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Redox properties of the nitronyl nitroxide antioxidants studied via their reactions with nitroxyl (HNO) and ferrocyanide

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Abstract

Nitronyl nitroxides (NNs) are the paramagnetic probes that capable of scavenging physiologically relevant reactive oxygen (ROS) and nitrogen (RNS) species, namely superoxide, nitric oxide (NO) and nitroxyl (HNO). NNs are increasingly considered as potent antioxidants and potential therapeutic agents. Understanding redox chemistry of the NNs is important for their use as antioxidants and as paramagnetic probes for discriminative detection of NO and HNO by electron paramagnetic resonance (EPR) spectroscopy. Here we investigated the redox properties of the two most commonly used NNs, including determination of the equilibrium and rate constants of their reduction by HNO and ferrocyanide, and reduction potential of the couple NN/hydroxylamine of NN (hNN). The rate constants of the reaction of the NNs with HNO were found to be equal to $(1-2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ being close to the rate constants of scavenging superoxide and NO by NNs. The reduction potential of the NNs and iminonitroxides (INs, product of NNs reaction with NO) were calculated based on their reaction constants with ferrocyanide. The obtained values of the reduction potential for NN/hNN ($E'_0 \approx 285\text{mV}$) and IN/hIN ($E'_0 \approx 495\text{mV}$) are close to the corresponding values for vitamin c and vitamin e, correspondingly. The “balanced” scavenging rates of the NNs towards superoxide, NO and HNO, and their low reduction potential being thermodynamically close to the bottom of the pecking order of oxidizing radicals, might be important factors contributing into their antioxidant activity.

Introduction

Nitronyl nitroxides, NNs, are widely recognized to be specific scavengers of nitric oxide and antagonists of NO action in biological systems [1-3]. They have been used in numerous studies to prove NO involvement in various physiological processes as well as for NO detection by EPR spectroscopy [4-8]. The reaction of NNs with nitric oxide has been well studied and the products of the reaction, iminonitroxide, IN, and NO_2 , clearly identified [1-4, 9-11]. Reactivity of the NNs towards nitroxyl, HNO, the product of one-electron reduction of NO, has been also reported [12, 13], and the reaction products were identified to be hydroxylamine of iminonitroxide, hIN, and NO_2 [13].

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Figure 1 shows the structures of the two NNs most commonly used in biological systems as NO scavengers, cPTIO and NN⁺, the mechanisms of their reactions with NO and HNO and illustration of the typical EPR spectral pattern for NN (quintet) and IN (septet). In general, the formation of different products in the reactions of NNs with NO and HNO allows for discriminative detection of NO and HNO using EPR technique. However, the observed fast reduction of the NNs and INs into diamagnetic hydroxylamines in biological samples makes these measurements difficult. A routine procedure of re-oxidation of the hcPTIO and hcPTI to the corresponding nitroxides by mild oxidizing agent, potassium ferricyanide, has been used [8] for quantification of the conversion of NN to IN in the reaction with NO. Here we demonstrate that this approach does not provide quantitative oxidation of hIN resulting in underestimation of the amount of IN formed and NO scavenged. Moreover this method does not allow for the discrimination of HNO and NO contributions in the EPR signal of reoxidized hIN.

We applied liposome-encapsulated membrane-impermeable NN⁺ probe for discriminative NO and HNO detection, the liposome membrane being permeable for both NO and HNO specimen but protective against biological reductants [13]. The rate constants of the reactions of cPTIO and NN⁺ with NO are high enough ($\approx 0.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [1, 2]) to justify their use as NO antagonist, and for accumulation sufficient product amounts for EPR detection in biological samples. The rate constant of the cPTIO reaction with HNO measured spectrophotometrically using NH₂OH as competitive reagent was found to be even slightly higher ($2.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [13]). In this work we performed EPR measurements of the rate constant of HNO reaction with NN⁺ and re-evaluated the rate constant of HNO reaction with cPTIO. To prevent oxidative decomposition of Angeli's salt used as HNO donor [14], these measurements were performed in the presence of the mild reducing agent, potassium ferrocyanide. The kinetics and equilibrium rate constants of the redox reactions of NNs and INs with potassium ferrocyanide were measured and taken into consideration for the quantitative data analysis.

NNs were found to be protective against oxidative stress in vitro [15, 16] and in vivo [17, 18] and are increasingly considered as potent antioxidants and potential therapeutic agents. The capacity of the NNs to scavenge a variety of reactive nitrogen (RNS) [10, 13] and oxygen (ROS) [10, 16, 19] species may be a key factor contributing to their antioxidant activity. Another important factor of antioxidant activity of NNs might be comparatively low reduction potentials of the NNs/hNNs and INs/hINs redox couples, the values evaluated in this work being thermodynamically close to the bottom of the pecking order of oxidizing radicals [20].

Materials and Methods

Reagents

Potassium ferricyanide, K₃Fe(CN)₆; sodium phosphate dibasic, Na₂HPO₄; sodium phosphate monobasic, NaH₂PO₄, were purchased from Fisher Scientific. Ascorbic acid; hydroxylamine, NH₂OH; diethylene triamine pentaacetic acid, DTPA, were bought from Acros Organics. Angeli's salt, Na₂N₂O₃; Proli NONOate; 2-(4-carboxyphenyl)-4, 5-dihydro-4, 4, 5, 5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide monopotassium salt, cPTIO,

were purchased from Cayman Chemicals. Potassium ferrocyanide, $K_3Fe(CN)_6 \cdot 3H_2O$ was purchased from Sigma-Aldrich. 4, 4, 5, 5-Tetramethyl-2- [4-trimethylammoniophenyl]-2-imidazoline-3-oxide-1-yloxy methyl sulfate (NN^+) and 4, 4, 5, 5-Tetramethyl-2- [4-trimethylammoniophenyl]-2-imidazoline-1-yloxy methyl sulfate (IN^+) were synthesized as previously described [2].

Stock solutions of Angeli's salt (AS) were prepared daily in 10 mM NaOH, concentration of AS was determined using extinction coefficient $8300\text{ M}^{-1}\text{cm}^{-1}$ on wavelength 248 nm [21]. All other solutions were prepared in 50 mM Na-phosphate buffer, pH 7, 0.5 mM DTPA. IN radical solutions ($cPTI$ or IN^+) were prepared from corresponding solutions of NNs ($cPTIO$ or NN^+) by titration with small aliquots of NO donor, Proli NONOate, until disappearance of the EPR signal of the NN. Water was purified using Milli-Q purification system.

EPR measurement

EPR spectra were acquired in 50- μl capillary tubes using an EMX X-band spectrometer (Bruker).

UV-visible studies

UV-visible spectroscopy studies were performed using Varian Cary Bio and Beckman-Coulter DU800 spectrophotometers equipped with thermostated cell holder. The solutions of hydroxylamines of nitroxides, $hcPTIO$, $hcPTI$, hNN^+ , and hIN^+ were prepared by their reduction using ascorbic acid. It was found that 1 mole of ascorbic acid reduces 2.5 mole of nitroxide in accordance with a previous observation that dehydroascorbic acid (two-electron oxidation product of ascorbic acid) also contributes to the nitroxide reduction [22].

In the studies of the reaction of AS with $cPTIO/hcPTIO$ mixture the solution of 400 μM $cPTIO$ and 200 μM $hcPTIO$ was prepared by addition of 80 μM ascorbic acid to 600 μM of initial $cPTIO$ solution. In the studies of AS with $hcPTIO$ the solution of 200 μM $hcPTIO$ was prepared by addition of 110 μM ascorbic acid to 200 μM of initial $cPTIO$ solution.

Stopped-flow studies

Stopped-flow studies were performed in quartz cuvette (lightpass 1 cm) using SFM300 (Bio-Logic) stopped-flow system equipped with MOS-200M rapid-kinetics optical registration system (Bio-Logic).

Calculations

Calculations were performed using MATLAB and Origin packages.

Results

Reversible reduction of NNs and INs by ferrocyanide

Figure 2 illustrates reduction of the nitroxides, NN^+ and IN^+ , to the corresponding hydroxylamines upon titration with potassium ferrocyanide measured from the decay of the EPR signal intensity. Almost stoichiometric reduction of the IN^+ and about 20% reduction of NN^+ even at 8-fold excess of the ferrocyanide over NN^+ were observed.

Figure 3 illustrates recovery of the nitroxides NN^+ and IN^+ from the corresponding hydroxylamines, hNN^+ and hIN^+ , upon titration with potassium ferricyanide measured from the increase of the EPR signal intensity. Almost stoichiometric recovery of the NN^+ and less than 40% recovery of IN^+ even at more than 10-fold excess of the ferricyanide over hIN^+ were observed. The data in Figures 2 and 3 clearly illustrate difference in redox properties of the NN/hNN and IN/hIN redox couples. To quantify these differences we measured equilibrium constants of their reactions with ferricyanide/ferrocyanide redox couple.

The reaction equilibrium between nitroxide/hydroxylamine and ferricyanide/ferrocyanide couple for NN and IN is described by the following reactions:

Equilibrium constants can be expressed as follows:

$$\frac{[hNN] \times [Fe(CN)_6]^{3-}}{[NN] \times [Fe(CN)_6]^{4-}} = K_{NN} \quad (3)$$

$$\frac{[hIN] \times [Fe(CN)_6]^{3-}}{[IN] \times [Fe(CN)_6]^{4-}} = K_{IN} \quad (4)$$

To find the equilibrium constant K_{NN} the dependence of NN EPR signal intensity on ferrocyanide concentration (Fig. 2) was linearized according to equation (5):

$$\frac{[\Delta NN]^2}{([NN]_0 - [\Delta NNH])} = K_{NN} \times ([Fe(CN)_6]^{4-}]_0 - [\Delta NN]) \quad (5)$$

where $[NN]_0 = 5 \times 10^{-4}$ M – concentration of NN before ferrocyanide addition in concentration $[Fe(CN)_6]^{4-}]_0$; $[NN]$ – concentration of NN which was reduced upon the ferrocyanide addition and equals to the concentrations of formed hNN and ferricyanide. The equilibrium constant K_{NN} equals to the slope of this linearized dependence (Fig. 4A) and was calculated to be $(5.5 \pm 0.6) \times 10^{-3}$ for NN^+ and $(5.3 \pm 0.4) \times 10^{-3}$ for $cPTIO$.

To find the equilibrium constant K_{IN} the dependence of IN EPR signal intensity on ferricyanide concentration (Fig.3) was linearized according to equation (6):

$$\frac{[\Delta IN] \times ([IN]_0 + [\Delta IN])}{([IN_T] - [IN]_0 - [\Delta IN])} = \frac{1}{K_{IN}} \times ([Fe(CN)_6]^{3-}]_0 - [\Delta IN]) \quad (6)$$

where $[IN]_0$ – concentration of IN before ferricyanide addition in concentration $[Fe(CN)_6^{3-}]_0$; $[IN]$ – concentration of IN formed via oxidation of hIN by ferricyanide; $[IN]_T$ is a total concentration of IN and hIN in the sample, 0.5 mM. The value of equilibrium constant K_{IN} equals to reciprocal of the slope of this linearized dependence and was calculated for cPTI and IN^+ to be equal to 20 ± 1 (Fig. 4B).

An about four thousands difference in equilibrium constants of the reaction of NN and IN reduction with ferrocyanide explains previously shown quantitative oxidation of hNN to NN by IN [11]. Indeed, the redox equilibrium between NN/hNN and IN/hIN redox couples is described by the equation:

with the equilibrium constant, $K_{INNN} = K_{IN}/K_{NN} \approx 4 \times 10^3$, therefore supporting a strong shift of the equilibrium to the right. This equilibrium is established in a few seconds supporting the fast oxidation of hNN by IN. The typical kinetics of hNN oxidation measured using spectrophotometric stopped-flow technique are shown in Figure 5A for hcPTIO oxidation by cPTI. The dependencies of the observed rate constants on the concentration of IN are shown in Fig. 5B yielding the bimolecular rate constants for the reaction of IN with hNN equal to (880 ± 10) for cPTI and $(180 \pm 10) \text{ M}^{-1}\text{s}^{-1}$ for IN^+ . Table 1 lists the equilibrium and bimolecular rate constants for the reactions of NNs and INs reduction by ferrocyanide, hNNs and hINs oxidation by ferricyanide, and NNs and INs reduction by hydroxylamines, hINs and hNNs, correspondingly. The equilibrium constants were measured by EPR and the bimolecular rate constants were measured using spectrophotometric stopped-flow technique.

Measurement of bimolecular rate constant of nitronyl nitroxide reaction with HNO

The kinetics of NNs reaction with HNO were studied by EPR using AS as a source of HNO. Note that AS undergoes accelerated decomposition in the presence of NNs [14]. This oxidative decomposition of AS is catalyzed by highly oxidizing NO_2 radical formed during the reaction of NNs with HNO (see Fig.1). An addition of potassium ferrocyanide as NO_2 scavenger (rate constant, $4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) [23] inhibits NO_2 -catalyzed AS decomposition resulting in more than 10-fold decrease of the rate of NNs decay in the presence of AS (Fig. 6). Ferrocyanide also inhibits NO_2 -induced oxidation of the NN and IN to the corresponding oxoammonium cations. However, partial reduction of the NN^+ into EPR-silent hNN^+ of about 10 % at equimolar 0.2 mM concentrations of the NN^+ and ferrocyanide was observed in quantitative agreement with the value of the corresponding equilibrium constant (Table 1).

To avoid significant NNs reduction by ferrocyanide and corresponding complication in the kinetics analysis, an addition of ferricyanide can be used. Therefore, the further measurements of the bimolecular rate constants of the NNs with HNO were performed in AS/ferrocyanide/ferricyanide system using NH_2OH as a competitive reagent (see Figure 7). The observed rate of NN^+ decay, 53.3 nM/s, in the presence of 50 μM AS and ferrocyanide/ferricyanide mixture (Fig. 7A, B) is in an agreement with the rate of HNO release by AS during its spontaneous decomposition (half lifetime about 16–18 min at 25 °C [15,16]) while

decrease in the initial NNs concentrations did not exceed 5% being close to the experimental error of the EPR measurements of the absolute radical concentration. The experimental data were analyzed according to the following equations taking into account the oxidation of HNO by ferricyanide with formation of nitric oxide [24, 25]:

$$V_0 = k_{NN} [NN] \times [HNO] + k_{FeIII} \times [Fe(CN)_6^{3-}] \times [HNO] + k_H [NH_2OH] \times [HNO] \quad (8)$$

$$V = k_{NN} \times [NN] \times [HNO] + k_{FeIII} \times [Fe(CN)_6^{3-}] \times [HNO] \quad (9)$$

$$\frac{V_0}{V} - 1 = \frac{k_H \times [NH_2OH]}{k_{NN} \times [NN] + k_{FeIII} \times [Fe(CN)_6^{3-}]} \quad (10)$$

where V_0 – rate of HNO generation which decays in the reactions with NN, ferricyanide (rate constant, k_{FeIII}) and NH_2OH (rate constant, $k_H = 0.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ [26]); V – rate of NN decay in the reactions with HNO and NO formed via HNO oxidation by ferricyanide (NN is a predominant scavenger of NO in the system). The dependencies of the term ($V_0/V - 1$) on NH_2OH concentration for NN^+ (measured at two different concentrations of ferricyanide) and cPTIO are shown in Figure 7B. Analyzing the data by the linear fits according to eq. (10) we obtained the values of the rate constants, $k_{NN}(NN^+) = (2.0 \pm 0.5) \times k_H = (0.8 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_{NN}(\text{cPTIO}) = (1.7 \pm 0.2) \times k_H = (0.7 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{FeIII} = (0.24 \pm 0.17) \times k_H = (0.96 \pm 0.07) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$.

Alternatively, we measured the bimolecular rate constant for cPTIO reaction with HNO based on competitive pathways of HNO decay via HNO dimerization to nitrous oxide and HNO scavenging by cPTIO. Figure 8 shows the concentration dependencies of the rates of cPTIO decay measured by EPR in AS/ferricyanide/ferricyanide system in argon- and air-saturated buffers that clearly indicate an increase of HNO scavenging rates with an increase of cPTIO concentration. In the absence of oxygen HNO decays via dimerization and competitive reactions with cPTIO and ferricyanide, while in the presence of oxygen an additional pathway for HNO decay via its reaction with oxygen has to be taken into account. Experimental data were analyzed according to the following equations:

$$V_0 = k_{AS} \times [AS] = k_{NN} \times [NN] \times [HNO] + k_{FeIII} \times [Fe(CN)_6^{3-}] \times [HNO] + k_{ox} \times [O_2] \times [HNO] + 2 \times k_D \times [HNO]^2 \quad (11)$$

$$V = k_{NN} \times [NN] \times [HNO] + k_{FeIII} \times [Fe(CN)_6^{3-}] \times [HNO] \quad (12)$$

$$V = \frac{k_{NN} \times [NN] + k_{FeIII} \times [Fe(CN)_6^{3-}]}{4 \times k_D} \times \left\{ \sqrt{(k_{NN} \times [NN] + k_{FeIII} \times [Fe(CN)_6^{3-}] + k_{ox} \times [O_2])^2 + \frac{8 \times k_{AS} \times [AS]}{k_D}} - (k_{NN} \times [NN] + k_{FeIII} \times [Fe(CN)_6^{3-}] + k_{ox} \times [O_2]) \right\} \quad (13)$$

where V_0 – rate of HNO generation during AS decomposition (the rate constant, k_{AS}) which is equal to the rate of HNO decay in the reactions with NN, ferricyanide, oxygen (rate

constant, k_{ox}) and due to its dimerization (rate constant, $k_D = 8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ [21]); V – rate of NN decay in the reactions with HNO and NO formed via HNO oxidation by ferricyanide. Fitting the experimental data by equation (13) (Fig. 8) yield close values of the rate constants obtained in oxygen-free and air-saturated solution, namely $k_{NN} = (1.7 \pm 0.5) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_{FeIII} = (2 \pm 1) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k_{AS} = (1.1 \pm 0.05) \times 10^{-3} \text{ s}^{-1}$ (which is in agreement with the rate of spontaneous AS decomposition [15,16]). The fitting also yield the value of $k_{ox} = (8 \pm 2) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$.

Discussion

Redox properties of NN/hNN and IN/hIN couples

Low reactive ROS and RNS, namely superoxide ($O_2^{\bullet-}$), NO $^{\bullet}$ and HNO, play important roles in various physiological and pathophysiological processes. At low concentrations they are utilized as signaling molecules, while at high concentrations and being in imbalance with antioxidants they may start free radical chain cascade resulting in generation of highly toxic ROS/RNS species such as hydroxyl and peroxy radicals, and peroxynitrite. Due to comparatively low reactivity and transient character of NO, HNO and superoxide, their scavenging and detection is difficult. The NNs, such as cPTIO and NN $^+$, were initially claimed to be selective NO scavengers and have been used in numerous biological systems as NO antagonists and for NO detection by EPR spectroscopy [2, 8, 11, 19]. However, subsequent studies questioned NNs specificity as NO scavenger revealing their high reactivity towards superoxide [10, 19] and HNO [12, 13]. The rate constants for the cPTIO reactions with ($O_2^{\bullet-}$), NO and HNO were found to be close to $10^4 \text{ M}^{-1}\text{s}^{-1}$ [2, 10, 13]. The previously reported rate constant of cPTIO reaction with HNO, $k_{NN}(\text{cPTIO}) = (2.2 \pm 0.4) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, was measured based on competition of cPTIO and NH_2OH for HNO using optical detection [13]. In this work we obtain similar value for the $k_{NN}(\text{cPTIO}) = (1.7 \pm 0.5) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ based on competition of HNO decay via dimerization and in reaction with cPTIO. This value of $k_{NN}(\text{cPTIO})$ and slightly lower value of $k_{NN}(\text{cPTIO}) = (0.7 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ found by EPR detection of cPTIO decay in HNO-generating systems in the presence of NH_2OH as competitive reagent, might be considered as being in a reasonable agreement taking into account complex composition of the experimental samples. The rate constant of the reaction of NN $^+$ with HNO was measured in this work for the first time and was found to be equal to $(0.8 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. Recently we proposed discriminative detection of NO and HNO [13] based on high reactivity of the NNs with these species and EPR-distinguishable products of the corresponding reactions (see Fig.1). In the proposed approach we have used liposome-encapsulated NN $^+$ to avoid fast reduction of the radical in biological systems, including that in the reaction with superoxide.

The “balanced” scavenging of the $O_2^{\bullet-}$, NO and HNO by the NNs with the similar rate constants of about $10^4 \text{ M}^{-1}\text{s}^{-1}$ might be a key factor contributing in the reported antioxidant activity of NNs [15-18]. Another important factor for the redox-active molecule to be an efficient antioxidant is the value of its reduction potential which has to be thermodynamically close to the bottom of the pecking order of oxidizing radicals to diminish its pro-oxidative activity [20]. In this work we determined equilibrium and bimolecular rate constants of the NNs and INs with ferrocyanide, and hNNs and hINs with

ferricyanide. Assuming reduction potential of the ferricyanide/ferrocyanide couple in 50 mM phosphate buffer, $E'_0(\text{ferri}/\text{ferrocyanide})=418\text{mV}$ [27], and using Nernst equation we calculated the values of reduction potential for NN/hIN and IN/hIN couples as follows:

$$E'_0(\text{NN}/\text{hNN})=E'_0(\text{ferri}/\text{ferrocyanide})+(RT/F)\times\ln K_{\text{NN}}=418\text{mV}+59\text{mV}\times\log(0.0053)=284\text{mV} \quad (14)$$

$$E'_0(\text{IN}/\text{hIN})=418\text{mV}+59\text{mV}\times\log(20)=495\text{mV} \quad (15)$$

The calculated value of $E'_0(\text{cPTIO}/\text{hcPTIO})=284\text{mV}$ ($E'_0(\text{NN}^+/\text{hNN}^+)=285\text{mV}$) is in a good agreement with the previously reported value of 270 mV measured for cPTIO electrochemically [10]. Note that reduction potentials of NN/hNN and IN/hIN redox couples are close to the values of natural antioxidants, vitamin C or ascorbate ($E'_0=282\text{mV}$) and vitamin E or α -tocopherol ($E'_0=500\text{mV}$), correspondingly. Not surprisingly, similar to the recycling of α -tocopherol via reduction of α -tocopherol radical by ascorbate [28], hNN recycle hIN via reduction of IN radical in reaction (7).

Both hydroxylamines, hNN and hIN, are oxidized by mild oxidizing agent, potassium ferricyanide ($E'_0=418\text{mV}$) to the corresponding radicals via reverse reactions (1) and (2) ($k_r\approx 3.4\times 10^3\text{ M}^{-1}\text{s}^{-1}$ for NN^+). Therefore, it is expected that hNNs will effectively scavenge highly oxidizing ROS and RNS such as hydroxyl ($E'_0=2.31\text{V}$ [29]), alkyl ($E'_0\approx 1.9\text{V}$), alkylperoxyl ($E'_0\approx 1\text{V}$) [30], and nitrogen dioxide $E'_0=0.99\text{V}$ [31]) radicals forming NNs. In its turn, NNs scavenge low-reactive $\text{O}_2^{\bullet-}$, NO and HNO species forming hNN, IN or hIN, correspondingly, with the similar rate constants of about $10^4\text{ M}^{-1}\text{s}^{-1}$. These redox cycles detoxify highly reactive oxidants and prevent radical chain formation by scavenging otherwise low-reactive radicals. The only reactive byproduct of these reactions is nitrogen dioxide radical, $\text{NO}_2^{\bullet}$, which is scavenged by hNN and hIN. An ability of hNN and hIN to scavenge $\text{NO}_2^{\bullet}$ radicals has been recently used for preventing $\text{NO}_2^{\bullet}$ -induced oxidative decomposition of AS and for discriminative detection of NO and HNO by EPR approach [13]. Figure 9 illustrates the redox cycling of the NNs that may provide a key mechanism underlying their antioxidant activity.

The nitroxides are considered as low molecular weight antioxidants and superoxide dismutase (SOD) mimics [32] with diverse therapeutic applications (for recent review see [33]). For the most nitroxide types, except oxazolidine nitroxides [34], their SOD-mimetic activity is attributed to redox cycling between nitroxide and its one-electron oxidation product, oxoammonium cation [35]. Due to high oxidizing potential of the oxoammonium cation (e.g., 0.81 V for oxoammonium cation of Tempol [35]) it is capable of oxidizing the target molecule that it was supposed to have protected, therefore resulting in pro-oxidative side effect of the nitroxide antioxidants [36]. Contrary to the redox cycle of these nitroxide SOD mimics [35], major intermediates of the NNs redox cycling (Fig. 9) are chemically inert hydroxylamines, hNN and hIN, and NNs and INs radicals with comparatively low reduction potentials. A combination of (i) unique capacity of the NNs to scavenge low-

reactive ROS and RNS, (ii) scavenging of highly reactive ROS and RNS by the hydroxylamines, and (iii) a chemical inertness of the NNs and the major products of their redox cycle, might be responsible for their reported antioxidant activity in various biological systems [15-18].

Acknowledgments

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List of abbreviation

AS	Angeli's salt
cPTI	2-(4-carboxyphenyl)-4, 5-dihydro-4, 4, 5, 5-tetramethyl-1H-imidazolyl-1-oxy monopotassium salt
cPTIO	2-(4-carboxyphenyl)-4, 5-dihydro-4, 4, 5, 5-tetramethyl-1H-imidazolyl-1- oxy-3-oxide monopotassium salt
DTPA	diethylene triamine pentaacetic acid
EPR	electron paramagnetic resonance
hcPTIO	hydroxylamine of cPTIO
hIN	hydroxylamine of iminonitroxide
hNN	hydroxylamine of nitronyl nitroxide
hNN⁺	hydroxylamine of NN ⁺
IN	imino nitroxide
IN⁺	4, 4, 5, 5-Tetramethyl-2- [4-trimethylammoniophenyl]-2-imidazoline-1-yloxy methyl sulfate
NN	nitronyl nitroxide
NN⁺	4, 4, 5, 5-tetramethyl-2- [4-trimethylammoniophenyl]-2-imidazoline-3-oxide-1- yloxy methyl sulfate
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase

Fig. 1.

The structures of the NNs, cPTIO ($\text{X}=\text{COOH}$) and NN^+ ($\text{X}=\text{N}^+(\text{CH}_3)_3$), the scheme of their reactions with NO and HNO, and typical EPR spectral pattern of NN (quintet, 1:2:3:2:1) and IN (septet, 1:1:2:1:2:1:1).

Figure 2. Reduction of the nitroxides, NN⁺ (●) and IN⁺ (○), to the corresponding hydroxylamines by potassium ferrocyanide illustrated by decrease of the EPR-measured radical concentrations upon titration with ferrocyanide.

Figure 3.

Oxidation of the hydroxylamines, hNN^+ (●) and hIN (○) to the corresponding nitroxides, NN^+ and IN^+ , by potassium ferricyanide measured by EPR and plotted as an increase of the radical concentrations, $\Delta[\text{Nitroxide}]$. The hydroxylamine solutions ($\approx 400 \mu\text{M}$) were prepared via reduction of the corresponding nitroxides by ascorbate ($\approx 160 \mu\text{M}$) as described in **Materials and Methods**

Fig 4.

A. The linearized dependence of NN reduction on potassium ferrocyanide concentration (see eq. (5)) for reaction between NN^+ (●) or cPTIO (○) and ferrocyanide. Lines represent fits yielding equilibrium constant equal to $(5.3 \pm 0.4) \times 10^{-3}$ and $(5.5 \pm 0.6) \times 10^{-3}$ for cPTIO and NN^+ , correspondingly. **B.** The linearized dependence of hIN oxidation on potassium ferricyanide concentration (see eq. (6)) for reaction between hIN^+ (□) or hcPTI (■) and ferricyanide. Lines represent fits yielding equilibrium constant equal to 20 ± 1 for both IN^+ and cPTI.

Fig. 5.

Oxidation of hcPTIO by cPTI. **A.** The kinetics of absorbance change at wavelength 560 nm measured after mixing of 200 μM hcPTIO with cPTI in concentration 0.76 ($\bullet$), 1.52 ($\square$) and 3.04 ($\circ$) mM at temperature 25 $^{\circ}\text{C}$. Solid lines represent monoexponential fits yielding observed rate constants, k_{obs} , equal to 0.625, 1.37 and 2.56 s^{-1} , correspondingly. **B.** The dependences of k_{obs} on IN concentration for cPTI ($\square$) and IN^+ ($\circ$). Lines represent fits yielding bimolecular rate constants for the reaction of IN with hNN equal to (880 ± 10) for cPTI ($\square$) and $(180 \pm 10) \text{ M}^{-1}\text{s}^{-1}$ for IN^+ ($\circ$).

Fig. 6.

The kinetics of NN^+ decay measured by EPR in the presence of Angeli's salt and various concentrations of ferrocyanide at 28 °C. Initial concentrations were as follows: NN^+ , 200 μM ; AS, 73 μM ; ferrocyanide, 0 mM ($\circ$), 0.1 mM ($\square$), 0.2 mM ($\bullet$) and 1 mM ($\blacksquare$). Lines represent the monoexponential approximations with time constant 80 s ($\circ$), 830 s ($\square$), 1010 s ($\bullet$), 1450 s ($\blacksquare$). Note significant decrease in initial NN^+ concentration of about 6% ($\square$), 10% ($\bullet$) and 17% ($\blacksquare$) due to reduction by ferrocyanide in a good agreement with the values calculated based on equilibrium constant (Table1). This reduction results in artificial overestimation of the time constant of the NN^+ decay at high concentration of ferrocyanide.

Fig. 7.

Measurements of the bimolecular rate constants of the NNs reaction with HNO in AS/ferrocyanide/ferricyanide system using NH_2OH as competitive reagent. **A.** The kinetics of NN^+ decay (initial concentration, 400 μM) in the presence of 50 μM Angeli's salt, 200 μM ferrocyanide, 200 μM ferricyanide in the absence (□) and presence of various concentrations of NH_2OH : 1mM (○), 2 mM (●) and 4 mM (■) measured by EPR at 25 °C. Lines represent linear fits yielding rates of NN^+ decay equal to 53.3 nM/s (□), 26.2 nM/s (○), 17.9 nM/s (●), 10.6 nM/s (■). **B.** The dependence of (V/V_0-1) term on NH_2OH concentration for NNs decay rates measured in the following systems: (□) described in (A); (○) same as (A) except 900 μM ferricyanide and 88 μM AS; (●) same as (A) except 400 μM cPTIO was taken instead of NN^+ and 75 μM AS. Lines represent linear fits yielding slopes equal to $1200 \pm 200 \text{ M}^{-1}$ (□), $1000 \pm 100 \text{ M}^{-1}$ (○), and $1350 \pm 100 \text{ M}^{-1}$ (●) which correspond to the rate constants $k_{\text{NN}}(\text{NN}^+) = (2.0 \pm 0.5) \times k_{\text{H}}$, $k_{\text{NN}}(\text{cPTIO}) = (1.7 \pm 0.2) \times k_{\text{H}}$ and $k_{\text{FeIII}} = (0.24 \pm 0.17) \times k_{\text{H}}$.

Fig. 8.

The dependences of the rates of cPTIO decay on the cPTIO concentration measured by EPR after AS addition in argon- (●) and air-saturated (□) solutions (0.25 mM of oxygen) at 25 °C. The samples contain 200 μM $K_4Fe(CN)_6$, 200 μM $K_3Fe(CN)_6$ and 85 μM AS (□) or 60 μM AS (●). Solid lines represent fits yielding the values of the rate constants, $k_{NN} = (1.7 \pm 0.5) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_{FeIII} = (2 \pm 1) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_{AS} = (1.1 \pm 0.05) \times 10^{-3} \text{ s}^{-1}$ and $k_{Ox} = (8 \pm 2) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ assuming HNO dismutation rate constant $k_D = 8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ [21].

Fig. 9. Redox cycling of the NNs

NNs scavenge low-reactive NO, HNO and $O_2^{\bullet-}$, species forming IN, hIN and hNN, correspondingly. IN is a subject for one-electron reduction, including that by superoxide, forming hIN. Both hNN and hIN scavenge highly oxidizing ROS/RNS, including $NO_2^{\bullet}$, byproduct of the reactions of NNs with NO and HNO.

Table 1

Equilibrium and bimolecular rate and constants for the redox reactions of NNs and INs.

Reaction	$k_b, M^{-1}s^{-1}$		$k_r, M^{-1}s^{-1}$		K_{eq}	
	cPTIO/cPTI	NN ⁺ /IN ⁺	cPTIO/cPTI	NN ⁺ /IN ⁺	cPTIO/cPTI	NN ⁺ /IN ⁺
(1)	2.1±0.3 ^a	19±2 ^a	400±40	3400±100	(5.3±0.4)×10 ⁻³	(5.5±0.6)×10 ⁻³
(2)	1020±10	3600±200	51±3 ^b	180±10 ^b	20±1	20±1
(7)	880±10	180±10	0.23±0.02 ^b	0.05±0.006 ^b	3800±300 ^c	3600±400 ^c

^a Calculated from K_{eq} and k_r using equation $k_f = K_{eq}k_r$;

^b calculated from K_{eq} and k_f using equation $k_r = k_f/K_{eq}$;

^c calculated from the values of K_{eq} for the reactions (1) and (2) using equation $K_{INNN} = K_{IN}/K_{NN}$.