactivation of the generated phenoxy radicals, antioxidant vitamin E (α-tocopherol) from the group of hindered phenols, operating in hy-

drophobic bioenvironment, was applied. The antioxidant activity of vitamin E was compared with that of the antiarrythmic drug Stobadine having a basic pyridoindole structure (Chart 1).



Stobadine

CHART 1

The study of the radical processes based on catecholamine at physiological temperature can contribute to better understanding of the initial step of "oxidative stress" (neurodegenerative diseases).

EXPERIMENTAL

The methods of preparing π-coordinated peroxy and σ-coordinated phenoxy radicals on diamagnetic transition metal chelates or metalloenzymes, as well as of continuously generated *tert*-butylperoxy radicals (*t*-BuOO$^{\bullet}$) have been described in previous papers[1–3] and in detail in a monograph[4].

The reaction producing peroxy radicals π-coordinated through one oxygen lone electron pair to the Co(III) chelate (L_2 = acetylacetonate) after electron transfer from [$Co^{II}(L_2)$] to the *tert*-butylhydroperoxide dimer in water-free benzene solution (2%) at 23 °C follows (Eq. (*1*)).

$$[Co^{II}(L_2)] + (ROOH)_2 \rightarrow [Co^{III}(OH)(L_2)(ROO^{\bullet})] + ROH \qquad (R = t\text{-Bu}) \qquad (1)$$

(*t*-BuOOH)$_2$ (92%) was distilled under vacuum, dried with P_2O_5 and added in ten-fold molar excess to the [Co^{II}(acetylacetonate)$_2$] solution in benzene. The resulting ESR signal of the π-coordinated *t*-BuOO$^{\bullet}$ radical is a singlet line with (typically) 2 mT shift to lower magnetic field at g = 2.0174.

The attack of neurotransmitters by the *tert*-butylperoxy radicals stabilized by the π-coordination results at ambient temperature in H-atom abstraction from one of the two aromatic OH groups of the studied catecholamine, producing sterically unhindered phenoxy radicals that remain stabilized by σ-coordination (5×10^{14} spin/0.2 ml). A part of the spin density of the unpaired electron on the phenoxy radicals is delocalized at the Co(III) centre. The interaction with the nuclear magnetic moment of ^{59}Co (I = 7/2) gives rise to the ESR octet signal at g = 1.9980.

For continuous generation of radicals from the neurotransmitters, applying the ISN (in statu nascendi) technique, the studied crystalline catecholamines were added to the [Co(acac)$_2$] solution (0.2 ml, benzene containing 0.05 ml acetone) in a cylindrical cell under an inert atmosphere, followed by addition of dry (*t*-BuOOH)$_2$ (0.05 ml) and bubbling the 10:1 benzene–acetone (v/v) solution for 5 min with argon or nitrogen before the ESR measurement at 23 °C.

High purity chemicals were obtained from Aldrich (dopamine), Galena Opava, Czech Republic (noradrenaline), Fluka (adrenaline), Slovakofarma Hlohovec, Slovak Republic (α-tocopherol), Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic (Stobadine).

A Varian E-3 spectrometer with 100 kHz modulation in the X-band region was used to record the ESR spectra. The measurements were carried out at room temperature in closed cylindrical quartz cells under a N_2 or Ar atmosphere. The ESR spectra were simulated with a Bruker ER/SRC-200 E spectral computer.

RESULTS

In general, peroxy radicals, free or coordinated, abstract readily at ambient temperature hydrogen from the OH group of dissolved phenols. Depending on the stability of the resulting phenoxy radicals, the corresponding phenolic compounds can be classified as hindered, partially hindered or unhindered. Hindered phenols having *tert*-butyl or two methyl groups in ortho positions and also a substituent in the para position, give stable free phenoxy radicals in non-polar solvents. Unhindered phenols, without bulky substituents in the ortho positions, transform to stable paramagnetic species only after coordination at the Co(III) centre. As proved also in this study, all three neurotransmitters give after the attack by the coordinated peroxy radicals ESR signals with basic hyperfine octet splitting due to the interaction with the ^{59}Co (I = 7/2) nucleus.

Figure 1 shows the ESR singlet of the initial *tert*-butylperoxy radicals on Co(III) (ca. 10^{14} spin/0.2 ml) (*1*) at g = 2.0147 and the subsequent octet signal (*2*) at g = 1.994 that appears after having added dopamine partially dissolved in DMSO. The partly resolved signal (linewidth 0.25 mT) can be simulated with a_{Co} = 1 mT and $a_{H_{(o)}}$ = 0.4 mT), see Chart 2.

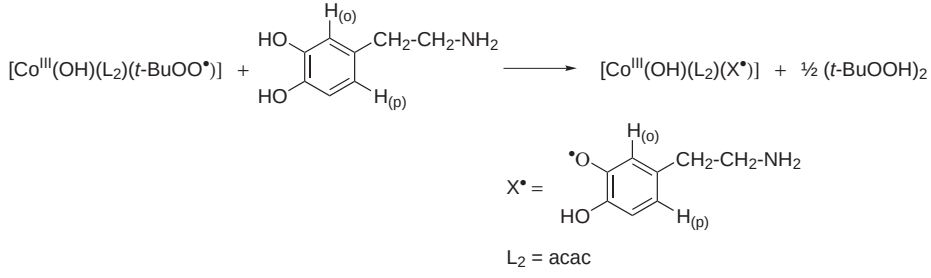


CHART 2

It has been proved for all so far studied complexes of phenoxy radicals σ-bound to Co(III) that the ^1H nucleus splitting the basis octet lines to doublets, is that in the ortho position to the oxygen carrying the unpaired electron[4].

Hydrophilic neurotransmitters are not particularly well soluble in non-polar media. Therefore, in order to generate their radicals in detectable amounts it is recommended to add some ethanol to the benzene solution. In this case the intensity of the octet signals due to Co^{III}(phenoxyl) is in the beginning of the reaction twice as high but the radical complexes gradually decompose and the free phenoxy radicals disappear by recombination. When vitamin E is present from the beginning of the peroxy radical attack in the solution of dopamine, only the ESR signal of the stable α-tocopheroxy radicals is detectable (Fig. 1, *3*).

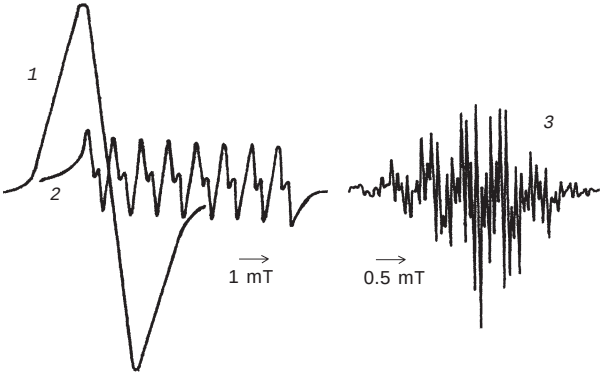


FIG. 1
ESR signal of *t*-BuOO$^{\bullet}$ radicals π-coordinated in [Co^{III}(OH)(acac)(*t*-BuOO$^{\bullet}$)] (*1*) and of σ-coordinated phenoxy radicals (*2*) generated after addition of dopamine (see Chart 2). Highly resolved ESR signal of α-Toc-O$^{\bullet}$ radicals (*3*) generated by the ISN technique in a 1:1 mixture of dopamine and vitamin E

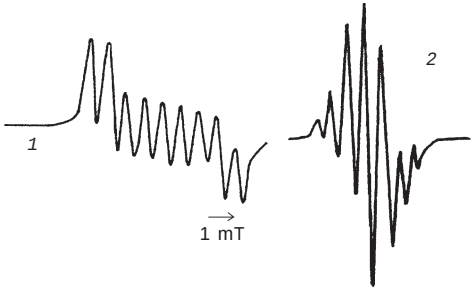
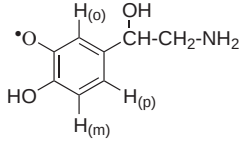


FIG. 2
ESR signal of the Co^{III}-phenoxy radical complex generated by addition of adrenaline to [Co^{III}(OH)(acac)(*t*-BuOO$^{\bullet}$)] (*1*); g = 1.9998. Septet ESR signal of α-Toc-O$^{\bullet}$ radicals generated by the ISN technique in simultaneous presence of adrenaline and vitamin E (1:1) (*2*); g = 2.0050.

This reactivity pattern also applies in the case of the other two studied neurotransmitters, noradrenaline and adrenaline (Fig. 2). After the attack of the coordinated *tert*-butylperoxy radicals, similar ESR spectra of the Co^{III}-coordinated phenoxy radicals are obtained, with basis splitting to 9 lines in both cases; however, in the presence of vitamin E, only the signal of α-tocopheroxy radicals is observed. The spin density on the Co(III) chelate is lower for the latter phenoxy radicals (a_{Co} = 0.67 mT) than in the case of dopamine (a_{Co} = 1 mT), as a result of introducing the third OH group in the catecholamine structure. The ESR spectra of the Co complexes could be simulated with the following hyperfine coupling constants: a_{Co} = 0.67 mT, $a_{H(o)}$ = 0.58 mT. The decrease of the concentration of the radical cobalt complexes with adrenaline in the presence of ethanol traces in the benzene solution during the recording time (10 min) led to a better resolved spectrum (Fig. 3). This allowed the determination of the hyperfine coupling constants $a_{H(p)}$ = 0.28 mT and $a_{H(m)}$ = 0.12 mT from the simulated spectrum (Chart 3).

$[Co^{III}(OH)(L_2)(X^{\bullet})]$

$X^{\bullet}$ =



L_2 = acac

CHART 3

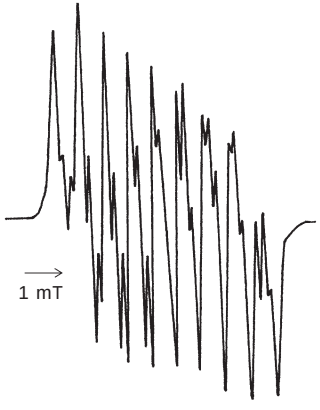


FIG. 3

Well-resolved ESR signal of the Co(III) complex with σ-bound phenoxy radicals of adrenaline (see Chart 3 and Fig. 2) at g = 1.994 recorded 10 min after adding a small amount of ethanol

In general, the free phenoxy radicals of the neurotransmitters liberated from their Co-complexes by increasing the polarity of the solution with ethanol, disappear instantaneously by recombination. On the other hand, if the decomplexation proceeds in the presence of α-tocopherol, the free phenoxy radicals of catecholamine immediately abstract the H-atom from the hindered OH group of vitamin E, thereby restoring the original structure of the neurotransmitters. If the primary radical attack is realized from the beginning in the presence of α-tocopherol, the generated free phenoxy radicals of the neurotransmitters are deactivated simultaneously with the highly reactive *t*-BuOO$^\bullet$ radicals. An important condition is the initial concentration ratio of the reactants.

Studies focused on the antioxidant efficiency of Stobadine, a pyridoindole antiarrhytmic drug[5], and on its beneficial effect on brain ischemia followed by reperfusion[6]. In this process the role of free oxygen-containing radicals causing brain lipide peroxidation is suppressed[7]. The pharmacodynamic properties of Stobadine have been summarized[8] and discussed from the point of view of its antioxidant effect[9].

The ability of Stobadine to form NO$^\bullet$ radicals on the indole nitrogen by the action of γ-radiation in the presence of oxygen was established for the first time from ESR spectra[10]. A well-resolved ESR signal was detected on oxidation of Stobadine with PbO_2 and (*t*-BuOOH)$_2$ by Staško et al.[11] As no hyperfine splitting from the proton in the β position to the nitroxy group could be detected, it was proposed that the intermediate radical converted by oxidation to nitrone and finally, after spin trapping of *t*-BuOO$^\bullet$ or *t*-BuO$^\bullet$, to a radical adduct (Chart 4).

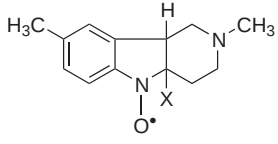


CHART 4

By testing the reactivity of Stobadine toward the coordinated *t*-BuOO$^\bullet$ radical (in benzene solution at laboratory temperature under an inert atmosphere), we have obtained a triplet ESR signal (a_N = 0.9 mT), with each line further split to another triplet by interaction with the two dominant protons on the indole moiety (a_H = 0.3 mT) (Fig. 4). When vitamin E (α-tocopherol) is added to this system, the signal of the Stobadine radical disappears and the typical septet signal of the more stable α-tocopheroxy radicals could be observed.

When the *t*-butylperoxy radicals attack the neurotransmitters (e.g., adrenaline) in the presence of Stobadine, the formation of the σ-coordinated phenoxy radicals of the neurotransmitters is prevented and only the ESR signal of the Stobadine radical appears. When, in addition, vitamin E is gradually added, the transformation of the Stobadine radicals to α-tocopheroxy radicals by H-abstraction proceeds practically immediately at ambient temperature. In the presence of both antioxidants, vitamin E and Stobadine, from the beginning of the peroxy radical formation, only the ESR signal of the most stable radical, α-tocopheroxyl, is detectable.

Only ESR results can explain the observation[12] that 5 M Stobadine had no effect on the vitamin E-deficient microsomes, and that its antioxidant efficiency is in a relation to the α-tocopherol presence. It must be stressed that commercial "E-vitamine" (α-tocopherol acetate) with the blocked reactive OH group cannot deactivate free radicals and, hence, the intensity of the singlet ESR signal (g = 2.0174) of the primarily formed π-coordinated peroxy radicals remains unchanged for many hours at ambient temperature. In bioenvironment, the enzymatic deesterification of α-tocopherol acetate is an important condition for exploring its antioxidant activity. Many papers have been published recently, proving that the precondition for partial conversion of α-tocopherol acetate to vitamin E is its hydrolysis with esterases[13,14].

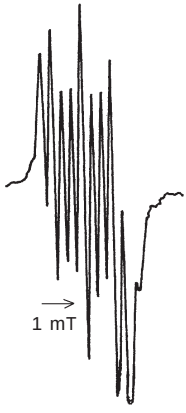


FIG. 4
ESR signal of free Stobadine radicals (see Chart 4) at g = 2.0060 generated by the addition of Stobadine (see Chart 1) to the solution of [$Co^{III}(OH)(acac)_2$(*t*-BuOO$^{\bullet}$)]

This paper deals with non-charged, highly reactive phenoxy radicals generated from catecholamines; negatively charged semiquinone anion radicals derived from catecholamine enzymatically oxidized in acetic acid were described by Kalyanaraman et al.[15]

DISCUSSION

One-electron transfer to paramagnetic O_2 triggers, under physiological conditions, the formation of free radicals with different reactivities, from the superoxide anion $O_2^{\bullet -}$ of low activity up to the highly reactive $HO^{\bullet}$ radical with the shortest mean lifetime and smallest operation space[1]. Such a redox step in living organisms proceeds either under enzymatic control in separated compartments without disturbing of the reductive bioenvironment, or the radicals are generated randomly in the presence of potential electron donors: transition metals with unpaired d-electrons[1], semiquinones in polar environment[16] or electrons from the respiration chain of mitochondria, when oxygen is not completely reduced to water in consequence of blocking the active center – COX.

It is assumed that the uncontrolled random radical reaction plays a fundamental role in some neurodegenerative diseases. So, elimination of dopamine production by dopamine-producing neurons[17] in the *substantia nigra* part of the brain, after the primary nitric oxide and superoxide radical attack of the overactive cells, leads to damage of many parts of the cell (membranes and critical proteins) and, when it cannot be any longer repaired, to its functional elimination and death[18]. In the case of the Parkinson disease, the lost of motion-controlling signals is chemically based on catecholamine structures[19].

Catalyzed oxidation of noradrenaline and adrenaline involving metal ions, has also been proposed to be the source of oxygen radicals in ischemia reperfusion injury[20]. Chronical increase of the catecholamine level in plasma and of the oxidation products has been proved in patients after heart attack[21] and other oxidation stress situations (e.g., stroke)[22].

It was shown that after a long oxidation, depending on increase in the oxygen pressure, which results in a higher rate of the $O_2^{\bullet -}$ and H_2O_2 formation in mitochondria, the cells are depleted of reduced glutathione (endogenous radical scavenging through SH groups) and enriched with inactive protein disulfide (–S–S–) groups resulting from recombination of $-S^{\bullet}$ radicals. This indirect experimental technique allowed to monitor the peroxidation of phospholipides, i.e. the radical attack to unsaturated fatty ac-

ids[23], and to measure simultaneously the antioxidant status and, so, the role of free radicals in the oxidation stress[20].

The gradual elimination of endogenous free radical deactivators during the thermal or oxidative stress (superoxide dismutase, catalase, Se-GSH, glutathione peroxidase) and of the exogenous deposit of natural antioxidants (vitamins E, C, A) leads to uncontrolled radical reactions which are often dominant in the induction period of different pathogenic processes: peroxidation of lipids[23] and membrane deterioration, carcinogenesis[24,25], aterosklerosis, ischemia reperfusion injury, myocard damage, inflammation – including accumulation of irreversible damages, aging[26]. Oxidative stress has been noted to be responsible for various autoimmune diseases such as muscle pathologies and degenerative arterial deseases.

It can be concluded that vitamin E and vitamin C (in aqueous media) are at the end of the antioxidant H-transfer cascade, stepwise deactivating highly reactive radicals ($HO^{\bullet}$, $HOO^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$) in hydrophobic bioenvironment (membranes, lipids, proteins, polyunsaturated fatty acids)[1,15,23–25]. Besides the local concentrations of different antioxidants and the oxygen status (e.g., pressure), also the acidity (pH) plays an important role. In particular, the superoxide anion radical $O_2^{\bullet -}$ formed in mitochondrial membranes in the presence of H^+ ions, produces $HOO^{\bullet}$ radicals ($O_2^{\bullet -} + H^+ \rightarrow HOO^{\bullet}$) that are more reactive than the peroxy radicals tested in this study. Importantly, a dominant role in uncontrolled radical reactions in biology must be ascribed to traces of transition metal ions and their chelates, namely those with unpaired d-electrons (Mn(II), Mn(IV), Fe(III), Co(II), Ni(I), Cu(II)), starting the dangerous sequences of the oxidative stress.

From this point of view, we will discuss the reactivity of peroxy radicals π-bound to vitamin B_{12} in the following paper[27].

The authors would like to express their gratitude to Dr O. Ondrejičková from the Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, for providing us with high-purity neurotransmitters, and to Ms M. Tkáčová for her help with the preparation of the manuscript.

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