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Capacity of Ascorbyl Palmitate to Produce the Ascorbyl Radical in Vitro: an Electron Spin Resonance Investigation

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Summary

This study aims to compare the electron spin resonance (ESR) spectra emitted by human blood loaded with either ascorbyl-6-palmitate (AP), a lipid-soluble derivative of ascorbic acid (AA), or with AA. Whole blood of a healthy male individual was equilibrated with equimolar concentrations of AP and AA of 200, 400, and 800 μmol/l. The intensity of the ESR signal, expressed as the peak-to-peak amplitude, reflects the amount of unpaired spins that are created due to the reducing action of AA and is proportional, in relative terms, to the amount of the ascorbyl radical formed. We found that the blood with AP emitted an ESR signal whose singlet shape, width, and location precisely correlate with the known characteristics of the ascorbyl radical *in vitro*. The signal magnitude increased linearly with increasing concentrations of AP and was similar to that of AA. We conclude that AP is biologically active, as it generates the ascorbyl radical, an action that also underlies the scavenging process by ascorbic acid. To this end, ascorbyl-6-palmitate might have potential advantages, due to its ability to penetrate biomembranes and to act at the lipid-related molecular target sites.

Key words

Ascorbate • Ascorbyl palmitate • Ascorbyl radical • Electron spin resonance spectroscopy • ESR signal

Introduction

Ascorbyl-6-palmitate (AP) is a lipid-soluble synthetic ester of ascorbic acid. It has been used as a preservative in foods (Baardseth 1999) and as an antioxidant in cosmetics and related products (Austria *et al.* 1997). Recently, AP is also a part of multivitamin preparations and its FDA status is acknowledged as safe due to the apparent lack of known toxicity. There is increasing interest in possible beneficial effects of AP, linked mostly to its antioxidant capacity, in various areas of research. These include anti-inflammatory effects consisting of inhibition of the oxidative burst and leukotriene formation by human neutrophils (Baader *et al.* 1988) or of protection of free fatty acids against oxidation in human low-density lipoproteins (Liu *et al.* 1998). Animal studies have shown a protective effect of ingested AP against free radical-linked hepatotoxicity of an acetaminophen overdose (Jonker *et al.* 1988, Mitra *et al.* 1988).

AP permeates through biological barriers and penetrates into neural tissues (Pokorski *et al.* 2003). AP has also been found to spread in erythrocyte membranes where it may retain its antioxidant action (May *et al.*

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1996, Ross *et al.* 1999). Such physical characteristics suggest that AP may possess advantages over ascorbic acid (AA) or ascorbate in terms of physiologic activity and may have a wider biomedical role. It is uncertain whether the production of free ascorbyl radicals by AP directly compares with that of AA in a biological system. In the present study, we addressed this issue using electron spin resonance (ESR) spectroscopy and taking advantage of the ease with which each ascorbate is oxidized in biological systems thus producing the ascorbate radical. We sought to determine the magnitude and characteristics of ESR signals emitted by human blood loaded *in vitro* with equimolar concentrations of AP and AA. We found that ESR signals produced by AP had the chracteristics typical for the ascorbyl radical and were equal to those of AA.

Methods

Compounds and solutions

The ESR signal was determined in whole human blood *in vitro*. The blood was withdrawn from a healthy male subject on a normal diet, aged 29 years. It was heparinized, protected from light, and kept refrigerated throughout the study period. AA was dissolved in 0.9 % NaCl and stock solutions were made of the sequentially doubling concentrations of 200, 400, and 800 μmol/l. AP was suspended in saline in equimolar concentrations by mild sonication for 15 min (sonifier UD11, TechPan, Warsaw, Poland). Both compounds were added, each in a final volume of 0.2 ml, to blood samples of 0.5 ml. The mixtures were allowed to equilibrate at room temperature of 22±1 °C for 30 min. This equilibration time was empirically set as rendering the optimal amplitude of ESR signals obtained from AP solutions. Shorter equilibration times decreased the amplitude, whereas longer ones, up to 60 min, caused no further increase. ESR signals of AA solutions were independent of the equilibration time within 10-60 min tested. Institutional Ethics Committee approved the study protocol.

ESR measurement conditions

All samples were frozen and stored in liquid nitrogen. At the time of analysis, samples were lyophilized at a pressure of $2x10^{-2}$ mm Hg and temperature of 40 °C (lyophilizator LGA 05, VEB MLW Leipzig, Germany), ground in small porcelain evaporators and 20 mg portions were placed in quartz tubes 5 mm in diameter after 60 min exposure in the air. The tubes were placed in a resonance chamber of an ESR spectrometer whose microwave spectrum was recorded. The spectrometer type SE/X 2544 (Radiopan, Poznań, Poland) equipped with a resonance chamber RCX 660 was used. ESR signals were recorded at room temperature with the following settings: microwave power of 3 mW, time constant of 1 s, scan rate of 20 mT/2 min, modulation frequency of 100 kHz/0.2 mT, and a microwave frequency of 9.45 GHz. These signals were used to calculate the peak-to-peak amplitude (arbitrary units). Diphenylpicrylhydrasil (DPPH) was employed as the standard to determine the *g* coefficient of the spectra recorded (g = 2.005). Care was taken to ensure constant conditions of humidity (24±2 %) and temperature (22±1 °C) during sample preparation, and the reproducibility of ESR spectral measurements was confirmed.

Control procedures

Measurements of ESR signals in blank samples containing 0.5 ml of blood and 0.2 ml of the saline alone constituted a basal control for the AA and AP solutions.

A set of samples was prepared in which the plasma alone was used instead of whole blood. The plasma fraction was isolated by centrifugation at 3000 x *g* and the supernatant was collected for the ESR measurement. In such samples, the ESR signal was either absent or its magnitude was negligible.

AP is a stable compound that resists washing procedures or osmotic lysis in erythrocyte ghosts (Ross *et al.* 1999). Nevertheless, it is possible that in the *in vitro* preparations, the compound could hydrolyze, especially because the sonication increased the temperature of the solution from 22 to 28 °C. Dissociation of the ascorbate moiety from the palmitic side chain could blur the interpretation of the study on AP because one could be dealing with the effects of AA itself. We checked the influence of sonication on the nature of the AP molecule, using thin-layer-chromatography (TLC). Solutions containing a suspension of 40 mg AP/100 ml 0.9 % NaCl, corresponding to the highest concentration used in the study, and ten times higher concentrations, namely 400 mg AP/100 ml 0.9 % NaCl, were subjected to the same sonication procedure. Solutions containing 40 mg AP/100 ml methanol, in which AP dissolves momentarily, and equimolar amounts of AA dissolved in both saline and methanol were used as references. Samples were analyzed on precoated HPTLC silica gel 60 Å plates (Merck KGaA, Darmstadt, Germany), using

benzene: methanol:acetic acid (16:3:1, v/v/v) as solvents. The plates were developed with 3 % $FeCl_3$ and 5 % $K_3[Fe(CN)]_6$ and the resolved ascorbate bands were immediately photocopied. We found that the AP bands, be it saline or methanol-solubilized, migrate in the same fashion and do not overlap with those of AA (data not shown).

Data evaluation

Individual data, depicting the peak-to-peak amplitude of ESR signals in arbitrary units, were collected from 6 samples analyzed in each experimental group (control plus three different concentrations of both AA and AP). The group values are expressed as mean ±S.D. The normality of data distribution was tested using the Shapiro-Wilk's test. The distribution was found normal in all groups. On the assumption that variances within groups are homogenous, statistical comparison of the signal amplitude across the four concentration groups for AA and AP was made by one-way analysis of variance (ANOVA) with Scheffe's *post hoc* test. Comparison between the effects of AA and AP on the signal amplitude at each concentration was made by the unpaired t-test. $P<0.05$ was considered significant.

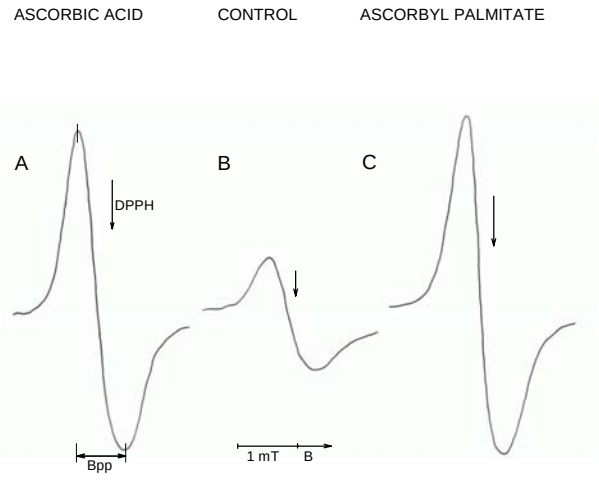

Fig. 1. ESR spectra of ascorbyl radicals obtained from blood samples equilibrated with ascorbic acid (**A**) and ascorbyl palmitate (**C**) in equimolar concentrations of 200 μmol/l. For comparison, control signal from the blood alone is shown in the middle panel (**B**). DPPH is diphenylpicrylhydrasil, used as the standard to determine the g coefficient. The letter B, at the bottom of the middle panel, denotes magnetic induction, the arrow shows the direction of its increase. Bpp is peak-to-peak magnetic induction that denotes the width of the signal (=0.8 mT). The peak-to-peak amplitude of the signal, which is proportional to the amount of unpaired spins, is similar for both compounds.

Results

An example of the original recordings of ESR signals from blood samples is shown in Figure 1. The figure compares the signals from the blood with 200 μmol/l AA added (panel A), blood plus an equimolar amount of AP (panel C), and from the blood alone (middle panel B) that contained the endogenous levels of ascorbate. The signals were asymmetrical singlets of nearly the same shape and location; Bpp, which is the peak-to-peak magnetic induction denoting the width of the signal, was 0.8 mT and g was 2.005. Such signal characteristics are in close agreement with those reported previously for the *in vitro* ascorbyl radical (Cimbolaityte *et al.* 1982, Mueller and Tannert 1986, Pokorski and Gonet 1997).

Signal intensity, estimated from the peak-to-peak amplitude, increased similarly after the admixture of both AP and AA and was, in this example, about threefold greater than that in the control blood. Since no endogenous AP is known to exist, the control blood signal must explicitly have originated from the endogenous AA. The intensity of the ESR signal depends on the number of unpaired spins that are formed during ascorbate oxidation and is thus proportional to the amount of the ascorbyl radical, which is formed in the process (Buettner and Jurkiewicz 1993). From the measured amplitude of the ascorbyl radical signal generated by the known concentration of ascorbic acid added to blood, the amount of the ascorbyl radical corresponding to the control blood signal's amplitude can be calculated. This amount, which reflects the content of ascorbic acid, the primary source of ascorbyl radical *in vivo* (Cimbolaityte *et al.* 1982, Gonet 1994) was about 63 μmol/l, which compares well with about 50 μmol/l found by a dinitrophenylhydrazine method in whole blood of healthy humans (Evans *et al.* 1982) or 50-60 μmol/l found by HPLC in plasma of male individuals in their twenties, supplemented with 100 mg AA/day (Levine *et al.* 1996). The consistent results in determining the blood ascorbate content from the intensity of *in vitro* ascorbate free radical signal highlight the accuracy of the ESR approach used.

Individual values of peak-to-peak amplitudes measured in all experimental groups, and their means are given in Table 1. Signal intensity increased linearly with increasing concentrations of AA or AP up to the 800 μmol/l tested. The increases differed significantly from the control group and from each other across the

three concentrations studied (ANOVA with Scheffe's test; $P<0.05$) in both AA and AP groups. On the average, signal amplitude increased by 219 %, 456 %, and 899 % for 200 µmol/l, 400 µmol/l, and 800 µmol/l AA, respectively. The respective increases for AP were 233 %, 442 %, and 919 %. There were inappreciable differences between the mean signal amplitudes provided by the two compounds at any given concentration studied.

Table 1. ESR signal intensity, measured as peak-to-peak amplitude in blood samples after equilibration with ascorbic acid and ascorbyl palmitate in equimolar concentrations.

Blood	Blood + 200 µmol/l		Blood + 400 µmol/l		Blood + 800 µmol/l	
Control	AA	AP	AA	AP	AA	AP
0.84	2.76	2.92	4.75	5.02	9.17	8.67
0.96	2.74	3.04	4.98	4.97	9.12	9.31
0.91	2.97	3.16	4.72	4.72	8.89	9.05
0.84	2.68	2.86	5.06	4.89	8.72	8.79
0.88	2.91	2.82	4.98	4.48	8.60	9.33
0.91	3.00	2.95	5.20	4.84	8.81	9.25
0.89±0.05	2.84±0.13	2.96±0.12	4.95±0.18	4.82±0.20	8.89±0.20	9.07±0.28

AA, ascorbic acid; AP, ascorbyl palmitate. Individual values are arbitrary units, means ± S.D. are given at the bottom of each column.

Discussion

This study demonstrates that blood equilibrated with AP emitted ESR signals that had all the characteristics of the ascorbyl radical *in vitro*. The magnitude and shape of the signal were similar to that of the ascorbyl radical provided by equimolar concentrations of AA and was concentration-dependent in a linear fashion. The similarity of ESR spectra is consistent with a similar autoxidation rate found for both compounds (Fleming *et al.* 1983). However, it would not be unreasonable to expect a different profile of ESR spectra for AP, since the palmitic chain hinders the formation of a double ring structure that develops when ascorbate is oxidized to ascorbyl radical (Fleming *et al.* 1983), which could modify the internal redox state of the compound. The lack of changes of ESR profiles, which has also been found in non-biological assays (Kim *et al.* 1993), supports the contention that the formation of this ring is not germane to ascorbate oxidation.

The increased ESR signal recorded in this study from the blood equilibrated with either AP or AA denotes the oxidation of the ascorbate moiety, which leads to formation of the ascorbyl radical. As oxidation intensifies, so does the ascorbyl radical formation. This oxidation reflects, in relative terms, the scavenging capacity of ascorbate (Buettner and Jurkiewicz 1993). Thus, ESR signal may be used as a marker, albeit indirect, of the scavenging capacity of ascorbate. Ascorbate scavenges a variety of free radicals both *in vivo* and *in vitro* (Rebec and Pierce 1994). In the blood preparation used, there were sufficient levels of endogenous metals, such as iron, to catalyze the autoxidation of ascorbate. Asorbate could also be involved in reduction and recycling of the tocopheroxyl radical back to α-tocopherol (Reiber *et al.* 1994) or react with almost every oxidizing radical that arises in a biological system, resulting in ascorbyl radical formation. This formation increases as a function of ascorbate concentration in aerated solutions. The ascorbate concentrations used were well below 10 mM, the concentration that exhibits saturation of the radical yield (Kim *et al.* 1993).

We used relatively high concentrations of ascorbate, but such concentrations are not supraphysiological, as active uptake mechanisms lead to even higher intracellular ascorbate enrichment in vital brain cells or in the eye (Reiber *et al.* 1994, Heath 1962). High ascorbate concentrations may actually enhance its antioxidant activity. Reiber *et al.* (1994) found that a high intracellular ascorbate (≈1 mM) in oligodendrocytes, combined with α-tocopherol, counteracts the prooxidant activity of low (<100 µM) extracellular ascorbate that induces lipid peroxidation *via* Fenton-like reactions.

The cell membrane is a structure most susceptible to oxidative damage. AP, on account of its lipophilicity, strongly penetrates into neural tissues (Pokorski *et al.* 2003) and is also able to spread in erythrocyte membranes. It may be supposed that although the ESR signal generated by AP is comparable to that of AA, the antioxidant protection exerted by AP in the lipid phase of the cell, the target area of signaling cascades, could be more extensive and effective. This might be of potential benefit in the pathological states that are aggravated by oxidative stress and lipid peroxidation damage to red or white blood cell lines, such as sepsis or leukemia (Bhattacharyya and Datta 2001, Ozturk *et al.* 2001). The potential for improved penetration capabilities of AP is still not sufficiently appreciated. The present work does not address this issue that remains to be explored in a further study.

We used adequate controls, including samples of whole blood with no compounds admixed, samples of plasma alone, which was devoid of erythrocytes, with the admixed compounds, and qualitative TLC screening, to verify the integrity of the AP molecule during the solubilization procedure. The TLC screening showed the selective localization of AP bands with no overlap with those of AA (data not shown). Since no signs of hydrolysis of the palmitate chain were detected, we believe that we had dealt with an intact ester molecule that exhibited a biologically relevant action. The need for the presence of erythrocytes and their contact with ascorbate, which causes membrane proteins to adsorb and stabilize the ascorbyl radical, corroborates previous findings on the generation of ESR ascorbate radical signals (Mueller and Tannert 1986, Ross *et al.* 1999). The reproducible patterns of ESR signals recorded and the predicted concentration-dependent changes in their magnitude suggest functional significance of the patterns we describe.

In summary, we believe that the ascorbate moiety of AP is biologically active because it produces ascorbyl radicals whose ESR spectra are indistinguishable from those of AA. This may help to explain the high scavenging capacity displayed by AP (Ross *et al.* 1999). Additionally, AP performance could be more effectively utilized, because in contrast to AA, AP is capable of spreading in biomembranes on account of its lipophilicity.

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Reprint requests

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