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Interactions of peroxynitrite with uric acid in the presence of ascorbate and thiols: Implications for uncoupling endothelial nitric oxide synthase

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Abstract

It has been suggested that uric acid acts as a peroxynitrite scavenger although it may also stimulate lipid peroxidation. To gain insight into how uric acid may act as an antioxidant, we used electron spin resonance to study the reaction of uric acid and plasma antioxidants with $ONOO^-$. Peroxynitrite reacted with typical plasma concentrations of urate 16-fold faster than with ascorbate and 3-fold faster than cysteine. Xanthine but not other purine-analogs also reacted with peroxynitrite. The reaction between $ONOO^-$ and urate produced a carbon-centered free radical, which was inhibited by either ascorbate or cysteine. Moreover, scavenging of $ONOO^-$ by urate was significantly increased in the presence of ascorbate and cysteine. An important effect of $ONOO^-$ is oxidation of tetrahydrobiopterin, leading to uncoupling of nitric oxide synthase. The protection of eNOS function by urate, ascorbate and thiols in $ONOO^-$-treated bovine aortic endothelial cells (BAECs) was, therefore, investigated by measuring superoxide and $NO^\bullet$ using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) and the $NO^\bullet$-spin trap $Fe[DETC]_2$. Peroxynitrite increased superoxide and decreased $NO^\bullet$ production by eNOS indicating eNOS uncoupling. Urate partially prevented this effect of $ONOO^-$ while treatment of BAECs with the combination of either urate with ascorbate or urate with cysteine completely prevented eNOS uncoupling caused by $ONOO^-$. We conclude that the reducing and acidic properties of urate are important in effective scavenging of peroxynitrite and that cysteine and ascorbate markedly augment urate's antioxidant effect by reducing urate-derived radicals.

Keywords: Urate; Peroxynitrite; eNOS; Ascorbate; Thiols; ESR; Superoxide; Nitric oxide

1. Introduction

Uric acid, the terminal oxidation product of purine metabolism, is present in human plasma largely as the monoanion urate ($pK_a = 5.4$) [1] and at much higher concentrations (200–500 μM) than those encountered in other primates because the enzyme urate oxidase is absent from human tissues [2]. Urate has been proposed to be an important plasma antioxidant [3]. Epidemiological studies, however, have demonstrated a positive correlation between plasma urate levels and the risk of cardiovascular diseases [4,5]. These correlations might be due to increased activity of xanthine oxidase [6] or co-morbid conditions such as obesity and diabetes, rather than pro-oxidative properties of urate itself. Urate has also been reported to have pro-oxidative properties in the setting of $ONOO^-$ mediated oxidative damage [7]. There is growing evidence, however, that urate is beneficial against $ONOO^-$ mediated damage and that it contributes to the total antioxidant defense pool in vivo [8–10]. The concentration of urate in brain tissue is much lower than heart tissue [11]. In studies of heart and brain tissues, proteins in heart homogenates were more resistant to nitration by $ONOO^-$ than brain, whereas protein nitration in heart homogenates depleted of urate by uricase treatment were similar to brain homogenates [11]. Moreover, the severity of multiple sclerosis correlates

Abbreviations: BAECs, bovine aortic endothelial cells; BH_4, tetrahydrobiopterin; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine; $CM^\bullet$, 3-methoxycarbonyl-proxyl; $CP^\bullet$, 3-carboxy-proxyl; CPH, 1-hydroxy-3-carboxy-2,2,5-tetramethyl-pyrrolidine; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; SIN-1, 3-morpholinosydnonimine; XXO, xanthine/xanthine oxidase

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inversely with serum urate levels and oral administration of uric acid inhibits $ONOO^-$ mediated MS progression [12]. The reaction of urate with $ONOO^-$ leads to formation of urate oxidation products that have been partially characterized as a nitrated urate derivative [13] and a urate-derived carbon-centered radical [7,14]. The latter product has been implicated in the pro-oxidative effect of urate during $ONOO^-$ exposure, and therefore, might play a role in the physiological properties of urate in vivo.

Although urate has frequently been employed as an $ONOO^-$ scavenger [13], the mechanisms whereby urate reacts with $ONOO^-$ are poorly understood. Further, the antioxidant properties of urate have partly been attributed to interactions with other antioxidants like ascorbate [17–20], however, these reactions also remain poorly defined. Previously, we have shown that tetrahydrobiopterin (BH_4) is an important target of $ONOO^-$ leading to uncoupling of endothelial NO synthase (eNOS) and endothelial dysfunction [15,16]. It is, therefore, important to know if urate can prevent $ONOO^-$ mediated uncoupling of eNOS. In this paper, we employed electron spin resonance (ESR) to examine reactions of $ONOO^-$ with urate in the presence and absence of ascorbate and cysteine as well as superoxide and NO production by endothelial cells treated with $ONOO^-$ in the presence and absence of urate.

Our data indicate that urate reacts very rapidly with $ONOO^-$, and this leads to formation of a carbon-centered radical. Common plasma antioxidants such as cysteine and ascorbate, which are less reactive with $ONOO^-$ than urate, are capable of reducing this carbon-centered radical, and therefore, contribute to the antioxidant properties of urate.

2. Experimental procedures

2.1. Chemicals and reagents

Uric acid, L-ascorbic acid, cysteine, 1H-purine, inosine, caffeine, histamine, hypoxanthine, xanthine and allantoin were obtained from Sigma–Aldrich (St. Louis, MO). Peroxynitrite was obtained from Cayman (Ann Arbor, MI). The peroxynitrite-donor SIN-1, the cyclic hydroxylamines 1-hydroxy-3-carboxy-2,2,5-tetramethyl-pyrrolidine (CPH), 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) and the spin trap 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO) were purchased from Alexis Corporation (San Diego, USA). Xanthine oxidase was purchased from Roche (Indianapolis, IN).

The concentration of $ONOO^-$ was determined spectrophotometrically from its absorbance at 302 nm in 0.1 M NaOH using molar extinction coefficient of 1670. Cell free experiments were performed in phosphate buffer saline (PBS: 0.9% NaCl, 50 mM phosphate, pH 7.4) containing 25 mM $NaHCO_3$. The ESR buffer consisted of sodium phosphate buffer with 2.35 g/L NaH_2PO_4, 7.61 g/L Na_2HPO_4, 0.15 g/L NaCl, 1 g/L glucose, 0.37 g/L KCl, 0.2 g/L $CaCl_2$, pH 7.4. This buffer was treated for 4 h with 50 g/L chelex 100, to minimize contamination with transition metals. Krebs–Hepes buffer (KHB) contained 5.786 g/L NaCl, 0.35 g/L KCl, 0.368 g/L $CaCl_2$, 0.296 g/L $MgSO_4$, 2.1 g/L $NaHCO_3$, 0.142 g/L K_2HPO_4, 5.206 g/L Na-Hepes, 2 g/L D-glucose, pH 7.35.

2.2. Preparation of spin probe stock solutions

Stock solutions of CPH and CMH (10 mM), dissolved in 0.9% NaCl containing 1 mM diethylenetriamine-pentaacetic acid (DTPA) and purged with argon, were prepared daily and kept under argon on ice. DTPA was used to decrease auto-oxidation of hydroxylamines catalyzed by trace amount of transition metals. CPH and CMH were used in a final concentration of 1 mM and EMPO was used in a final concentration of 20 mM.

2.3. Cell culture and treatments

Bovine aortic endothelial cells (BAECs; cell systems, Kirkland, WA) were cultured on 100 mm plates in Media 199 (M199; Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) as previously described [21]. Confluent BAECs from passage 4 to 7 were used for ESR experiments. Cell suspensions were used for ROS measurement. For this purpose, BAECs were scraped from the cultured dish in 1 mL of Krebs–Hepes buffer, centrifuged at 1800 rpm for 10 min and resuspended in PBS containing 25 mM bicarbonate. Peroxynitrite (0.27 mM) was added to the cell suspension as a bolus and vortexed. To test if urate can protect eNOS against $ONOO^-$, the suspended cells were divided in two Eppendorff tubes and one portion incubated with 100 μM urate before $ONOO^-$ treatment. ESR measurements of $O_2^{\bullet -}$ were made 3 min later. Some of these cell suspensions were incubated with L-NAME (1 mM) or PEG–SOD (50 U/mL) for 5 min and then the spin probe CMH added and vortexed. Superoxide production was determined by inhibition with 50 U/mL PEG–SOD while superoxide generated by uncoupled eNOS was measured as L-NAME (1 mM) inhibited $CM^{\bullet}$ nitroxide formation. Urate (50–100 μM) did not interfere with CMH detection of $O_2^{\bullet -}$ generated by xanthine and xanthine oxidase.

2.4. Measurements of nitric oxide with $Fe(DETC)_2$

$NO^{\bullet}$ production by BAECs was measured using $Fe(DETC)_2$ as previously described [22,23]. Cells on 100 mm plates were incubated for 2 h in ESR buffer with the $ONOO^-$-donor SIN-1 (0.5 mM), in the presence or absence of antioxidants. The cells were then washed twice with KHB and some plates were incubated with 10 μM BH_4 for 15 min before adding $Fe(DETC)_2$. After incubation with $Fe(DETC)_2$, the media was aspirated and the cells were harvested with a rubber policeman in Krebs–Hepes

buffer and aspirated into 1 mL syringes which were frozen immediately in liquid nitrogen.

2.5. ESR measurements

Oxidation of the spin probes CPH and CMH by reactive oxygen species (ROS) leads to formation of the stable nitroxide radicals 3-carboxy-proxyl ($CP^{\bullet}$) and 3-methoxy-carbonyl-proxyl ($CM^{\bullet}$), which can be detected by ESR spectroscopy [24–26]. The amount of nitroxide formed is proportional to the concentration of the reacted oxidant species. The concentration of these nitroxides was determined from the ESR amplitude according to a calibration curve using standard solutions of 3-carboxyproxyl radical. ROS formation was measured from the kinetics of nitroxide accumulation by following the ESR amplitude of the low-field component of ESR spectra. The rate of superoxide radical formation was determined by measuring the superoxide dismutase (SOD) inhibited nitroxide generation.

The reactivity of various peroxynitrite scavengers was studied by examining competition with CPH during bolus exposures to $ONOO^-$. Urate and other $ONOO^-$ scavengers compete with CPH to react with $ONOO^-$. The reactivity of each scavenger with bolus $ONOO^-$ was determined using the formula:

$$\left(\frac{A_0}{A}\right) - 1 = \frac{k_{SCAV}}{k_{CPH}} \times \frac{c_{SCAV}}{c_{CPH}} \quad (1)$$

where A_0 is the ESR amplitude in absence of $ONOO^-$ scavengers, A the ESR amplitude in presence of $ONOO^-$ scavengers and k the reaction rate constant and c is concentration.

The free radical intermediates from the reaction $ONOO^-$ with urate, ascorbate and cysteine were detected with the nitrone spin trap EMPO and partially by direct ESR spectroscopy without a spin trap. The reaction of a radical with EMPO yields a spin adduct with a characteristic ESR spectra and allows the identification of the original radical [27].

ESR samples were placed in 100 μL capillary and measured at room temperature using a field scan with the following ESR settings: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 164 ms, time constant 164 ms. Peroxynitrite and ROS production by BAECs were detected by following the low-field peak of the nitroxide ESR spectra using time scans with the following ESR settings: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 1.3 s, time constant 5.2 s.

Frozen cell samples were measured in a finger Dewar filled with liquid nitrogen at 77 K in field scan with following ESR settings: field sweep 160 G, microwave frequency 9.39 GHz, microwave power 20 mW, modulation amplitude 3 G, conversion time 655 ms, time constant 5242 ms, receiver gain 1×10^4, number of scans 4. Under these conditions, the $NO^{\bullet}$–$Fe(DETC)_2$ complex yields a 3-line ESR spectra whose amplitude is proportional to amount of bioactive $NO^{\bullet}$ produced in cells [22,23,28].

2.6. Computer simulation of ESR spectra

Programs for simulation of ESR spectra and spin trap database are readily available through the Internet (http://epr.niehs.nih.gov/). Details of this computer simulation program have been described elsewhere [29]. Hyperfine-coupling constants are expressed as an average of ESR parameters obtained from computer simulation. The ESR spectrum of the ascorbyl radical was simulated with hyperfine-coupling constant on proton $a_H = 1.8$ G (line-width 0.11 G). Computer simulation of the EMPO radical adducts contained components of EMPO/$^{\bullet}$OH ($a_N = 13.4$ G, $a_H = 14.6$) and three urate-derived radical adducts: EMPO/$^{\bullet}R_1$ ($a_N = 15.1$ G, $a_H = 21.7$), EMPO/$^{\bullet}R_2$ ($a_N = 14.4$ G, $a_H = 18.7$) and EMPO/$^{\bullet}R_3$ ($a_N = 14.5$ G, $a_H = 16.3$).

2.7. Statistical analysis

Data are presented as mean ± standard error. For comparison of two groups, a two-tailed t-test was employed using Excel software. Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Specificity of antioxidant reactions of urate: comparison of reactivity with $ONOO^-$ and superoxide

In initial experiments, we compared the scavenging efficiency of urate and ascorbate at physiological concentrations using bolus $ONOO^-$ and xanthine/xanthine oxidase (XXO) as source for superoxide. CPH alone did not yield an ESR signal (Fig. 1A) while xanthine and xanthine oxidase, as well as bolus $ONOO^-$ mixed with CPH, resulted in a prominent ESR signal (Fig. 1B and G). Experiments with superoxide dismutase (SOD) and decomposed $ONOO^-$ served as controls and confirmed that $ONOO^-$ or superoxide mediated the respective ESR signal (Fig. 1C and H). Oxidation of CPH by superoxide was not affected by the presence of urate (Fig. 1D), indicating that urate is not capable of scavenging $O_2^{\bullet -}$. In contrast, ascorbate (25 μM) was almost as potent as SOD in inhibition of ESR signal in the superoxide generating X/XO system (Fig. 1E). The reactions between $ONOO^-$ and either ascorbate or urate were very different than those between $O_2^{\bullet -}$ and these scavengers. Urate strongly inhibited the $ONOO^-$ signal while ascorbate did not (Fig. 1I and J). These data show that urate was very effective in scavenging of $ONOO^-$ but not superoxide, while ascorbate was highly reactive with $O_2^{\bullet -}$ but not with $ONOO^-$.

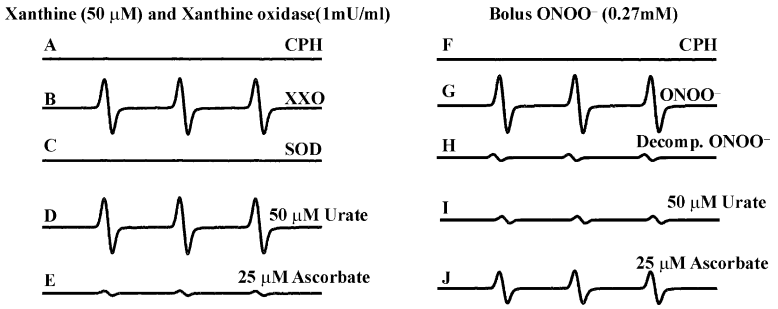


Fig. 1. Reaction of urate or ascorbate with $ONOO^-$ and superoxide. (A) CPH (0.1 mM) in PBS with 25 mM $NaHCO_3$ at pH 7.4. (B) ESR signal of CPH mixed with xanthine (50 μM) and xanthine oxidase (1 mU/mL) as source for superoxide generation. (C) SOD-inhibited (50 U/mL) ESR signal in the mixture of CPH and xanthine/xanthine oxidase (XXO) and confirms superoxide mediated $CP^•$ formation. (D) ESR signal of CPH, XXO in the presence of urate (50 μM) or (E) ascorbate (25 μM). Both antioxidants compete with CPH in the reaction with superoxide or $ONOO^-$ and inhibit $CP^•$ formation detected by ESR. (F) ESR spectra of CPH (0.1 mM) and bolus 0.27 mM $ONOO^-$ (G) in the presence of 50 μM urate (I) or 25 μM ascorbate (J). CPH or decomposed $ONOO^-$ in buffer serve as controls (F and H). ESR signal (%) were compared to ESR amplitude of XXO or $ONOO^-$, which were set as 100%.

3.2. Reaction of $ONOO^-$ with urate and other antioxidants

Because previous studies have indicated that peroxynitrite readily oxidizes urate in human plasma [14], we hypothesized that the reactivity of $ONOO^-$ with urate would exceed that of $ONOO^-$ with physiological concentrations of other common intracellular antioxidant small molecules. Reactions between $ONOO^-$ and urate, cysteine, ascorbate or tetrahydrobiopterin (BH_4) were therefore studied. Boluses of $ONOO^-$ (0.27 mM) were added to reaction mixtures containing other potential $ONOO^-$ scavengers and CPH. In the absence of any scavenger, the reaction of $ONOO^-$ with CPH resulted in formation of $CP^•$, detected as a strong ESR signal (Fig. 1G). By using the ESR amplitudes for the respective reactions as described in the materials and methods and formula (1), the relative reactivities of the antioxidant scavengers with peroxynitrite were calculated (Fig. 2). In contrast to urate, cysteine and ascorbate exhibited substantially less reactivity with peroxynitrite (Fig. 2). Urate was almost 3 times faster than cysteine and 16 times faster than ascorbate in reacting with $ONOO^-$ (Fig. 2). Xanthine but not other purine-analogs also reacted with peroxynitrite (Fig. 3). Histamine (Scheme 1) also reacted with peroxynitrite (Fig. 3). It has been previously reported that one electron oxidation of urate produces a radical located primarily on the five-membered ring of the purine structure [43], which may suggest that reaction with the five-member ring of the purine structure plays a major role in $ONOO^-$ scavenging (Fig. 3). The reactivity of ascorbate combined with cysteine was less than that of urate alone (Fig. 2). In contrast, urate in combination with ascorbate, cysteine or with both antioxidants together was three times more efficient in $ONOO^-$ scavenging than urate alone (Fig. 2). In previous studies, we found that tetrahydrobiopterin (BH_4) is highly reactive with $ONOO^-$ and that its reaction rate constant exceeds those of ascorbate or thiols with $ONOO^-$ [15]. We therefore also used BH_4 as an $ONOO^-$ scavenger for comparison. The reactivity of $ONOO^-$ with BH_4 was close to that of urate, but higher than that of ascorbate or cysteine (Fig. 2). The reactivity of urate combined with ascorbate and cysteine, however, exceeded the reactivity of BH_4 with $ONOO^-$ (Fig. 2).

These data indicate that $ONOO^-$ scavenging by urate significantly increases in the presence of ascorbate and thiols. In the physiological concentrations employed, neither urate, ascorbate or thiols alone may prevent the reaction of $ONOO^-$ with BH_4. Urate combined with ascorbate and thiols, however, may almost completely inhibit this reaction.

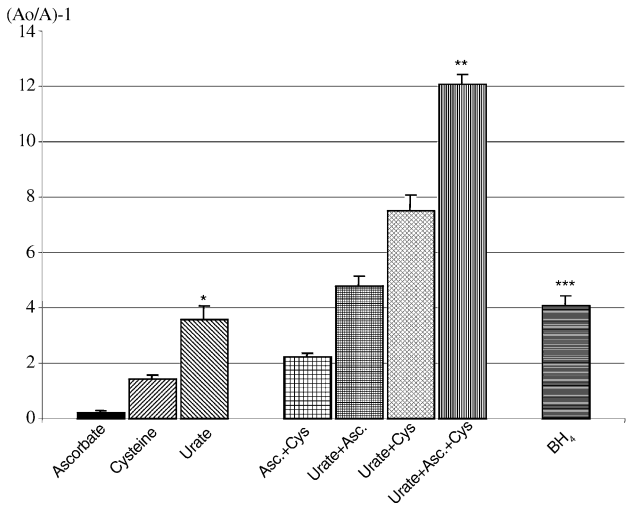


Fig. 2. Reactivity of potential $ONOO^-$ scavengers with bolus $ONOO^-$. Inhibition of $CP^•$ nitroxide formation by bolus $ONOO^-$ (0.27 mM) in the presence of different peroxynitrite scavengers and their combination. Reactivity was calculated using the ESR amplitude $(A_0/A) - 1 = k_{SCAV}/k_{CPH} \times [scav]/[CPH]$ as described in material and methods. BH_4 concentration was 10 μM and CPH concentration 0.1 mM. The reactivity of urate was significantly different from that of the ascorbate (25 μM) combined with cysteine (25 μM) ($^*p = 0.01$, $n \geq 5$ experiments). The combination urate (100 μM), ascorbate (25 μM) and cysteine (25 μM) was found to have the highest reactivity with $ONOO^-$ ($^{**}p = 0.003$ vs. BH_4; $^{***}p = 0.005$ vs. urate + Cys).

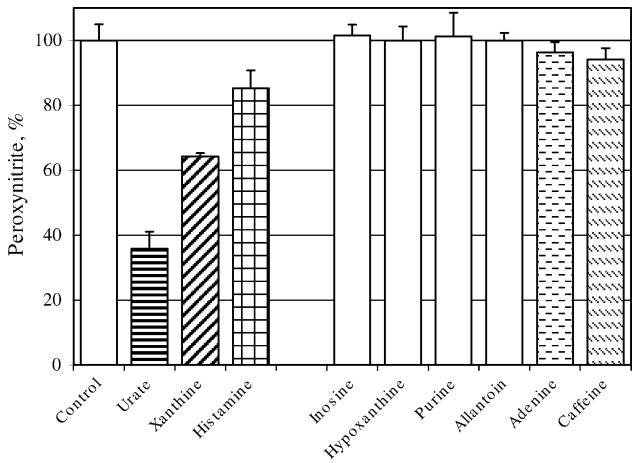


Fig. 3. Effect of structural analogs of uric acid on CPH oxidation by $ONOO^-$. Various analogs of uric acid (0.1 mM) were mixed with the spin probe CPH (0.1 mM) and the reaction was initiated by bolus $ONOO^-$. Peroxynitrite was measured using the ESR signal of $CP^{\bullet}$ nitroxide; 100% is ESR signal in the absence of urate or its analogs. Urate was a more effective scavenger of $ONOO^-$ than xanthine or histamine ($p = 0.01$ for urate vs. xanthine; $p = 0.001$ for urate vs. histamine, $n = 4$ experiments).

3.3. Formation of free radical intermediates from the reaction of urate with $ONOO^-$

It has been shown that the oxidation of urate by $ONOO^-$ leads to formation of urate-derived radical intermediates [14], which might be responsible for the pro-oxidant effects of urate in the presence of $ONOO^-$ [7]. We therefore performed experiments using the spin trap EMPO to detect free radical intermediates formed during the reaction between $ONOO^-$ and urate. EMPO alone or mixed with urate did not yield an ESR signal (Fig. 4A and B),

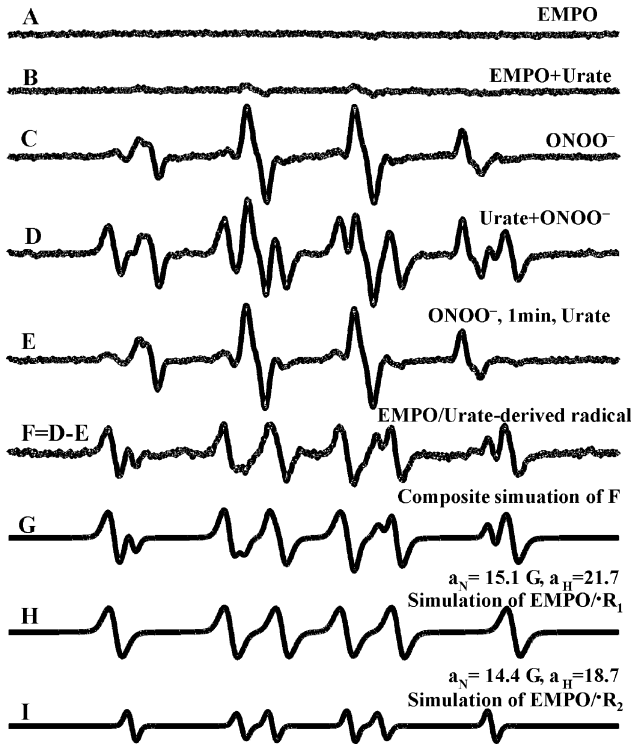
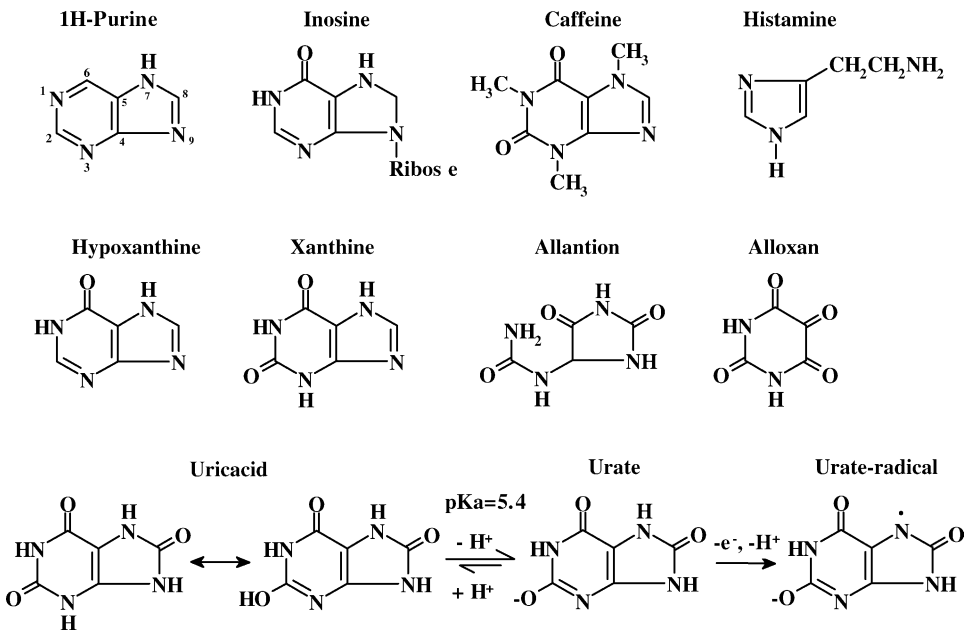


Fig. 4. Formation of the carbon-centered radical as intermediate in the reaction of urate with bolus $ONOO^-$. (A and B) EMPO (20 mM) as well EMPO plus urate (1 mM) did not yield an ESR spectra. (C) ESR signal of EMPO and bolus $ONOO^-$ (0.27 mM). (D) ESR spectra of EMPO and $ONOO^-$ in the presence of urate (1 mM). (E) Urate (1 mM) was added 1 min following initiating the reaction of EMPO with bolus $ONOO^-$. (F) ESR spectra obtained from the subtraction of spectra D and E. (G) Composite computer simulation of spectra F. (H and I) Computer simulations of EMPO/radical adducts contained components of two urate-derived radicals adducts: $EMPO/^{\bullet}R_1$ ($a_N = 15.1$ G, $a_H = 21.7$) and $EMPO/^{\bullet}R_2$ ($a_N = 14.4$ G, $a_H = 18.7$).



Scheme 1. Chemical structures of uric acid and purine-analogs.

while EMPO vortexed with bolus $ONOO^-$ yielded a typical 4-line ESR spectra characteristic of an EMPO/OH adduct (Fig. 4C). Peroxynitrite added to EMPO in the presence of urate resulted in formation of new EMPO/radical adducts observed as additional peaks in the ESR spectra (Fig. 4D). In control experiments where urate was added 1 min following the reaction of $ONOO^-$ and EMPO, we did not observe urate-derived radical adducts (Fig. 4E). These experiments confirmed that the reaction of urate with $ONOO^-$ produces urate-derived radicals. After subtraction of spectra E from D (Fig. 4D and E), we obtained an EMPO/urate-derived radical adduct spectra (Fig. 4F). Computer simulation of this spectrum revealed the presence of two EMPO/urate radical adducts (Fig. 4G). The composite simulation consisted of components $EMPO^{\bullet}/R_1$ and $EMPO/^{\bullet}R_2$ (Fig. 4H and I), which have hyperfine-coupling constants consistent with urate-derived carbon-centered radicals.

Taken together these spin trapping experiments confirm that oxidation of urate by $ONOO^-$ results in formation of urate-derived carbon-centered free radical intermediates. With computer simulation we identified two urate-derived radicals, which could contribute to oxidative damage.

3.4. Urate oxidation by $ONOO^-$ and interactions with ascorbate and thiols

The above data show that ascorbate is only marginally effective in scavenging peroxynitrite (Fig. 1J). In prior studies, however, it has been reported that $ONOO^-$ treatment of human plasma consumes ascorbate [14]. Using direct ESR spectroscopy we performed experiments with urate, $ONOO^-$ and ascorbate to examine interactions between these compounds. PBS buffer as well ascorbate in buffer did not yield an ESR signal (Fig. 5A). Bolus addition of $ONOO^-$ (0.27 mM) to ascorbate resulted in a 2-line ESR spectrum (Fig. 5B). Neither decomposed $ONOO^-$ nor NaOH as the solvent for $ONOO^-$ produced an ESR signal when exposed to ascorbate (data not shown). Computer simulation of spectra B confirmed assignment of the ESR spectrum of the reaction between ascorbate and $ONOO^-$ as the ascorbyl radical (Fig. 5D). Interestingly, bolus $ONOO^-$ addition to ascorbate in the presence of urate decreased the ESR signal intensity of the ascorbyl radical (Fig. 5C) indicating that urate effectively scavenged $ONOO^-$ and thus decreased ascorbyl radical generation by $ONOO^-$.

Based on these findings, we next sought to determine if ascorbate might interact with urate-derived radicals formed in the reaction of $ONOO^-$ with urate, which were previously detected by EMPO (Fig. 4F). Urate oxidized by bolus $ONOO^-$ yielded a carbon-centered radical (Fig. 6A). In the presence of ascorbate, however, the ESR spectrum revealed additional peaks (Fig. 6B), while the intensity of ESR components of the original urate-derived radical adducts was decreased. In a control sample where ascorbate was added 1 min after the reaction of urate with $ONOO^-$ (Fig. 6C), the previously observed ESR lines in Fig. 6B became smaller implying partial reduction of radical adduct by ascorbate. With computer simulation,

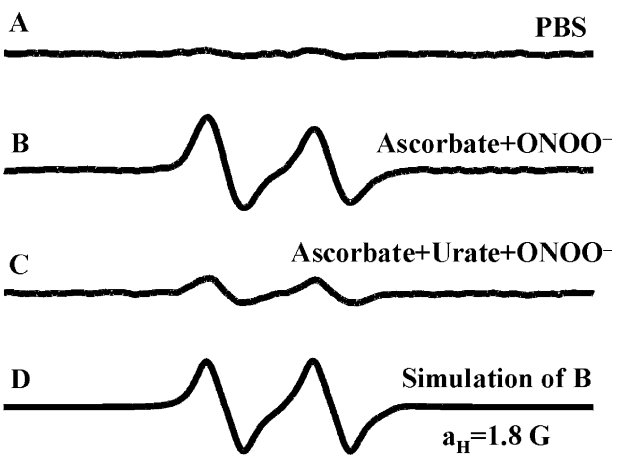


Fig. 5. Formation of ascorbyl radical in the reaction of ascorbate with bolus $ONOO^-$. (A) PBS containing 25 mM $NaHCO_3$ at pH 7.4. (B) ESR spectra of the reaction mixture ascorbate (25 μM) and bolus $ONOO^-$ (0.27 mM) measured by direct ESR spectroscopy. (C) Inhibition of ascorbyl radical formation in the presence of urate (50 μM). (D) Computer simulation of spectra B (a_H = 1.8 G, line-width 0.1 G) for verification of ascorbyl radical.

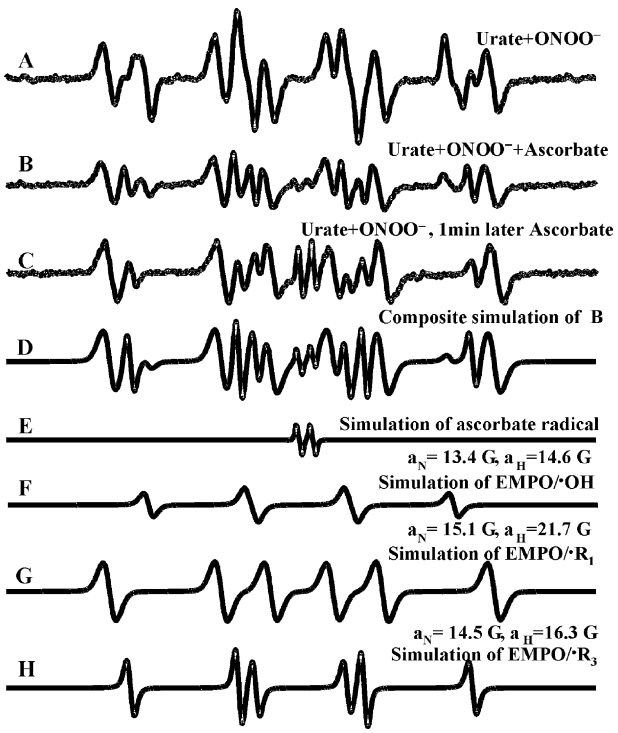


Fig. 6. Interaction of urate-derived radicals with ascorbate. (A) ESR spectra of EMPO (20 mM) mixed with urate (50 μM) and bolus $ONOO^-$ (0.27 mM). (B) ESR signal of EMPO, urate, $ONOO^-$ in the presence of ascorbate (25 μM). (C) Ascorbate was added 1 min after the reaction of EMPO, urate and $ONOO^-$. (D) Computer simulation of the ESR spectrum (B). (E) Computer simulation of ascorbyl radical component. (F) Computer simulation of $EMPO/^{\bullet}OH$ component. (G) Simulation of $EMPO/^{\bullet}R_1$ radical adduct. (H) Computer simulation of $EMPO/^{\bullet}R_3$ component.

we analyzed the different radical components (Fig. 6D–H) formed upon reactions of urate, $ONOO^-$ and ascorbate (Fig. 6B). The ascorbyl radical (Fig. 6E) was identified as one component. The EMPO/$^{\bullet}$OH adduct was identified as second component (Fig. 6F). Beside the component EMPO/$^{\bullet}R_1$ (Fig. 6G), which has already been identified in Fig. 4H, an EMPO/$^{\bullet}R_3$ adduct was identified as a new component of this reaction (Fig. 6H). Thus, ascorbate reduces formation of the intermediate R1 that results from the reaction of urate with $ONOO^-$, and completely prevents formation of R2, while introducing a new intermediate R3. Experiments with bolus $ONOO^-$ and urate in the presence of cysteine showed that cysteine decreased formation of the EMPO/urate-derived radical adduct (data not shown) but the changes were not as pronounced as in the presence of ascorbate.

We next studied the effect of ascorbate and cysteine on formation of urate-derived radicals caused by 1 mM SIN-1, which releases both superoxide and nitric oxide, forming $ONOO^-$ (1 μM/min as estimated using ESR and the spin probe CPH). EMPO mixed with SIN-1 resulted in formation of traces of an EMPO/OH adduct (Fig. 7C), while addition of urate yielded a spectra compatible with a combination of EMPO/urate-derived radical adducts (Fig. 7D). The presence of cysteine and ascorbate, however, inhibited formation of EMPO/urate adduct while EMPO/OH adduct was not affected by cysteine (Fig. 7E and F). The combination of both antioxidants inhibited the EMPO/urate-derived radical adduct more than either alone (Fig. 7G). These data provide further support of co-operation between antioxidants to inhibit formation of urate-derived free radical intermediates.

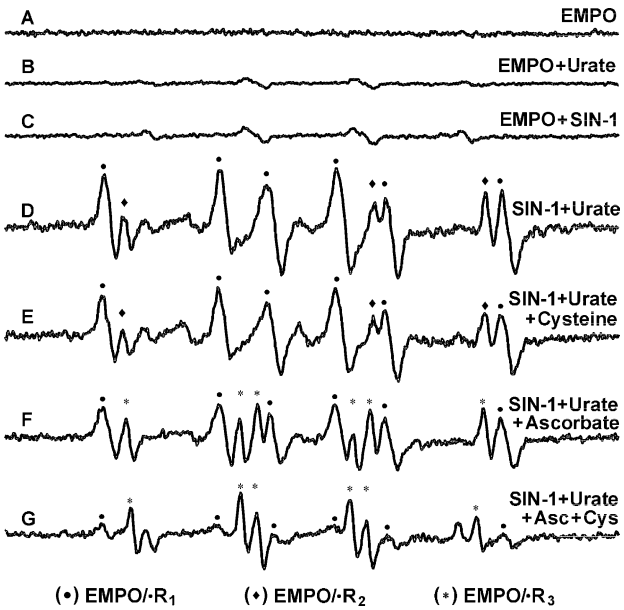


Fig. 7. Interaction of urate-derived radicals with ascorbate and cysteine. (A) ESR spectra of EMPO (20 mM). (B) Spectra of EMPO with urate (1 mM). (C) ESR signal of EMPO and SIN-1 (1 mM). (D) Spectra of EMPO, SIN-1 and urate with formation of EMPO/$^{\bullet}R_1$ (•) and EMPO/$^{\bullet}R_2$ (◆) radical adducts. (E) ESR spectra of EMPO, SIN-1 and urate in the presence of cysteine (25 μM) that yields EMPO/$^{\bullet}R_1$ (•) and EMPO/$^{\bullet}R_2$ (◆) radical adducts. (F) ESR spectra of EMPO, SIN-1 and urate in the presence of ascorbate (25 μM) with formation of a new radical component EMPO/$^{\bullet}R_3$ (*) radical adduct. (F) ESR spectra of EMPO, SIN-1 and urate in the presence of ascorbate and cysteine that shows EMPO/$^{\bullet}R_3$ (*) radical adduct.

3.5. Effect of urate on eNOS uncoupling by $ONOO^-$-donor SIN-1

The above data indicate that both ascorbate and cysteine inhibit formation of potentially damaging radical intermediates formed by the reaction of $ONOO^-$ with urate. Their interactions might therefore improve the ability of urate to act as an antioxidant in vivo. In endothelial cells, an important effect of $ONOO^-$ is oxidation of tetrahydrobiopterin, a critical co-factor for nitric oxide synthase [15]. This leads to a condition referred to as eNOS uncoupling, associated with a reduction in the nitric oxide production and an increase in $O_2^{\bullet-}$ production. We therefore performed additional studies to determine if urate, ascorbate and cysteine are able to prevent eNOS uncoupling upon exposure to $ONOO^-$ using $ONOO^-$-donor SIN-1 (0.5 mM) to model physiological production of $ONOO^-$ (0.5 μM/min). Superoxide production by BAECs was estimated by SOD-inhibitable accumulation of $CM^{\bullet}$ nitroxide radical followed by the amplitude of the low-field component of the ESR spectra. Untreated cells demonstrated minimal SOD-inhibitable $CM^{\bullet}$ accumulation (Fig. 8A) and no inhibition by L-NAME implying coupled eNOS. In contrast, cells exposed to peroxynitrite robustly increased superoxide production, which was inhibited by L-NAME and BH_4 indicating superoxide generation by uncoupled eNOS (Fig. 8B). Urate partially prevents this effect of $ONOO^-$ on superoxide production, while the combination of urate, ascorbate and cysteine further inhibited stimulation of superoxide production by in $ONOO^-$ (Fig. 8C and D). In contrast to the inhibitory effect of L-NAME on $O_2^{\bullet-}$ production in $ONOO^-$-treated cells, L-NAME increased CMH-oxidation in cells exposed to $ONOO^-$ in the presence of urate or urate plus ascorbate and cysteine (Fig. 8G and H). Thus, exposure of cells to these scavengers restored the pattern of response to L-NAME to that observed in control cells (Fig. 8E). Inhibition of signals by SOD in these experiments confirmed that they were due to CMH oxidation of $O_2^{\bullet-}$ (Fig. 8I). BH_4 addition to $ONOO^-$-treated cells also diminished endothelial cell superoxide production, in keeping with the concept that eNOS uncoupling was due to BH_4 oxidation (Fig. 8J).

The activity of eNOS was also determined by measuring $NO^{\bullet}$ production in BAECs using the $NO^{\bullet}$-specific colloid spin trap $Fe(DETC)_2$. Because $Fe(DETC)_2$ cannot be used in cell suspensions, and bolus $ONOO^-$ can only be used in cell suspensions, we examined the effect of SIN-1-generated $ONOO^-$ on cellular $NO^{\bullet}$ production. Colloid $Fe(DETC)_2$ in a cell free sample did not yield an ESR signal (data not shown). SIN-1 treatment significantly decreased BAEC $NO^{\bullet}$ production observed in controls

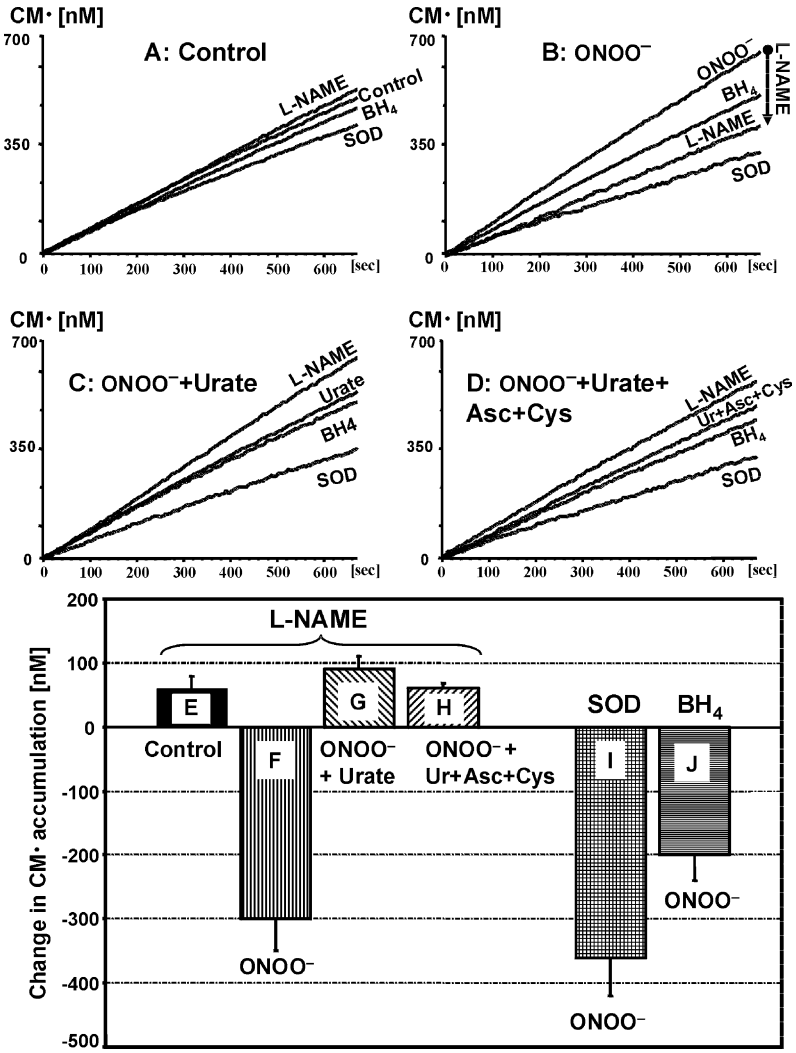


Fig. 8. Uncoupling of eNOS by $ONOO^-$. Effects of urate, ascorbate and cysteine on ROS production in $ONOO^-$-treated BAECs. (A) ROS production as $CM^•$ accumulation in untreated control BAECs and after L-NAME or SOD pre-incubation. (B) ROS production in bolus $ONOO^-$ (0.27 mM)-treated cells. Arrow indicates inhibition of ROS production by L-NAME plotted in (F). (C) ROS production in cells exposed to $ONOO^-$ in the presence of urate (100 μM). (D) ROS production in BAECs exposed to $ONOO^-$ in the presence of urate (100 μM), ascorbate (25 μM) and cysteine (25 μM). (E) Increase of $CM^•$ accumulation by L-NAME in untreated cells. (F) Inhibition of $CM^•$ accumulation (equals superoxide production) by L-NAME in $ONOO^-$-treated cells. (G and H) Increase of $CM^•$ accumulation by L-NAME in cells exposed to $ONOO^-$ in the presence of urate or urate combined with ascorbate and cysteine. (I and J) Inhibition of superoxide production by SOD and BH_4 in $ONOO^-$-treated cells. Inhibition of ROS production by L-NAME, SOD and BH_4 demonstrates uncoupling of eNOS, while increase in ROS production by L-NAME (E, G, H) shows the presence of coupled eNOS. Data are presented as mean ± standard error, $n \geq 4$ experiments.

cells (Fig. 9A and B). In contrast, $NO^•$ production was preserved in BAECs exposed to SIN-1 in the presence of urate, although less then that observed in control cells (Fig. 9A and B). Urate in the presence of either ascorbate or cysteine or both antioxidants, however, preserved $NO^•$ production to values similar those that observed in control cells (Fig. 9A and B), which were significant. BH_4 supplementation after SIN-1 exposure partially recovered eNOS activity (Fig. 9A).

It should be noted that treatment of BAECs with BH_4 unlikely interfered with either superoxide measurements or nitric oxide detection. To avoid such interference BH_4 containing media was removed from cells prior to measurements. Further, supplementation of control BAECs with BH_4 did not change the amount of detected ROS (Fig. 8A) and NO production [15]. The concentration of BH_4 is too low (~1 μM, rate constant $10^5\ M^{-1}\ s^{-1}$) to compete with CMH spin probe (1 mM, rate constant $10^4\ M^{-1}\ s^{-1}$) for superoxide and it definitely cannot compete with NO, which reacts with superoxide with nearly diffusion-limited rate [7]. Thus, direct scavenging of superoxide by intracellular BH_4 was likely insignificant in these studies. Moreover, loading cells with PEG–SOD, which may restore bioactive NO to the normal level under oxidative stress [22,23,28] did not increase NO production in $ONOO^-$-treated BAECs. This supports eNOS uncoupling rather than inactivation of NO by superoxide in $ONOO^-$-treated cells.

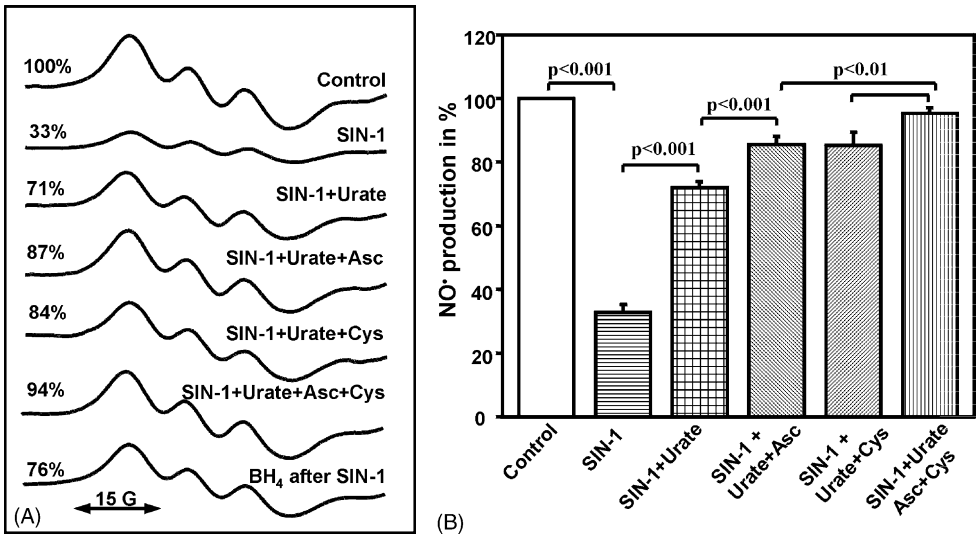
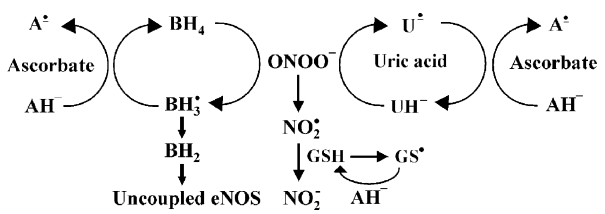


Fig. 9. Prevention of $ONOO^-$ mediated eNOS uncoupling by urate, ascorbate and cysteine. (A) ESR spectra of $NO^•$–$Fe(DETC)_2$ in untreated control bovine aortic endothelial cells; in SIN-1 (0.5 mM)-treated cells; in cells exposed to SIN-1 and urate (100 μM); in cells treated with SIN-1, urate and ascorbate (25 μM); in cells treated with SIN-1, urate and cysteine (25 μM); in cells exposed to SIN-1 in the presence of urate, ascorbate and cysteine; and in cells incubated with BH_4 (10 μM) after the treatment with SIN-1. $NO^•$ signal in control cells was set as 100%. (B) $NO^•$ production in percentage is presented in columns. ESR signals of $NO^•$–$Fe(DETC)_2$ were significantly different with $p < 0.01$–0.001, $n \geq 4$ experiments.

4. Discussion

It has been previously shown that urate is an efficient scavenger of nitrogen dioxide in the plasma [30], while the efficiency of reactions with $ONOO^-$ and superoxide radical had not been studied. In the present study, we demonstrated that $ONOO^-$ reacts with physiological concentrations of urate approximately 15 times faster than it does with ascorbate and 3 times more rapidly than with cysteine (Fig. 2), while urate does not scavenge superoxide (Fig. 1). Previous spin trapping studies did not take into account effect of CO_2 on reactions of $ONOO^-$ [36]. This work used buffers containing 25 mM $NaHCO_3$ at pH 7.4 to ensure physiological conditions that may be responsible for modulation of $ONOO^-$ reactivity by CO_2 [36]. Using EMPO spin-trapping experiments, carbon-centered free radicals were detected as intermediate products of the reaction between $ONOO^-$ and urate, which in turn reacted with ascorbate or cysteine. Both of these antioxidants inhibited formation of the carbon-centered radical adducts. Comparison of $ONOO^-$ scavenging by various purine derivatives revealed a unique reactivity of urate (Fig. 3), which was shared with xanthine and histamine but not other analogs (Scheme 2), indicating that the five-membered ring of the purine structure likely plays a major role in the $ONOO^-$ scavenging. Finally, we demonstrated that these interactions have in vivo relevance by examining their ability to preserve eNOS function in endothelial cells. In these experiments, the combination of urate and either ascorbate or cysteine was effective in preventing uncoupling of eNOS by $ONOO^-$.



Scheme 2. Possible interactions of $ONOO^-$, urate, ascorbate and cysteine.

By examining the competition between the CPH and various antioxidants, we were able to compare the rate constants of $ONOO^-$ reactions with urate, ascorbate and cysteine. The rate constants of $ONOO^-$ reactions with ascorbate and cysteine have been previously determined to be 236 and 10^3 M^{-1} s^{-1} [31–33]. Our data indicate that the rate constant of urate's reaction with $ONOO^-$ is close to the rate constant of cysteine with $ONOO^-$. This combination of a high rate constant and the high concentration of urate in human plasma (200 μM urate versus 25 μM cysteine) suggests that urate likely plays a critical role in scavenging peroxynitrite in the extracellular space. The role of urate in scavenging of intracellular peroxynitrite requires further investigation.

It has been previously described that tautomers of uric acid (Scheme 1) form the urate anion ($pK_a = 5.4$) through N3 deprotonation [42] and that one electron oxidation of urate produces a radical located primarily on the five-membered ring of the purine structure [43]. We assume that the reaction of peroxynitrite with urate causes a similar one electron oxidation of five-membered ring. Furthermore, a one electron oxidation of xanthine has been reported to occur at the C8-position, which is located on the five-membered ring [42]. Interestingly, we found that histamine with the five-membered ring also reacted with peroxynitrite (Fig. 3). The dramatic

difference in the reactivity of purine derivatives with peroxynitrite is likely associated with their redox potentials. The most efficient scavenger, uric acid has the lowest standard potential of 260 mV [44] followed by xanthine (590 mV, pH 13) and hypoxanthine (780 mV). Indeed, hypoxanthine, with a redox high potential of 780 mV [44], did not react with peroxynitrite (Fig. 3). Purine-analogs with redox potentials higher than 700 mV (purine, adenine, inosine) also did not scavenge peroxynitrite (Fig. 3).

We found that scavenging of $ONOO^-$ by urate was significantly increased in the presence of ascorbate and cysteine (Fig. 2). Reaction of the urate radical with ascorbate was previously described by Maples and Mason [43]. Our data indicate that a co-operative interaction exists between urate, ascorbate and cysteine in scavenging $ONOO^-$. This was confirmed by the effect of ascorbate and cysteine on free radical intermediates observed in the EMPO spin trapping experiments (Figs. 4, 6, 7). The aromatic structure and location of the uncoupled electron on nitrogen makes it difficult to detect a urate radical with spin traps. It is, therefore, likely that the spin trap EMPO detected secondary urate-derived radicals. It has been previously suggested that oxidation of urate to alloxan is an important step in the generation of urate-derived radicals [7,14]. Our preliminary data support generation of carbon-centered radicals from alloxan, suggesting that cysteine and ascorbate may inhibit decomposition of urate to alloxan. Taken together, our results suggest that the effect of either cysteine or ascorbate is to convert the urate-derived radicals back to urate thus increasing the ability of the latter to scavenge $ONOO^-$ and decreasing formation of free radical intermediates (Scheme 2) that might have importance for the antioxidant properties of urate. Thus, while urate-derived radicals might cause damage [7], plasma ascorbate and thiols can effectively eliminate these radicals and improve the antioxidant effects of urate. Given these considerations, it is likely that the reaction between urate and $ONOO^-$ has different consequences depending on its microenvironment and the level of co-existent antioxidants such as ascorbate and cysteine.

In previous studies, we have shown that an important effect of $ONOO^-$ is oxidation of the endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH_4), leading to uncoupling of the enzyme [15,34]. In these studies, we found that ascorbate and thiols are relatively ineffective in preventing BH_4 oxidation by $ONOO^-$. In contrast to these direct effects of either ascorbate or cysteine, these agents seemed to markedly augment the effect of urate in preventing $ONOO^-$ mediated eNOS uncoupling. Previously, it was shown that ascorbate can act as a ''free radical sink'' reducing the intermediate $BH_3^\bullet$ radical, which was formed upon the reaction of BH_4 with peroxynitrite [15]. Ascorbate has high reactivity with secondary radical intermediates derived from $ONOO^-$ mediated oxidation. While $O_2^{\bullet -}$ scavenging [35] and recycling BH_4 [15] might be a mechanism for improvement in endothelium-dependent vasodilatation our current data would indicate that another effect of Vitamin C might involve recycling of urate and protection from urate-derived radicals.

Our studies provide insight into the debate whether urate is pro- or antioxidant. In previous studies of human plasma treated with $ONOO^-$, urate-based radicals have been suggested to increase oxidative damage [13,14]. Urate has also been shown to amplify $ONOO^-$ mediated oxidation of liposomes and LDL [7]. Additionally, urate has been demonstrated to increase inactivation of α1-antiproteinase [36] and alcohol dehydrogenase promoted by irradiation [37]. Urate, however, was shown to be an effective inhibitor of $ONOO^-$ mediated nitration [11], and an additional protection against $ONOO^-$ by urate might be due to interaction with heme proteins [38]. Our current data support protection of endothelial cell by urate from $ONOO^-$ mediated damage by direct $ONOO^-$ scavenging.

Epidemiological studies have demonstrated a positive correlation between plasma urate levels and the risk of cardiovascular diseases [4,5], however, such correlations might be associated with increased activity of xanthine oxidase [6] or co-morbid conditions such as obesity and diabetes, rather than pro-oxidative properties of urate itself. Moreover, administration of urate to humans has been shown to prevent the increase in plasma isoprostanes caused by exercise [39]. We have also shown that increasing uric acid levels in hypercholesterolemic mice prevents oxidative inactivation of the Cu/Zn-containing superoxide dismutases [40]. Recently, it has been suggested that the neuro-protective effect of urate is due to $ONOO^-$ scavenging [9]. Several clinical studies have shown that administration of urate or its precursor inosine can hinder multiple sclerosis progression [12]. It is now well established that numerous common diseases such as hypercholesterolemia, hypertension, diabetes and heart failure are associated with a loss of $NO^\bullet$ production by the endothelium, a condition commonly referred to as endothelial dysfunction [41]. In many of these conditions, eNOS uncoupling by $ONOO^-$ or similar oxidants seems to be present, leading to an increase in endothelial cell $O_2^{\bullet -}$ production and a decrease in $NO^\bullet$ production. Of note, the urate precursor inosine can be administered orally and is effective in inhibiting $ONOO^-$ mediated MS progression [12]. It is interesting to speculate that increasing urate levels in vivo would prove protective against $ONOO^-$ mediated disorders.

Acknowledgments

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