THE INVOLVEMENT OF URIC ACID IN THE SCAVENGING OF NITROXIDE RADICALS BY ASCORBATE

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Abstract

The reaction kinetics, measured by electron paramagnetic resonance (EPR) was used to study the involvement of uric acid in the reactions of nitroxide radical (TEMPONE) scavenging by ascorbate (PBS, pH 7.4, and T=37^0 C). In absence of iron ions, the oxygen reoxidation of hydroxylamine was slow and the addition of uric acid did not change the observed kinetics. On the other hand, in presence of iron ions the reoxidation starts to recover the nitroxide radical. This reaction rate is strongly enhanced by uric acid. In fact there are two hypotheses, the first ascribes more efficient iron complexes with uric acid, by which the reoxidation rate increases. Especially the competition between the phosphate and uric acid ligands might be important. However, uric acid could be directly involved in scavenging the hydroxyl radicals, and herewith influence the reaction rate. No scavenging activity of uric acid in UV irradiated aqueous solutions was found. The trapped adducts of the hydroxyl and the superoxide radicals show the same concentration in presence and absence of uric acid.

Introduction

It is generally assumed that uric acid is a powerful antioxidant and a scavenger of singlet oxygen and radicals [1]. The role of urate is discussed in a broader context of the plasma antioxidant activity [2].

We tried to assess the reducing potential of human plasma using the reduction kinetics of nitroxide spin probes. If the role of ascorbic acid was easy to study we could not find any correlation in the content of uric acid in plasma and its

reduction kinetics. Therefore we decided to study the system of nitroxide reduction by ascorbate in presence of uric acid.

Experimental

The reaction rate of the spin probe TEMPONE (2,2,6,6-tetramethyl-1-oxylpiperidine-4-one, Fig.1) with sodium ascorbate was measured in PBS (phosphate buffered saline) pH 7.4 at 37^0 C. The initial concentrations at the instant of merging the spin probe and the freshly prepared ascorbate solution are:

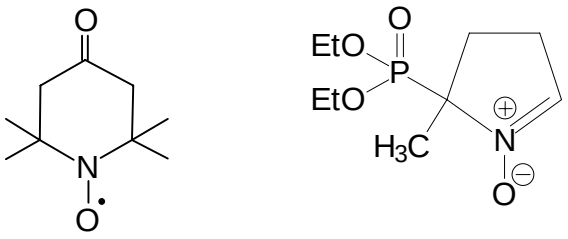
a) $c_{SL}{}^0 = 0.0\ 167$ mM, $c_{ASC}{}^0 = 0.167$ mM.

b) same as a) in presence of uric acid $c_{UA}{}^0 = 0.06$ mM.

c) same as a) in presence of ferrous ions $c_{Fe}{}^0 = 0.02$ mM.

d) same as b) with the same addition of ferrous ions as in c).

In all measurements the initial concentration of oxygen was about 0.2 mM.



TEMPONE DEPMPO

Fig. 1 The structural formulas of the spin probe TEMPONE and the spin trap DEPMPO.

EPR measurements have been performed on a Bruker 300 ES spectrometer at 9.3 GHz, 100 kHz modulation frequency, 10 mW microwave power. The samples were stored in glass capillaries, 1 mm inner diameter.

Reduction kinetics

EPR spectra were recorded sequentially in 2 minute intervals, altogether the measurement lasted 14 minutes, and the amplitudes of the first hyperfine component (m=1) (Fig.2) were plotted as the relative intensity against time (Fig.3). Each experiment was repeated three times, and the standard deviations are assigned by the bar.

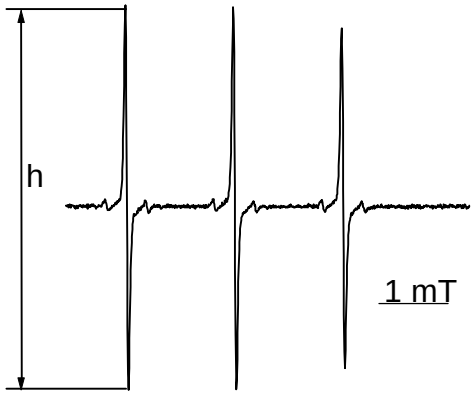


Fig. 2. The EPR spectrum of TEMPONE in the PBS buffer solution at pH 7.4 and 37^0C.

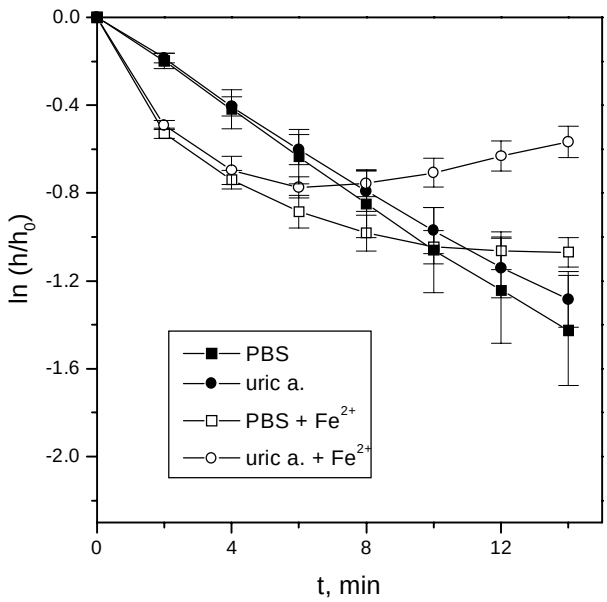


Fig. 3 The logarithmic plot of the relative amplitudes h/h_0 of the EPR first hyperfine line of TEMPONE against time in the reaction of TEMPONE with ascorbate in presence of the identified reactants. The experiment was performed in PBS buffer at 37^0 C.

The same experiment with the same initial concentrations of the reagents was also performed in the Tris-HCl buffer pH=7.4, at 37^0 C. The results of the kinetic study are given in Fig.4.

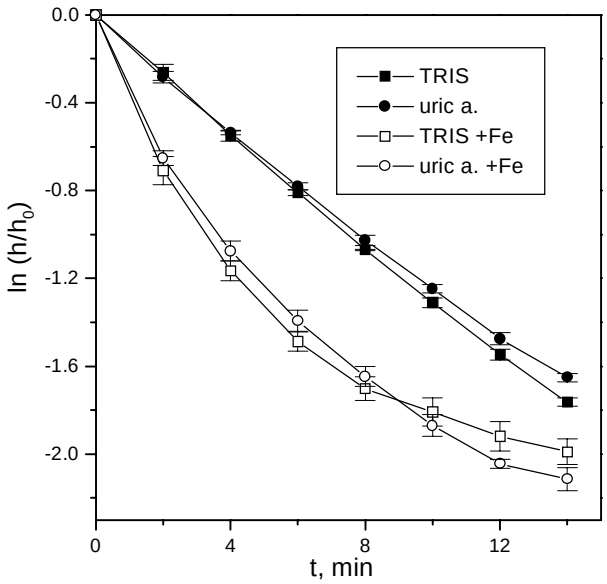


Fig. 4 The logarithmic plot of the relative amplitudes of the EPR first hyperfine line of TEMPONE against time in the reaction of TEMPONE with ascorbate in presence of the identified reactants. The experiment was performed in Tris-HCl buffer at 37^0 C.

Kinetics model

The main reaction between nitroxide, ascorbate, oxygen, and iron ions are cast by a system of differential equations describing the reaction rates.

$$dc_{SL}/dt = -k_1 c_{SL} c_{AH_2} - k_2 c_{SL} c_{AH\cdot} + k_3 c_{SLH} c_{O_2} + k_4 c_{SLH} c_{Fe^{3+}}$$

$$dc_{SLH}/dt = k_1 c_{SL} c_{AH_2} + k_2 c_{SL} c_{AH\cdot} - k_3 c_{SLH} c_{O_2} - k_4 c_{SLH} c_{Fe^{3+}}$$

$$dc_{AH_2}/dt = -k_1 c_{SL} c_{AH_2} - k_5 c_{AH_2} c_{Fe^{3+}}$$

$$dc_{AH\cdot}/dt = k_1 c_{SL} c_{AH_2} + k_5 c_{AH_2} c_{Fe^{3+}} - k_2 c_{SL} c_{AH\cdot} - k_6 c_{AH\cdot} c_{Fe^{3+}}$$

$$dc_{O_2}/dt = -k_3 c_{SLH} c_{O_2} - k_7 c_{O_2} c_{Fe^{2+}}$$

$$dc_{Fe^{3+}}/dt = k_7 c_{O_2} c_{Fe^{2+}} - k_4 c_{SLH} c_{Fe^{3+}} - k_5 c_{AH_2} c_{Fe^{3+}}$$

Here the indexes *SL*, *SLH*, AH_2, $AH^{\bullet}$, O_2, Fe^{2+}, and Fe^{3+} of the concentrations *c* describe the spin probe, reduced spin probe, ascorbate, ascorbate radical, oxygen, ferrous and ferric ions. The meaning of the rate constants, k_i, is evident from the equations. The system was solved with initial conditions of the experiment and the reaction rate constants were estimated from the best fits of the calculated and experimental kinetics curves. The Runge-Kutta integration algorithm was used.

Spin trapping experiment

The oxygen radical trapping was performed at room temperature (20^0 C) by DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide, Fig.1) purchased from OXIS International Inc. Portland OR. The initiation of radicals was triggered by the Fenton reaction with the reactants hydrogen peroxide, ferrous ions, and DEPMPO in the PBS solution: c^0_{H2O2} = 0.03 mM, $c_{Fe}{}^0$ = 0.5 mM, $c_{UA}{}^0$ = 0.03mM, $c_{DPMPO}{}^0$ = 0.025 M.

The EPR spectrum of adducts was recorded one minute after mixing of the reactants (Fig.5). The spectrum of the hydroxyl radical adduct did not differ in the presence or absence of uric acid, either in shape or in intensity.

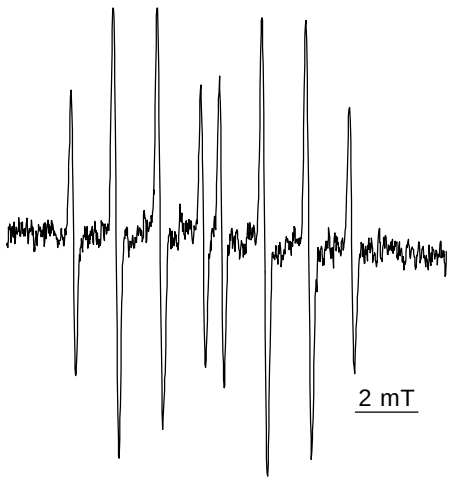


Fig. 5. A typical spectrum of the hydroxyl adduct with DEPMPO of the Fenton reaction initiation experiment at 20^0C.

In the second experiment, UV irradiation (λ=365 nm, 6W) was used to initiate the reaction in the samples: the aqueous dispersions of liposomes (90 mg phosphatidylcholine

and 10 mg phosphatidylserine in 2 ml water) with 0.05M DEPMPO, in presence or absence of 0.06mM uric acid.

After two hours of irradiation in an open test tube, the sample was transferred into the glass capillary for EPR measurement.

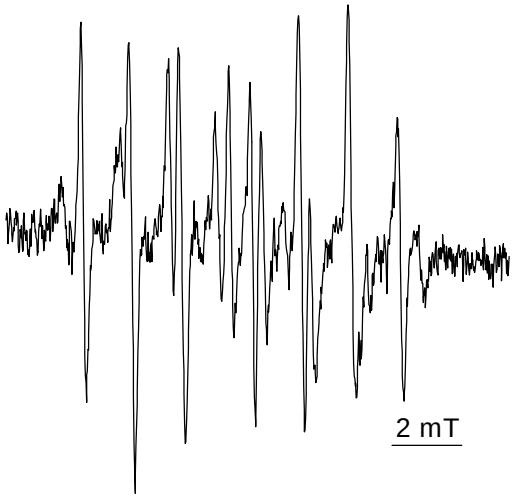


Fig.6. Superimposed spectra of the radical adducts with DEPMPO in the UV radiation initiation experiment. The concentration ratio between the hydroxyl, superoxide and ethyl radical adducts is 0.2, 0.3, and 0.5, respectively.

Results and Discussions

The experiments in which the interference of uric acid in the radical scavenging was measured, gave clear evidence to assess its antioxidant activity. The kinetic curves show that the nitroxide reoxidation rate in presence of iron ions is enhanced if uric acid is added (Fig.3). Simple reasoning might explain the effect. The iron phosphates are sparingly soluble in the aqueous phase. Therefore uric acid might produce a new complex which enhances the reaction with respect to the phosphate complexes. This consideration is in accord with the experiment performed in Tris-HCl buffered solutions where the kinetic curves in presence of iron show fast reduction, which does not depend on the presence of uric acid (Fig.4).

Table I. The reaction rate constants evaluated from the model describing the reduction rate of the spin probe TEMPONE by ascorbate in PBS in absence or presence of ferrous ions and / or uric acid.

Reaction rate constants, l mol $^{-1}$ min^{-1}

Sample	k_1	k_2	k_3	k_4	k_5	k_6	k_7
PBS	650	1000	30	-	-	-	-
Uric a.	650	1000	80	-	-	-	-
PBS+Fe	1570	1000	680	100	1000	500	700
Uric a.+Fe	1750	1000	680	14000	1000	500	4800

From the model it is evident that iron increases the rate of the reoxidation of hydroxylamine to nitroxide by oxygen, while this reoxidation via the iron ions was significantly enhanced by the action of uric acid. The influence of iron was known from previous studies [3].

The formed uric acid complex with the iron ions is more efficient in the reoxidation of hydroxylamine than the corresponding complex with phosphate, (Table I, Fig. 3). On the other hand, the effect of uric acid was not significant in the experiment where PBS was replaced by Tris-HCL buffer (Fig. 4). In presence of iron ions the reduction rate of nitroxide is enhanced, though the reoxidation rate of hydroxylamine by uric acid does not occur. Therefore, it can be assumed, that the iron Tris-complex, does not support the hydroxylamine reoxidation. Additionally the effect of uric acid is prevented. This result supports similar reasoning, about the uric acid complexes[4].

The involvement of uric acid in the reactions with oxygen radicals could not be proven. The EPR spectra of the spin trap adducts showed any difference in the line shapes or the relative proportions as well the total concentrations of the trapped radicals with respect to presence or absence of uric acid.

Conclusions

In our study of the involvement of uric acid in the reaction between ascorbate and nitroxide we found that uric acid accelerates the spin probe hydroxylamine reoxidation reaction by oxygen which is convened by the presence of iron ions.

The very short-lived oxygen radical producing systems Fenton reaction and UV irradiation have not been influenced by the presence of uric acid.

Acknowledgement

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Povzetek

Raziskali smo vpliv sečne kisline na kinetiko redukcije nitroksidnih radikalov z askorbatom. Uporabili smo metodo elektronske paramagnetne resonance (EPR) in spinski označevalec TEMPON. Reakcije so potekale v PBS oziroma Tris-HCl pufru pri pH 7,4 in T= 37^0C. V odsotnosti železovih ionov je bila reoksidacija hidroksilamina počasna in dodatek sečne kisline ni povročil sprememb hitrosti reakcije. V prisotnosti železovih ionov je reoksidacija hitrejša, prisotnost sečne kisline pa bistveno pospeši reoksidacijo hidroksilamina. Dejansko sta tu dve možnosti in sicer, da sečna kislina tvori železov kompleks, ki je bolj aktiven v smislu hitrosti redoks reakcije-reoksidacije kot železovi fosfati, oziroma da sečna kislina mimo tega posega v hitrost reakcije neposredno. Glede na poskus v Tris –HCl pufru, kjer pospeševalnega efekta sečne kisline ni, je prva hipoteza bolj verjetna. To potrjujeta tudi oba poskusa s spinskimi lovilci, kjer ni bilo opaziti vpliva sečne kisline na tvobo kisikovih radikalov, z vzbujanjem tvorbe kisikovih radikalov s Fentonovo reakcijo, oziroma z obsevanjem z UV žarki.