RESEARCH COMMUNICATION

Oxidative denitrification of *N*^ω-hydroxy-L-arginine by the superoxide radical anion

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The superoxide radical anion ($O_2^{\cdot -}$) produced during the catalytic activity of nitric oxide synthase (NOS) and cytochrome *P*-450 has been implicated in the oxidative denitrification of hydroxyguanidines ($>C=NOH$). The reactivity of the radiolytically generated $O_2^{\cdot -}$ radical with *N*^ω-hydroxy-L-arginine (NHA) is pH dependent and appears to parallel the prototropic equilibrium of the hydroxyguanidino group ($>C=NOH \rightleftharpoons >C=NO^- + H^+$; $pK = 8$). The *N*^ω-hydroxyguanidino group is more reactive towards $O_2^{\cdot -}$ when deprotonated but exhibits negligible reactivity when protonated. Based on a model, the rate constant for the

reaction of the $O_2^{\cdot -}$ with NHA was estimated as $k(O_2^{\cdot -} + >C=NO^-) \approx 200\text{--}500\text{ M}^{-1}\text{ s}^{-1}$, which is probably too low to compete with $O_2^{\cdot -}$ reactions with $NO^\bullet$ or superoxide dismutase, which occur many orders of magnitude faster. The oxidative elimination of NO from NHA by $O_2^{\cdot -}$ was not accompanied by the formation of L-citrulline. Since only 21% of NHA will exist in the deprotonated $>C=NO^-$ form at physiological pH, it is unlikely that oxidative denitrification of NHA by cytochrome *P*-450 or NOS-derived $O_2^{\cdot -}$ radicals will prove a major free-radical pathway to $NO^\bullet$ and L-citrulline.

INTRODUCTION

NO synthases (NOSs; EC 1.14.13.39) are cytochrome *P*-450-type haemoproteins [1], which catalyse the oxidation of L-arginine to $NO^\bullet$ with the concomitant formation of L-citrulline [2]. Since *N*^ω-hydroxy-L-arginine (NHA) was identified as a stable intermediate in the L-arginine/ $NO^\bullet$ pathway [3], the mechanism of the NOS-catalysed conversion of NHA to $NO^\bullet$ and L-citrulline has been a matter for debate [4]. To account for the electron transfer, it has recently been suggested that a hydrogen-atom transfer from the *N*^ω-hydroxyguanidino group ($>C=NOH$) of NHA to the peroxyhaem complex, $[FeOO^\bullet]^{2+}$, is the most likely possibility, based on thermodynamic considerations [5]:

Subsequent nucleophilic attack of the hydroperoxyl species $[FeOOH]^{2+}$ on the iminoxyl radical ($>C=NO^\bullet$) is believed to account for the generation of L-citrulline ($>C=O$) and $NO^\bullet$ via the reaction:

It has also been proposed that one-electron oxidation may occur at some other point during the biosynthesis of $NO^\bullet$ [4]. For example, superoxide dismutase has been shown to enhance the formation of free $NO^\bullet$ from L-arginine by NOS, by catalysing the oxidation of an intermediate nitrogen oxide species, possibly nitroxyl (NO^-) to $NO^\bullet$ [6]. Evidence has also been obtained for the formation of NO^- during the hydrogen peroxide-supported oxidation of NHA by NOS [7]. It has been shown that calmodulin binding to NOS occurs in response to elevated Ca^{2+} concentrations, and this enables flavin-to-haem interdomain electron transfer [8,9]. Haem reduction is associated with increased NADPH oxidation and results in superoxide radical anion ($O_2^{\cdot -}$)

production. Substrates for NOS, including L-arginine and NHA, have been shown to inhibit calmodulin-dependent $O_2^{\cdot -}$ or H_2O_2 production by neuronal NOS [10,11].

Exogenous NHA causes nitrite production in vascular smooth-muscle cells devoid of NOS activity [12]. Moreover, cytochrome *P*-450 and peroxidases are also capable of catalysing the oxidative denitrification of NHA to $NO^\bullet$ and citrulline [13,14]. Both observations indicate that NHA may liberate nitrogen oxides via NOS-independent pathways, which may also be exploited by non-physiological hydroxyguanidines. Human promyelocytic leukaemia HL60 cells, which contain both myeloperoxidase and cytochrome *P*-450 enzymes [15], can metabolize hydroxyguanidine to $NO^\bullet$ and urea [16]. Cytochrome *P*-450 can catalyse the oxidative denitrification of hydroxyguanidine and related compounds, including aldioximes, ketoximes, amidoximes and guanidoximes [17,18]. The formation of $O_2^{\cdot -}$ after the decoupling of the peroxyhaem complex, $[FeOO^\bullet]^{2+} \rightarrow Fe^{3+} + O_2^{\cdot -}$, in cytochrome *P*-450 is believed to represent an important free-radical pathway to the oxidative denitrification of NHA [19]. Indeed, the $O_2^{\cdot -}$ radical has been shown to cleave oxidatively the $>C=NOH$ bond in amodioximes and hydroxyguanidines to produce NO_2^- and NO_3^- ions [20]. In light of the possible involvement of $O_2^{\cdot -}$ in the enzymic conversion of L-arginine to $NO^\bullet$ [21], the mechanism of the oxidative denitrification of NHA by radiolytically generated $O_2^{\cdot -}$ radicals was investigated.

MATERIALS AND METHODS

Chemicals and reagents

NHA, *S*-nitroso-*N*-acetyl-L-penicillamine and NOR-1 were obtained from Alexis Biochemicals. $HCOONa$ and potassium salts were obtained from Merck. Water was purified with a Milli-Q system and saturated with oxygen (British Oxygen Company) for approx. 10 min before irradiation.

Abbreviations used: NOS, nitric oxide synthase; $O_2^{\cdot -}$, superoxide radical anion; NHA, *N*^ω-hydroxy-L-arginine; $[FeOO^\bullet]^{2+}$, peroxyhaem complex; $ONOO^-$, peroxynitrite; OPA, o-phthalaldehyde; $Fe(III)$ -cyt c, ferricytochrome c.

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Radiolytic generation of $O_2^{\cdot-}$ radicals

The $O_2^{\cdot-}$ radical was generated by radiolysis of O_2 -saturated aqueous solutions containing 0.1 M $HCOONa$, 100 μM EDTA and 1 mM phosphate buffer. Under these experimental conditions the primary radical species are rapidly converted into $O_2^{\cdot-}$ via the reactions [22]:

In this system the radiation chemical yield, $G(O_2^{\cdot-})$, is 0.68 $\mu M \cdot J^{-1}$ [23]. Steady radiolysis produces radicals at a readily varied but quantifiable rate; pulse radiolysis with spectrophotometric detection enables the kinetic properties of radicals to be measured directly [24]. The pulse radiolysis facility at the Gray Laboratory Cancer Research Trust has been described previously [25]. The $O_2^{\cdot-}$ radicals were generated by 30 ns electron pulses, which produced doses of 5–10 Gy determined by KSCN dosimetry. The kinetics of the $O_2^{\cdot-}$ radical-induced reduction of ferricytochrome *c* [Fe(III)-cyt *c*] were evaluated at 550 nm, utilizing a tungsten lamp and a photodiode detector, which facilitated measurements approx. 0.1 s after the electron pulse. For product analysis, steady-state γ -radiolysis experiments were performed in gas-tight vials, using a 2000 Ci ^{60}Co γ -ray source at dose rates of 0.9–26 $Gy \cdot min^{-1}$, as determined by Fricke dosimetry [26]. Samples were analysed immediately post-irradiation in order to minimize the hydrolysis of NHA, particularly at pH > 8.5.

Identification of products derived from NHA

NO was determined following autooxidation to NO_2^- ions by ion-chromatography system 2 as described previously [27]. The remaining products from the oxidation of NHA were determined by ion-pair reverse-phase HPLC on a LiChrospher 100 RP-18 (5 μm) 125 mm $\times$ 4 mm column (LiChroCart), with the eluent 1.5% acetonitrile/6 mM pentane sulphonic acid/10 mM H_3PO_4 /10 mM KH_2PO_4 at a flow rate of 1.4 $ml \cdot min^{-1}$. Detection of products containing primary amino groups was achieved spectrophotometrically (at 214 nm) and following post-column derivatization by 5 mM *o*-phthalaldehyde (OPA)/12 mM 2-mercaptoethanol/0.4 mM potassium borate at pH 10. The combined flows were passed through a 1 ml coil at 40 $^\circ C$, and OPA-derivatized products were detected by fluorescence (λ_{ex} at 345 nm, λ_{em} at 550 nm) [28].

RESULTS AND DISCUSSION

Figure 1 shows a typical product profile obtained after radiolysis (approx. 100 Gy at 0.94 $Gy \cdot min^{-1}$) of an O_2 -saturated aqueous solution containing 100 μM NHA, 0.1 M $NaHCO_2$, 100 μM EDTA and 1 mM phosphate buffer at pH 8.2. An identical product profile has been observed for the oxidation of NHA by free radicals that mimic peroxidase catalytic activity [30], suggesting that the $O_2^{\cdot-}$ radicals perform a one-electron oxidation of the *N*^o-hydroxyguanidino group of NHA. The concentration of NHA (Figure 1, peak a) was found to decrease with increasing radiation dose, and new products were formed in a concentration proportional to dose (Figure 2). The formation of NO_2^- ions (Figure 1, peak f) during radiolysis was ascribed to the auto-oxidation of $NO\cdot$ in aqueous solution [31,32] and was indicative

Figure 1 Products formed by the oxidative denitrification of NHA by the $O_2^{\cdot-}$ radical

Product profile obtained on the steady-state γ -radiolysis (approx. 100 Gy at 0.94 $Gy \cdot min^{-1}$) of an O_2 -saturated aqueous solution containing NHA (100 μM) and $HCOONa$ (0.1 M) in phosphate buffer (1 mM) at pH 7.9. Peak designation: a, NHA; b, L-citrulline; c, NHA/ $NO\cdot$ adduct; d, L-arginine; e, putative cyclized carbodiimide (not amenable to OPA derivitization); f, NO_2^- ; and g, NO_3^- .

Figure 2 Oxidative degradation of NHA by the $O_2^{\cdot-}$ radical

Decline of NHA and formation of products after steady-state γ -radiolysis of 0.94 $Gy \cdot min^{-1}$ of an O_2 -saturated aqueous solution containing NHA (100 μM) and $HCOONa$ (0.1 M) in phosphate buffer (1 mM) at pH 7.9. Oxidative denitrification of NHA by the $O_2^{\cdot-}$ radical does not generate L-citrulline.

of the one-electron oxidative denitrification of NHA by the $O_2^{\cdot-}$ radical anion:

The peroxyxynitrite ion ($ONOO^-$) can be generated in aqueous solution after the conjugation of $NO^{\cdot}$ and $O_2^{\cdot-}$ radicals [33]:

Although $ONOO^-$ can rearrange to NO_3^- ions (which was not detected) the possibility of it generating small quantities of NO_2^- ions under these experimental conditions cannot be ruled out.

The addition of *S*-nitroso-*N*-acetyl-L-penicillamine or NOR-1 (recognized NO donor molecules) to NHA was found to generate a product that co-eluted with peak c in Figure 1. Both NHA and hydroxyguanidine have previously been shown to form adducts with NO [34–36], and peak c was identified as the NHA/ $NO^{\cdot}$ adduct from its retention time. Nevertheless, the radiation chemical yield of the NHA/ $NO^{\cdot}$ adduct was insignificant ($< 1\%$ of the radical yield), and it was concluded that the decline in NHA during radiolysis was essentially entirely due to the reaction with the $O_2^{\cdot-}$ radical.

In marked contrast to previously published observations of the cytochrome *P*-450-catalysed oxidation of NHA, no evidence was obtained for the formation of L-citrulline [13], which did not increase with increased production of $O_2^{\cdot-}$ (Figure 2), although small but variable quantities were present in unirradiated samples (Figure 1, peak b). L-Arginine was also present in unirradiated samples (Figure 1, peak d) but also did not increase with radiation dose, thereby ruling out the possibility of reduction of NHA to L-arginine by $O_2^{\cdot-}$.

However, a dose-dependent increase in peak e in Figure 1 did occur. As noted previously [29,30], peak e was not amenable to OPA derivatization when detected by HPLC. The α - NH_2 group is a most unlikely target for an $O_2^{\cdot-}$ radical and must therefore become masked by re-arrangement of an unstable intermediate formed on the reaction of $O_2^{\cdot-}$ with the *N*^o-hydroxyguanidino group of NHA. In previously proposed mechanisms for the L-arginine/ $NO^{\cdot}$ pathway, one-electron oxidation of NHA was believed to result in homolytic cleavage of the $>C=NOH$ bond to generate an unstable carbodiimide that hydrolyses to L-citrulline [37,38]. Similarly, homolytic cleavage of the $>C=NOH$ bond by the $O_2^{\cdot-}$ radical could also be envisaged, resulting in an unstable carbodiimide that would react with the α - NH_2 group to generate a guanidine group fused into a 7-membered ring. Although peak e in Figure 1 remains to be fully characterized, similar cyclization reactions of sterically unhindered carbodiimides [39] supports our argument.

pH and dose-rate dependence of superoxide reactivity toward NHA

Increasing pH increases the lifetime of superoxide by an order of magnitude per pH unit above approx. pH 5. This arises because the prototropic equilibrium [eqn. (9)] has a $pK_{[eqn.(9)]}$ of 4.8 and the $O_2^{\cdot-}$ radical reacts with $HO_2^{\cdot}$, but the reactivity of two $O_2^{\cdot-}$ radicals in aqueous solution is negligible [40].

The efficiency of decomposition of NHA and production of NO_2^- on reaction of the $O_2^{\cdot-}$ radical with NHA varied with pH and with dose rate (i.e. rate of generation of $O_2^{\cdot-}$ radicals). At pH 7.9 and a dose rate of $0.94 \text{ Gy} \cdot \text{min}^{-1}$ (radicals produced at $\sim 11 \text{ nM} \cdot \text{s}^{-1}$), the superoxide is expected to exist exclusively in

Table 1 Radiation chemical yields, G ($\mu\text{M} \cdot \text{J}^{-1}$), of products generated by the reaction of the $O_2^{\cdot-}$ radical with NHA

Products were analysed by HPLC and HPIC after steady-state γ -radiolysis of O_2 -saturated aqueous solutions containing NHA ($100 \mu\text{M}$), $HCOONa$ (0.1 M), EDTA ($100 \mu\text{M}$) and phosphate buffer (1 mM) at pH 7.9.

Products	G ($\mu\text{M} \cdot \text{J}^{-1}$)	
	Dose rate ...	$0.94 \text{ Gy} \cdot \text{min}^{-1}$ $26 \text{ Gy} \cdot \text{min}^{-1}$
NHA*	0.33	0.046
NO_2^-	0.28	0.042
NO_3^-	Not detected	Not detected
L-Citrulline	Not detected	Not detected
NHA/ $NO^{\cdot}$ adduct	Detected	Detected
L-Arginine	Not detected	Not detected
Cyclic carbodiimide	Detected	Detected

*Degradation of NHA

Figure 3 Effect of pH and rate of $O_2^{\cdot-}$ radical production on the oxidation of NHA

Steady-state γ -radiolysis of O_2 -saturated aqueous solution containing NHA ($100 \mu\text{M}$) and $HCOONa$ (0.1 M) in phosphate buffer (1 mM) was performed between pH 6 and 10 at low ($\bullet$) ($0.94 \text{ Gy} \cdot \text{min}^{-1}$) and high ($\circ$) ($25.9 \text{ Gy} \cdot \text{min}^{-1}$) dose rates (d.r.). The lines show predictions based on the model involving reaction of $HO_2^{\cdot}/O_2^{\cdot-}$ [eqn. (10a) and eqn. (10b)] competing with reaction between $O_2^{\cdot-}$ and NHA [eqn. (12)].

the deprotonated $O_2^{\cdot-}$ radical form and competing radical-radical reactions would be minimized. Nevertheless, $G(-\text{NHA}) = 0.33 \mu\text{M} \cdot \text{J}^{-1}$ represents only approx. 48 % of that expected from $G(O_2^{\cdot-}) = 0.68 \mu\text{M} \cdot \text{J}^{-1}$ (Table 1). At a higher dose rate of $25.9 \text{ Gy} \cdot \text{min}^{-1}$ the decomposition of NHA was reduced significantly, to approx. 7 % relative to the $O_2^{\cdot-}$ radical. NO_2^- formation, indicative of oxidative denitrification of NHA by $O_2^{\cdot-}$, paralleled the loss of NHA at different pH values and dose rates almost quantitatively (Table 1).

The data from Figure 3 suggest that the $O_2^{\cdot-}$ radical is more reactive towards the free base of NHA than towards its protonated conjugate, since the decomposition yield rises steeply around pH > 7.5 , and, denoting NHA as substrate X, deprotonation of XH^+ occurs with a $pK_{[eqn.(11)]}$ of 8.0 [41]:

An attempt to obtain a rate constant for the reaction of $O_2^{\cdot-}$ with NHA was made using a competition method with Fe(III)-cyt *c* as a standard [22]. The reduction of Fe(III)-cyt *c* to ferrous cytochrome *c* by the $O_2^{\cdot-}$ radical is accompanied by a decline in absorption at 550 nm, the kinetics of which are readily measurable by pulse radiolysis. A series of pulse radiolysis experiments (approx. 5 Gy) were performed on O_2 -saturated aqueous solutions containing 50 μ M Fe(III)-cyt *c*, 0.1 M HCOONa and 1 mM potassium phosphate buffer at pH 8.5 with various concentrations of NHA up to 200 μ M. However, even 200 μ M NHA did not prevent the $O_2^{\cdot-}$ radical-induced bleaching at 550 nm, indicating that $k_{[eqn.(12)]} \ll 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

In order to estimate the rate constant for the reaction of the $O_2^{\cdot-}$ radical with NHA, $k_{[eqn.(12)]}$, we first assumed reactivity towards XH^+ could be neglected, i.e. $k_{[eqn.(13)]} \ll k_{[eqn.(12)]}$:

This was deduced from Figure 3, which clearly shows that at pH 6, where 99% of NHA exists as XH^+ and 96% of the superoxide is deprotonated, no reaction was observed:

and that the reactivity of $HO_2^{\cdot}$ could be ignored at pH > 7. Then analysing reactions (9)–(12), utilizing the steady-state approximation ($d([HO_2^{\cdot}] + [O_2^{\cdot-}])/dt = 0$) and denoting $k_{[eqn.(10)]_{obs}}$ as the pH-dependent rate constant for superoxide decay, eqn. (10a) and eqn. (10b) yield:

$$[O_2^{\cdot-}] \approx \{[(k_{[eqn.(12)]}[X])^2 + 4 k_{[eqn.(10)]_{obs}} \times k_0]^{\frac{1}{2}} - k_{[eqn.(12)]}[X]\} / 2 k_{[eqn.(10)]_{obs}}$$

where k_0 is the rate of production of $O_2^{\cdot-}$, or the dose rate ($\text{Gy} \cdot \text{s}^{-1}$) $\times G(O_2^{\cdot-})$ ($\text{M} \cdot \text{J}^{-1}$). The expected rate of destruction of X is then given by

$$-d[X]/dt = k_{[eqn.(12)]} [O_2^{\cdot-}] \times [X]$$

from which the concentration change per unit dose is easily calculated.

Loss of NHA depended on the rate of superoxide generation (dose rate), as shown in Figure 3: the lower the $O_2^{\cdot-}$ flux, the greater the loss of NHA. This strongly suggests that $HO_2^{\cdot}/O_2^{\cdot-}$ was competing with the reaction with NHA, as assumed in the kinetic model. Figure 3 shows that up to pH ~ 8, the experimental data were consistent with $k_{[eqn.(12)]} \approx 200\text{--}500 \text{ M}^{-1} \cdot \text{s}^{-1}$. However, at higher pH values the loss of substrate was much lower than expected, reaching a maximum of ~ 0.4 $\mu\text{M} \cdot \text{Gy}^{-1}$ at the lowest dose rate (only ~ 59% of the radical yield). This may be explained by reaction (12) being best represented as an equilibrium, involving superoxide addition/elimination involving base-catalysed decomposition of the intermediate and hence restoration of the substrate at higher pH values:

Base-catalysed elimination of superoxide from peroxy radicals is well established [42].

In view of the uncertainties, further speculation is inappropriate. However, we feel the data provide evidence that the free base of NHA is more reactive towards superoxide than its conjugate acid, and that the rate constant for the reaction of the $O_2^{\cdot-}$ radical with the free base is unlikely to exceed a few hundred $\text{M}^{-1} \cdot \text{s}^{-1}$.

Proposed mechanism for oxidative denitrification of NHA by superoxide

The proposed reaction mechanism for the reaction of $O_2^{\cdot-}$ with NHA is shown in Figure 4. Product analysis has established that $O_2^{\cdot-}$, despite being a relatively weak oxidant [$E^0(O_2^{\cdot-}, H^+/HO_2^{\cdot})$

Figure 4 Proposed reaction scheme for the oxidative elimination of $NO^{\cdot}$ from NHA by the $O_2^{\cdot-}$ radical

Nucleophilic addition of the $O_2^{\cdot-}$ radical to the deprotonated N^w -hydroxyguanidino group of NHA generates an unstable intermediate species that eliminates NO without the concomitant formation of L-citrulline.

= +1 V] [43], can promote the oxidative denitrification of NHA. However, the reactivity of $O_2^{\cdot-}$ radicals towards NHA is facilitated by deprotonation of the N^w -hydroxyguanidino group according to the prototropic equilibrium reaction ($>C=NOH \rightleftharpoons >C=NO^- + H^+$; $pK = 8$). By analogy with the nucleophilic addition reaction of the $FeOOH^{2+}$ with the iminoxyl radical ($>C=N-O^{\cdot}$) proposed in the NOS-catalysed oxidation of NHA [4], we propose that deprotonation facilitates the nucleophilic addition of the $O_2^{\cdot-}$ radical to the $>C=NO^-$ group to generate an unstable adduct radical ($NHAOOH^{\cdot}$) via step A (see Figure 4). A similar reaction has been proposed for the reaction of the $O_2^{\cdot-}$ radical with NHA and non-physiological hydroxyguanidines, guanidoximes and amidoximes [18–20,44]. The decay of this radical does not, however, generate L-citrulline via step C, which has been previously shown to occur during the oxidative denitrification of NHA by the catalytic activity of cytochrome *P*-450 [13]. Alternatively, $O_2^{\cdot-}$ appears to generate what is tentatively identified as a cyclized carbodiimide and NO via step B. Steps A and B represent an overall one-electron oxidation of NHA by $O_2^{\cdot-}$, which provides the electron necessary for the generation of $NO^{\cdot}$.

Conclusions

Both NOS and cytochrome *P*-450 have been shown to catalyse the oxidative denitrification of NHA to $NO^{\cdot}$ and L-citrulline. At the physiological pH of 7.4, only 21% of NHA exists in the deprotonated $>C=NO^-$ form, and therefore only a fraction of NHA produced by NOS would be amenable to oxidative denitrification by $O_2^{\cdot-}$ produced during cytochrome *P*-450 and NOS catalytic activity. The rate constant $k(O_2^{\cdot-} + >C=NO^-) = 200\text{--}500 \text{ M}^{-1} \cdot \text{s}^{-1}$ estimated from this work is very slow and would be unable to compete with reactions such as the conjugation of the $O_2^{\cdot-}$ and $NO^{\cdot}$ radical, which occurs many orders of magnitude faster ($k = 6.7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) [45], or superoxide dismutase-catalysed dismutation of $O_2^{\cdot-}$. We therefore conclude that the oxidative denitrification of NHA by the $O_2^{\cdot-}$ radical is unlikely to contribute to the formation of $NO^{\cdot}$ or L-citrulline

during either NOS or cytochrome *P*-450 catalysis. Nevertheless, the *pK* of the *N*^ω-hydroxyguanidine group is clearly an important parameter that influences the reactivity toward the O₂^{•−} radical. This pathway may prove more important in the oxidative denitrification by cytochrome *P*-450 of non-physiological hydroxyguanidines and related compounds with different prototropic characteristics [18,20].

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