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SPIN TRAPPING OF FREE RADICALS IN HOMOGENATES OF HEART FROM SELENIUM AND VITAMIN E DEFICIENT RATS

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The formation of unstable free radical species in homogenates of heart from selenium and/or vitamin E deficient rats has been investigated by electron spin resonance spectroscopy using the spin trap α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone. There was only a small amount of free radical formation by heart from control or selenium deficient animals. However, the amounts and rates of formation of free radicals were significantly higher in animals with vitamin E deficiency and higher still in those with combined selenium and vitamin E deficiency.

KEY WORDS: Spin trapping, electron spin resonance spectroscopy, free radicals, heart, selenium, vitamin E.

INTRODUCTION

Selenium (Se) and vitamin E have important functions in the antioxidant systems of cells.[1] Se is a component of the cytosolic enzyme glutathione peroxidase (Se-GSHpx), which metabolises potentially injurious hydrogen peroxide and lipid hydroperoxides. Vitamin E, a lipid-soluble antioxidant, acts as a free radical scavenger in the cell membrane.[2] In combined Se and vitamin E deficiency in animals, pathological conditions probably arise from oxidative and free radical attack on cell components.[1,2] Since many free radicals are highly reactive and consequently exist for only very short times, evidence for their involvement in disease processes in Se and vitamin E deficiency has come indirectly from measurements of products formed by free-radical mediated degradation of polyunsaturated fatty acids.[3–5]

In the present work we have used α-(4-pyridyl-1-oxide)-n-*tert*-butylnitrone (4-POBN) as a spin trap for the direct demonstration of production of unstable free radicals in heart tissue from Se and vitamin E deficient rats.

MATERIALS AND METHODS

Animals and diets

Four groups of male Hooded Lister rats of the Rowett Institute strain were fed from weaning a semisynthetic diet based on Torula yeast,[6] with cod liver oil (15 g/kg) (BP

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grade) replacing lard (15 g/kg) in order to increase the content of unsaturated lipid. The basal diet contained (-Se-vitE) < 0.01 mg Se/kg and < 1 mg α-tocopherol/kg and was supplemented as appropriate with 0.1 mg Se/kg as Na_2SeO_3 and/or 200 mg α-tocopherol/kg as α-tocopherol acetate. After six weeks the rats were anaesthetised with ether and their hearts removed, blotted free of residual blood, and immediately frozen in liquid nitrogen. The tissue was then stored at −40°C before use in the spin trapping studies.

Experimental procedures

Se-GSHpx activity in heart homogenates and Se content of the diet were determined as described previously.[7] Vitamin E concentrations were determined in hexane extracts of homogenate and diet by the fluorimetric method of Taylor *et al.*[8] Plasma pyruvate kinase (PK) activity was determined using a test kit, (Boehringer Lewes, Sussex, U.K.) and plasma lipid hydroperoxides as thiobarbituric acid reactive substances by the method of Satoh.[9]

Hearts were homogenised in 5 ml 0.05 M potassium phosphate buffer (pH 7.4), using a blade homogeniser. Incubations (final volume 4.4 ml containing 1.0 ml hom-

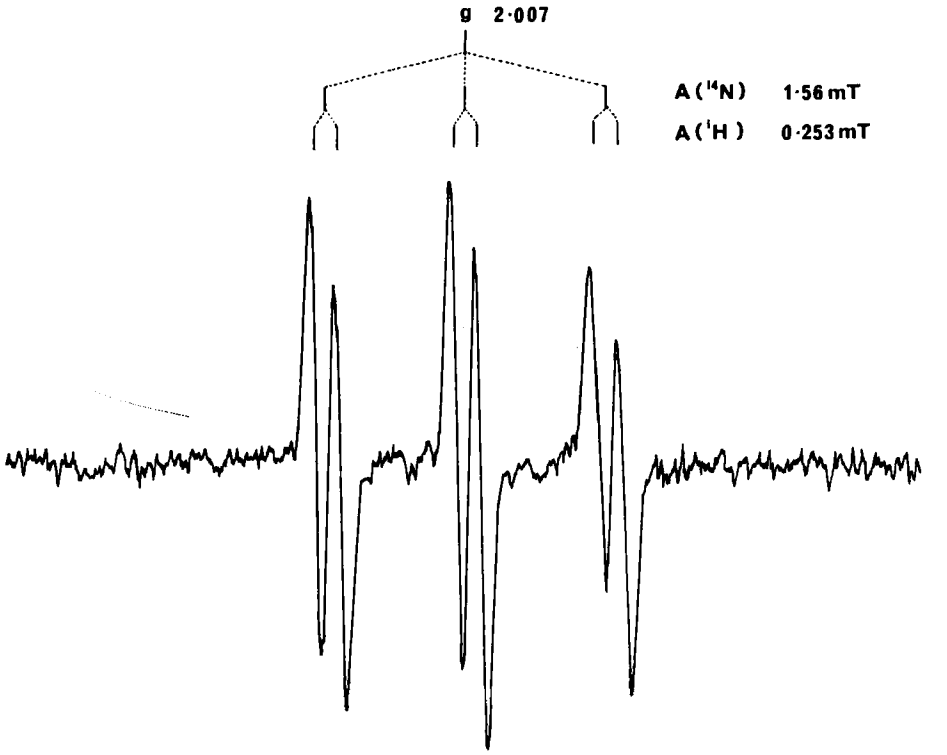

FIGURE 1 ESR spectrum obtained when − Se − vitamin E heart homogenate was incubated for 15 min with Fe/ADP, NADPH and 4-POBN as described in the methods section. Instrumental parameters were as follows: time constant 2 sec, modulation amplitude 0.2 mT, modulation frequency 100 kHz, power 10 mW, microwave frequency 9.5 GHz and gain 1 × 10^5. The hyperfine coupling constants are shown on the spectrum.

ogenate) were carried out in 5 ml "Reacti-vials" (Pierce U.K., Cambridge, U.K.). The incubations were open to the atmosphre, constantly stirred at 25°C and also contained (final concentrations) 6.8 μM $FeSO_4$, 2.3 mM ADP and 0.218 mM NADPH, to initiate peroxidation and 45 mM 4-POBN as the spin trap. All components of incubations were dissolved in phosphate buffer. ESR spectra, in 0.25 ml samples of incubations, were recorded at regular intervals with a Varian E104A X-band spectrometer using a flat quartz cell. Operating conditions are given in the legend to Figure 1.

RESULTS AND DISCUSSION

Feeding of the − Se and − vitE diet to rats decreased Se-GSHpx activity and vitamin E concentrations in the heart, and increased plasma PK activity and the concentration of fatty acid hydroperoxides (Table I). Rats given the − Se + vitE showed decreased heart Se GSHpx activity and those given the + Se − vitE diet had decreased heart vitamin E concentration. These groups only had small increases in plasma PK activity. Increases in PK in Se/vitamin E deficiency are known to result from release of the M1 isoenzyme of PK.[10] Since this isozyme occurs only in skeletal and heart muscle and in brain, its occurrence in plasma is indicative of some degree of damage in these tissues. Increases in fatty acid hydroperoxides generally result from free-radical mediated reactions and they can cause further radical production.[11] Spin trapping increased the concentration of free radicals in heart homogenates by the formation of more stable 4-POBN adducts which were detected by ESR spectroscopy (Fig. 2). No evidence of increased free radicals in hearts of − Se − vitE rats was observed directly or in presence of 4-POBN alone. However when Fe/ADP and NADPH were added to incubations, to enhance radical formation, major increases occurred. Thus the results in this report probably reflect the increased tendency of hearts from rats with combined Se/vitamin E deficiency or vitamin E deficiency to form free radicals which will react with 4-POBN, rather than endogenous radical formation.

TABLE I

Heart vitamin E concentration and GSHpx activity and plasma PK activity and lipid hydroperoxide concentration

Group:	+ Se + vitE	+ Se − vitE	− Se + vitE	− Se − vitE
vitE μg/mg protein	0.151[a] ± 0.013	0.025[b] ± 0.003	0.151[a] ± 0.002	0.028[b] ± 0.007
GSHpx Units/ mg protein	0.372[a] ± 0.084	0.282[a] ± 0.026	0.014[b] ± 0.002	0.016[b] ± 0.001
PK Units/l plasma	58.6[b] ± 2.8	89.0[c] ± 5.1	89.0[c] ± 3.7	2803[a] ± 575
Plasma lipid hydroperoxide OD 540 nm	0.031[d] ± 0.002	0.046[b] ± 0.002	0.031[d] ± 0.004	0.086[c] ± 0.019

Animals and diets are described in materials and methods. Results are mean ± SEM with 5 animals/group, lipid hydroperoxides are expressed as OD at 540 nm/ml plasma, produced by reaction of the hydroperoxides with thiobarbituric acid. Groups without common superscripts within row are significantly different: a vs b or c $P < 0.001$, b vs c $P < 0.02$, c vs d $P < 0.005$. Groups with common superscripts or b vs d are not significantly different.

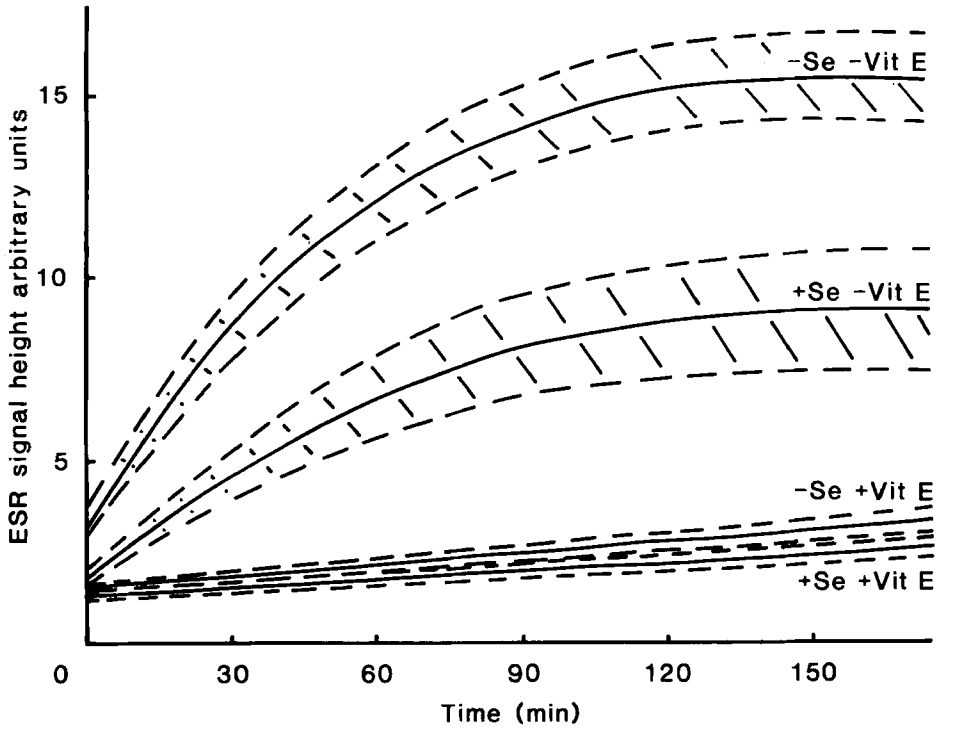

FIGURE 2 Time course of development of ESR spectra obtained when heart homogenates from +Se + vitE, −Se + vitE, +Se − E and −Se − VitE rats were incubated with Fe/ADP, NADPH and 4-POBN as described in the methods section. Instrumental parameters are given in Figure 1. Shown are best fit curves derived from 6 to 9 animals/group. Shaded area represents the SEM.

Vitamin E deficiency was necessary for the demonstration of any appreciable free radical/4-POBN adduct formation. This may mean that the radical trapped was being formed in the membrane fraction of the cell, where vitamin E is normally found associated with the lipid components. Se deficiency alone did not significantly increase the formation of radical reacting with 4-POBN. This does not exclude the possibility of the formation of radicals which could not react with the 4-POBN or the formation of an unstable 4-POBN adduct which immediately decomposed. Se deficiency did, however, increase radical adduct formation when imposed upon vitamin E deficiency suggesting that radical formation in the membrane fraction may have been stimulated by a compound formed in Se deficient homogenate. Based upon the hyperfine splitting constants (Fig. 1) the radical responsible for 4-POBN-adduct formation is probably oxygen or carbon centred.

Thus, in rats with combined Se and vitamin E deficiency there was evidence for tissue damage, increased concentrations of lipid peroxides in plasma and increased formation of free radicals in heart homogenates. Raised free-radical concentrations were also observed with heart homogenates from vitamin E deficient rats. The results are more direct evidence for the hypothesis that free radicals are involved in tissue damage in vitamin E deficiency which may be further exacerbated by Se deficiency.[2] However, although free radical formation was increased in heart tissue after oxidative

stress (Fe/ADP) this is not absolute proof of the involvement of free radicals in disease processes. The free radicals may have been formed as a consequence of tissue damage. Proof of the involvement of free radicals in the initiation of disease processes would be more convincing if increased radical formation could be demonstrated *in vivo* prior to the onset of tissue damage.

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