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Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide

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Both nitric oxide (NO) and superoxide are generated by macrophages, neutrophils and endothelial cells. It has been postulated that the generation of these two radicals under physiological conditions can lead to the formation of peroxynitrite and (as a result of the homolytic lysis of this molecule) the production of hydroxyl radicals. We have used 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), a sydnonimine capable of generating both NO and superoxide simultaneously, to test this hypothesis. SIN-1 (1 mM) generated superoxide and NO at rates of 7.02 μM/min and 3.68 μM/min respectively in phosphate-buffered saline, pH 7.2, at 37 °C. Incubation of SIN-1 with both deoxyribose and sodium benzoate resulted in the formation of malondialdehyde (MDA). In addition, the incubation of SIN-1 with sodium benzoate resulted in the production of compounds with fluorescence emission spectra characteristic of hydroxylated products. Both the production of MDA and the generation of fluorescent compounds were inhibited by the hydroxyl radical scavenger mannitol. In all the above respects, SIN-1 mimicked the production of hydroxyl radicals from the ascorbate-driven Fenton reaction. Catalase had no effect on the SIN-1-dependent generation of MDA, and superoxide dismutase was partially inhibitory. SIN-1 produces an oxidant with the properties of the hydroxyl radical by a mechanism clearly different to that of the Fenton reaction. We conclude that the simultaneous production of NO and superoxide from SIN-1 results in the formation of hydroxyl radicals.

INTRODUCTION

Oxidative stress is a phenomenon associated with many disease states, e.g. inflammation [1] and reperfusion injury [2]. It has been proposed that, under many circumstances, the production of hydroxyl radicals may contribute to the pathophysiology of these conditions [3]. The mechanism generally invoked for hydroxyl radical production involves the presence of superoxide, hydrogen peroxide and either iron or copper ions. This is shown in the sequence of reactions (1)–(3):

$$O_2^{\cdot-} + M^{n+} \longrightarrow O_2 + M^{(n-1)+} \quad (1)$$

$$O_2^{\cdot-} + M^{(n-1)+} + 2H^+ \longrightarrow H_2O_2 + M^{n+} \quad (2)$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + OH^- + {}^{\cdot}OH \quad (3)$$

The transition metal ion (M^{n+}) is reduced by superoxide (eqn. 1) and the reduced ion donates an electron to another molecule of superoxide to give hydrogen peroxide (eqn. 2). This then reacts with reduced metal ions from eqn. (1) to produce hydroxyl radicals (eqn. 3) in what is referred to as the Fenton reaction. Recently, a novel mechanism for hydroxyl radical production, which is not dependent on the presence of transition metal ions, has been proposed [4]. This involves the production of peroxynitrite arising from the reaction of nitric oxide (NO) with superoxide, as shown in reactions (4)–(7):

$$NO^{\cdot} + O_2^{\cdot-} \longrightarrow ONOO^- \quad (4)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (5)$$

$$ONOOH \longrightarrow {}^{\cdot}OH + NO_2^{\cdot} \quad (6)$$

$$2H^+ + O_2^{\cdot-} + O_2^{\cdot-} \longrightarrow H_2O_2 + O_2 \quad (7)$$

The combination of NO with superoxide proceeds, at 20 °C, with a rate constant of 3.7×10^7 $M^{-1} \cdot s^{-1}$ [5]. The reaction will thus effectively compete with the dismutation of superoxide (reaction 7), the rate constant of which has been calculated to be 5×10^5 $M^{-1} \cdot s^{-1}$ at pH 7 and 25 °C [6]. Peroxynitrite has a pK_a of 7.5 [4] and will be, therefore, substantially protonated (reaction 5) at physiological pH, to produce peroxynitrous acid. One route for the decomposition of this molecule is thought to be homolytic cleavage (reaction 6) to produce hydroxyl radical and nitrogen dioxide.

NO is produced by vascular endothelial cells on stimulation with acetylcholine [7], and acts as a potent vasodilator [8]. In preparations of aortic rings it was found that superoxide dismutase (SOD) increased the half-life of endothelial-derived NO, suggesting that in this preparation there is a basal level of superoxide production [9], and that the potency of NO as a vasodilator is decreased in the presence of superoxide. It is probable that the interaction between superoxide and NO can occur *in vivo*, and it has been suggested that the vascular endothelium has the ability to regulate the effects of NO by generating superoxide [10]. In some pathological situations, macrophages and neutrophils, recruited to a site of injury, can be activated to produce both superoxide and nitric oxide as part of the inflammatory response [11]. It is possible, therefore, to envisage the formation of peroxynitrite in both normal and pathological states *in vivo*.

Peroxynitrite has a half-life of 1.9 s at pH 7.4, and has been shown to be the source of a strong oxidizing agent with properties identical to those of the hydroxyl radical [4]. These studies, however, were performed with pure peroxynitrite, and the possibility that reactions (4)–(6) can occur upon the simultaneous production of superoxide and NO has not yet been investigated in depth. In this study we have used 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1) as a model for the continuous release of superoxide and NO that occurs *in vivo*. SIN-1, the active

Abbreviations used: NO, nitric oxide; PBS, phosphate-buffered saline; MDA, malondialdehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; SIN-1, 3-morpholinosydnonimine *N*-ethylcarbamide; DTPA, diethylenetriamine penta-acetic acid; oxyHb and metHb, oxy- and met-haemoglobin respectively; IC_{50}, concn. causing half-maximal inhibition.

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metabolite of the vasodilatory drug molsidomine, is hydrolysed to an open-ring form (SIN-1A) that undergoes an oxygen-dependent release of NO with the concomitant production of superoxide [12,13].

We show here that the release of both NO and superoxide from SIN-1 results in the production of hydroxyl radicals by a mechanism distinct from that of the Fenton reaction.

MATERIALS AND METHODS

Measurement of oxygen consumption

The rate of oxygen consumption by SIN-1 was measured in a Clark oxygen electrode. SIN-1 (1 mM) and diethylenetriamine penta-acetic acid (DTPA, 0.1 mM) were added to phosphate-buffered saline (PBS) at 37 °C in the chamber of the electrode. The rate of oxygen uptake was measured in the presence and the absence of SOD (50 units/ml).

Measurement of NO production

The rate of NO production was monitored by measuring the rate of conversion of oxyhaemoglobin (oxyHb) to methaemoglobin (metHb) using a Cary 210 spectrophotometer. SIN-1 (1 mM) was added to oxyhaemoglobin (6 μM) in PBS at 37 °C. Absorbance changes were measured at 401 nm with oxyHb as an optical reference [14]. The molar absorption coefficient for metHb/oxyHb at 401 nm was determined by oxidation of oxyHb by sodium nitrite and found to be 45 $mM^{-1} \cdot cm^{-1}$.

SIN-1 incubations

SIN-1 (1 mM) was incubated in PBS containing DTPA (0.1 mM), for 2 h at 37 °C. Solutions also contained a hydroxyl radical detector molecule [either deoxyribose (1 mM) or sodium benzoate (1 mM)] and appropriate concentrations of either hydroxyl radical scavengers (citrate, ethanol, mannitol or desferrioxamine) or the enzymes catalase and SOD.

Ascorbate-driven Fenton reaction

This system was used as a positive control for hydroxyl radical production and consisted of $FeCl_3$ (0.1 mM), ascorbate (0.1 mM), EDTA (0.1 mM) and H_2O_2 (1.0 mM) in PBS. Samples were incubated for 2 h at 37 °C.

Thiobarbituric acid-reactive substances (TBARS) assay

Malondialdehyde (MDA) and other TBA-reactive material, produced from the oxidation of deoxyribose and sodium benzoate, was detected using the TBARS assay. To 1 ml of 0.5% TBA in 50 mM-H_2SO_4 was added 1 ml of sample. This was then placed in a boiling-water bath for 10 min. After the solution had cooled, the absorbance at 532 nm was determined. MDA concentrations were obtained by reference to a standard curve prepared from MDA produced by the acid hydrolysis of tetraethoxypropane. We are aware that the oxidation of deoxyribose and especially of sodium benzoate results in the production of TBARS other than MDA, which may also absorb at 532 nm. However, for ease of comparison with the literature, we have reported the concentrations of all such materials as MDA equivalents. The relative abilities of scavenger molecules to inhibit the production of MDA in both the SIN-1- and the Fenton-reaction-dependent oxidations were assessed. The concentration of scavenger required to inhibit MDA production by 50% (IC_{50}) was determined from the plot of the reciprocal of the concentration of MDA versus scavenger concentration [15].

Detection of fluorescent products from sodium benzoate

Samples containing sodium benzoate (1 mM) as the detector compound were incubated with either SIN-1 (1 mM) or the reagents described above to drive the Fenton reaction, and the fluoresence emission spectrum was recorded. With the excitation wavelength set at 300 nm, a broad emission peak centred at 410 nm was observed. All fluorescence measurements are given as a percentage of control samples that did not contain scavengers or enzymes.

Materials

Ca^{2+}- and Mg^{2+}-free PBS was obtained from Gibco; desferrioxamine was a gift from Ciba Laboratories; EDTA, $FeCl_3$, sodium citrate and ethanol were obtained from BDH; deoxyribose, catalase, sodium benzoate, TBA, SOD (from bovine erythrocytes), DTPA, hydrogen peroxide, sodium nitrite, mannitol, ascorbate and tetraethoxypropane (TEP) were obtained from Sigma. SIN-1 was obtained from Cassella AG. OxyHb was prepared from human erythrocytes. The units of activity for catalase and SOD are those defined by the supplier.

RESULTS AND DISCUSSION

Generation of oxidants by SIN-1

The auto-oxidation of 1 mM-SIN-1 was monitored in the oxygen electrode in PBS at 37 °C. After a short lag phase, presumably due to the hydrolysis of SIN-1 to SIN-1A [12], the rate of oxygen uptake was measured as 10.03 ± 0.95 μM/min (mean $\pm$ S.D., $n = 3$). Superoxide has been shown to be a product of SIN-1 oxidation. To confirm this, we added SOD to the chamber of the oxygen electrode. A decrease in oxygen consumption of 35%, to 6.54 ± 0.32 μM/min (mean $\pm$ S.D., $n = 3$), was observed in the presence of 50 units of SOD/ml. This suggests that 70% of the oxygen uptake can be ascribed to the production of superoxide. However, SOD has been shown to stabilize SIN-1A [12]. It is also evident that superoxide can act autocatalytically in this system by promoting the oxidation of SIN-1A. For this reason the rate of superoxide formation in the absence of SOD is probably higher than estimated.

The rate of production of NO from SIN-1 (1 mM) was monitored by measuring the rate of conversion of oxyHb to metHb [14], and was determined to be 3.68 ± 0.05 μM/min (mean $\pm$ S.D.) at 37 °C, in reasonable agreement with the value reported in the literature of 2.39 μM/min [12].

The concomitant production of both NO and superoxide by SIN-1 raises the possibility that significant amounts of peroxynitrite may be formed from the combination of these two radicals [5,16]. It follows, therefore, that SIN-1 may be capable of generating hydroxyl radicals from the homolytic lysis of peroxynitrite [4]. SIN-1 auto-oxidation is thus a good system for examining the consequences of the simultaneous production of these two free radicals.

Oxidation of deoxyribose

Hydroxyl radical has been shown to produce TBARS from deoxyribose with an absorbance spectrum identical to that of the MDA adduct [15]. To test whether SIN-1 is also capable of producing hydroxyl radicals, it was incubated with deoxyribose at 37 °C and the TBARS test for the generation of MDA was performed. An adduct with a characteristic absorbance maximum at 532 nm was indeed detected, consistent with the formation of MDA, showing that SIN-1 is capable of producing an oxidizing agent potent enough to degrade deoxyribose. The progress curve for the production of MDA in this reaction system is complex (Fig. 1), consisting of a fast initial phase and a slower second phase. The change in rate may be a reflection of oxygen availability, the slower rate being limited by the diffusion of oxygen into the reaction mixture. An incubation time of 2 h was used in all subsequent experiments.

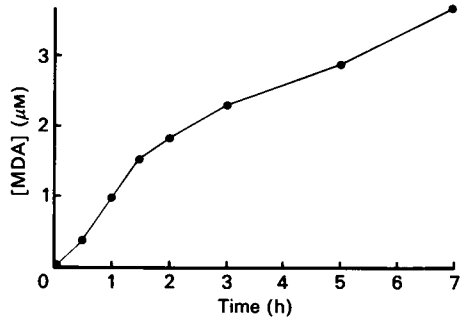

Fig. 1. SIN-1-dependent decomposition of deoxyribose to form MDA

SIN-1 (1 mM), DTPA (0.1 mM) and deoxyribose (1 mM) were incubated in PBS at 37 °C. Samples were taken at the times shown and the concentration of MDA was determined by reaction with TBA.

Table 1. Effect of metal ion chelators on the production of MDA from deoxyribose in the presence of ascorbate and H_2O_2

Metal chelators (0.1 mM) were assessed for their ability to inhibit the production of MDA from deoxyribose (1 mM) in the presence of H_2O_2 (1 mM) and ascorbate (0.1 mM). Samples were incubated in PBS for 2 h at 37 °C and MDA was measured after reaction with TBA. Values are reported as means ± S.E.M. for three experiments.

Chelator	[MDA] (μM)	Inhibition (%)
None	4.27 ± 0.07	–
EDTA	3.11 ± 0.04	27
DTPA	0.04 ± 0.01	99
Desferrioxamine	0.23 ± 0.01	95

Mechanism of the generation of the SIN-1-derived oxidant

Any system capable of generating superoxide is also capable of producing the hydroxyl radical if catalytic quantities of 'Fenton-active' transition metal ions (e.g. iron and copper) are also present in solution [17]. It is possible, therefore, that the SIN-1-dependent oxidation of deoxyribose is mediated by hydroxyl radicals produced by the interaction of superoxide with transition metal ions endogenous to the buffers (eqns. 1–3). The ability of the reaction buffer to support the formation of hydroxyl radicals was assessed by incubating deoxyribose with H_2O_2 and ascorbate in PBS for 2 h at 37 °C. Table 1 shows that PBS is capable of supporting the production of MDA, and that this can be inhibited by the addition of metal ion chelators. EDTA was only partially inhibitory, but both DTPA and desferrioxamine inhibited 95% or more of the MDA production. In the case of SIN-1 it would be expected that superoxide rather than ascorbate would act as the reducing agent to drive the Fenton reaction. It has been shown that, in a system dependent on superoxide for the production of hydroxyl radicals, DTPA is only 5% as efficient as EDTA in sustaining this reaction [18]. In agreement with this result, we found that DTPA inhibited hydroxyl radical formation due to endogenous transition metals contaminating our buffers (Table 1). Desferrioxamine has been shown to be a potent hydroxyl radical scavenger, independent of its iron-chelating ability [19], and can also scavenge peroxynitrite [4]. For these reasons DTPA was chosen to inhibit Fenton-derived radical production in subsequent experiments. DTPA (100 μM) inhibited 20% of SIN-1-dependent MDA production from deoxyribose (results not shown), suggesting that some transition-metal-dependent generation of hydroxyl radical can occur under the conditions employed for the SIN-1 experiments reported below.

To determine whether superoxide and/or hydrogen peroxide are involved in the mechanism of SIN-1-dependent MDA production, the effects of SOD and catalase at several concentrations, and in combination, on the yield of MDA were measured. Table 2 shows the extent to which MDA production in both the SIN-1 and the ascorbate-driven Fenton reaction systems was inhibited by these two enzymes. The mechanism of production of oxidant in the two systems is clearly different. Catalase (50 units/ml) completely inhibited the production of MDA from the ascorbate-driven Fenton reaction, whereas SOD (25 units/ml) only partially decreased the amount of MDA produced. However, SOD had a greater effect on SIN-1-dependent MDA production and catalase was completely ineffective. The inhibitory effect of SOD in the SIN-1 system is clearly a function of enzyme concentration; however, even at a concentration of 500 units of SOD/ml, MDA production was only inhibited by 43%. The combination of both SOD and catalase had no additional effect on SIN-1-dependent MDA production compared with that of SOD alone (Table 2). These results give credence to the hypothesis that the SIN-1-derived oxidant is not produced by the same mechanism as that of the Fenton reaction, which has an absolute requirement for hydrogen peroxide.

Effect of hydroxyl radical scavengers

The possibility that the SIN-1-dependent degradation of deoxyribose was mediated by the production of hydroxyl radicals

Table 2. MDA production from deoxyribose arising from the auto-oxidation of SIN-1 or the Fenton reaction: effects of SOD and catalase

The production of MDA from deoxyribose (1 mM) in the presence of SOD or catalase was measured after reaction with TBA. Samples were incubated in PBS for 2 h at 37 °C in the presence of either SIN-1 (1 mM) and DTPA (0.1 mM) or $FeCl_3$ (0.1 mM), EDTA (0.1 mM). ascorbate (0.1 mM) and H_2O_2 (1 mM). MDA concentrations are reported as means ± S.E.M. for three experiments. n.d., not determined.

	SIN-1		Fe/EDTA/ascorbate/H_2O_2	
Additions	[MDA] (μM)	Inhibition (%)	[MDA] (μM)	Inhibition (%)
None	2.32 ± 0.07	–	16.0 ± 0.3	–
SOD (25 units/ml)	1.77 ± 0.05	24 ± 1	14.4 ± 0.3	10 ± 2
SOD (50 units/ml)	1.70 ± 0.05	27 ± 1	14.7 ± 0.3	8 ± 2
SOD (500 units/ml)	1.33 ± 0.01	43 ± 1	14.4 ± 0.04	10 ± 3
Catalase (50 units/ml)	2.32 ± 0.05	0	1.15 ± 0.05	93 ± 1
Catalase (500 units/ml)	2.28 ± 0.04	2 ± 2	0.46 ± 0.05	97 ± 1
Catalase (50 units/ml) + SOD (500 units/ml)	1.28 ± 0.08	45 ± 3	n.d.	n.d.

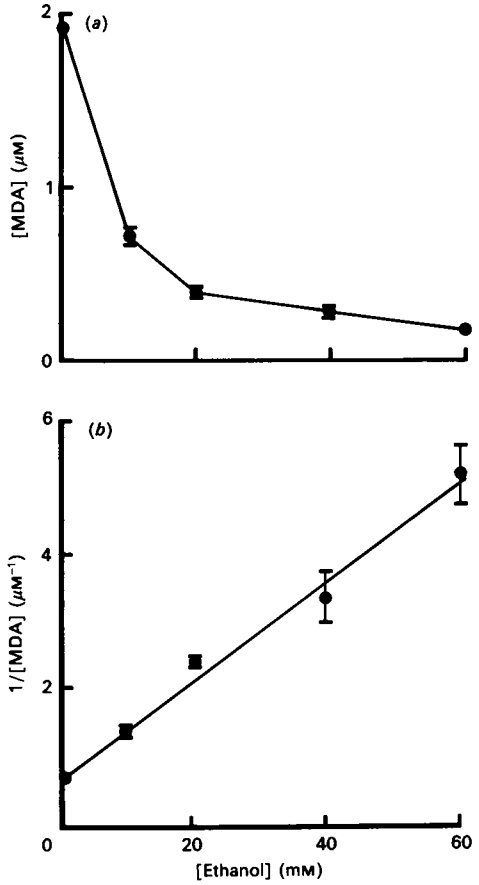

Fig. 2. Inhibition of SIN-1-dependent MDA production by ethanol

Deoxyribose (1 mM) was incubated with SIN-1 (1 mM) and DTPA (0.1 mM) in PBS for 2 h at 37 °C. Ethanol (0–60 mM) was added to the samples before the incubation period. After 2 h the concentration of MDA was determined by reaction with the TBA. (*a*) Dependence of the concentration of MDA as a function of ethanol concentration. A plot of the reciprocal of the MDA concentration versus the concentration of ethanol was linear, as shown in (*b*), and the concentration of ethanol required to give 50% inhibition of MDA formation can be calculated from a linear regression fit of the data. All data points are representations of the means ± S.E.M. from three experiments.

Table 3. Inhibition of MDA production, arising from the auto-oxidation of SIN-1 and the Fenton reaction, by hydroxyl radical scavengers

Hydroxyl radical scavengers were tested for their ability to scavenge the oxidant produced by either SIN-1 (1 mM) or $FeCl_3$ (0.1 mM), ascorbate (0.1 mM), EDTA (0.1 mM) and H_2O_2 (1 mM). Reaction mixtures, containing deoxyribose (1 mM) were incubated for a period of 2 h at 37 °C. The concentration of MDA was determined after reaction with TBA. The IC_{50} was calculated from a plot of the reciprocal of MDA concentration versus the concentration of scavenger (see Fig. 3). The errors represent the S.D. of the regression line fitted to the data.

Scavenger	IC_{50} (mM) SIN-1	IC_{50} (mM) Fe/EDTA/ascorbate/H_2O_2
Citrate	35±6	8.9±0.5
Mannitol	4.5±0.5	3.1±0.4
Ethanol	8.5±3.2	2.1±0.2
Desferrioxamine	0.63±0.14	0.11±0.02

was further investigated by using molecules capable of scavenging this radical [15]. Fig. 2(*a*) demonstrates the effect of increasing scavenger concentration (in this case ethanol) on the amount of MDA formed during incubation of SIN-1 with deoxyribose. All compounds tested were capable of scavenging the oxidant produced by SIN-1. The hyperbolic dependence of MDA yield on scavenger concentration is indicative of direct competition between the scavenger and the deoxyribose molecules. A plot of the reciprocal of MDA generated against scavenger concentration (Fig. 2*b*) is linear, and can be used to calculate the IC_{50} for MDA production. The IC_{50} values for citrate, mannitol, ethanol and desferrioxamine using both SIN-1 as a source of oxidant and the ascorbate-driven Fenton reaction system are shown in Table 3. All four compounds were able to scavenge the oxidant produced by the Fenton reaction, as expected. It is clear that the oxidant produced by SIN-1 can also be effectively scavenged by these compounds. We note that interpretation of the effects of desferrioxamine is complicated by the fact that this compound is a potent iron chelator and may also react with peroxynitrite [4]. It is clear that the SIN-1-produced oxidant is of comparable reactivity to the hydroxyl radical.

Sodium benzoate oxidation

Hydroxyl radicals have been shown to be capable of degrading sodium benzoate to TBARS as well as producing hydroxylated products (e.g. 2- and 3-hydroxybenzoate) with characteristic fluorescence emission spectra [17,20]. Table 4 shows the results of the oxidation of sodium benzoate by both SIN-1 and the ascorbate-driven Fenton reaction, detected both by the formation of TBARS and production of fluorescent products. The production of TBARS from sodium benzoate in both oxidation systems was only about 20% of that produced from deoxyribose. This may reflect the fact that TBARS are a minor component of hydroxyl-radical-dependent benzoate oxidation and that other compounds, such as hydroxylated products, are also generated. The formation of TBARS from benzoate was inhibited by mannitol in both oxidation systems (Table 4). Incubation of sodium benzoate with SIN-1 also resulted in the production of fluorescent molecules with emission maxima at 410 nm, typical of hydroxylated products. The ascorbate-driven Fenton reaction resulted in a higher intensity of fluorescence than the SIN-1 system, and the presence of mannitol decreased the fluorescence intensity in both cases (Table 4). The effects of catalase and SOD are in reasonable agreement with those shown earlier in the deoxyribose oxidation system (Table 2). We note that, in the Fenton reaction, catalase inhibited benzoate hydroxylation by approx. 90%, while SOD had a relatively smaller effect (35% inhibition). Conversely, during the auto-oxidation of SIN-1, SOD inhibited the production of hydroxylated products whereas catalase had little effect.

Summary

It is clear that SIN-1 is capable of producing a strong oxidant that exhibits many of the properties generally attributed to the hydroxyl radical. This oxidant is capable of generating TBARS from deoxyribose and sodium benzoate. It has been suggested that the product of the Fenton reaction with iron complexed in solution with phosphate in the buffers or perhaps deoxyribose, in contrast to iron chelated with EDTA, may not be the hydroxyl radical but some other iron-derived oxidant [21], or that the hydroxyl radicals produced locally by iron bound to deoxyribose are not released into free solution [22]. Our results show that ethanol inhibits the SIN-1-dependent degradation of deoxyribose, making it unlikely that transition metals endogenous to our buffers play an important role in this reaction, and demonstrating that the oxidant is released into solution. In

Table 4. Formation of MDA and fluorescent derivatives from the oxidation of sodium benzoate mediated by the Fenton reaction and the auto-oxidation of SIN-1

Sodium benzoate (1 mM) was used to detect the presence of hydroxyl radicals in both the SIN-1 and the ascorbate-driven Fenton reaction systems. Samples containing either SIN-1 (1 mM) or $FeCl_3$ (0.1 mM), EDTA (0.1 mM), ascorbate (0.1 mM) and H_2O_2 (1 mM) were incubated in PBS for a period of 2 h at 37 °C. After incubation, MDA was measured after reaction with TBA. Fluorescence intensity at 410 nm was measured using an excitation wavelength of 300 nm. MDA concentration is expressed as mean ± S.E.M. for three experiments, and fluorescence intensity is expressed as a percentage of control values. n.d., not determined.

	[MDA] (μM)		Fluorescence (% of control)	
Addition	SIN-1	Fe/EDTA/ascorbate/ H_2O_2	SIN-1	Fe/EDTA/ascorbate/ H_2O_2
None	0.48 ± 0.01	3.84 ± 0.08	100	100
Mannitol (20 mM)	0.09 ± 0.01	1.10 ± 0.16	23	22
SOD (50 units/ml)	n.d.	n.d.	48	65
Catalase (50 units/ml)	n.d.	n.d.	84	11

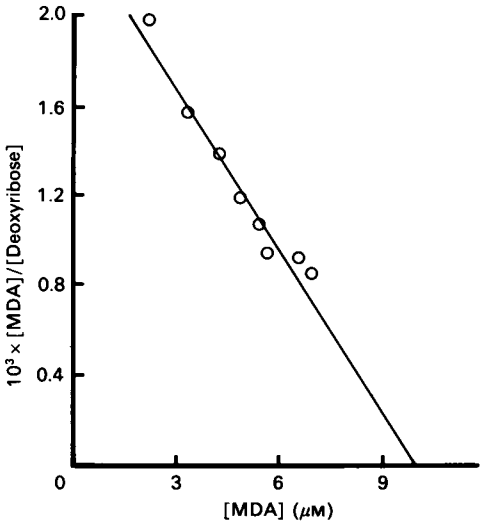

Fig. 3. Determination of the maximum yield of MDA from deoxyribose oxidation by SIN-1

SIN-1 (1 mM) and DTPA (0.1 mM) were incubated with deoxyribose (0–8 mM) in PBS at 37 °C for a period of 2 h. MDA was determined after reaction with TBA. The data are plotted according to the Scatchard formulation, and a linear regression fit gives a yield of 10 μM-MDA at infinite deoxyribose concentration.

addition, it is clear that desferrioxamine only inhibits the SIN-1-dependent degradation of deoxyribose at concentrations far in excess (Table 3) of that required to chelate transition metals contaminating our buffers (Table 1). Consistent with the hypothesis that the hydroxyl radical is mediating the decomposition of these molecules, the production of MDA was inhibited by known hydroxyl radical scavengers such as mannitol. The oxidant can also produce, from benzoate, fluorescent molecules with emission spectra characteristic of hydroxylated products. In all these respects the behaviour of this species mimics the oxidant generated by the ascorbate-driven Fenton reaction, a system known to produce hydroxyl radicals. However, unlike the ascorbate-driven Fenton reaction, the production of hydroxyl radicals from SIN-1 is not inhibited by catalase, and yet exhibits partial inhibition by SOD (Tables 2 and 4). This suggests that the oxidant is produced by a pathway that is both transition-metal- and hydrogen-peroxide-independent. It might be expected that SOD would completely inhibit the production of hydroxyl radicals that occurs as a consequence of the reaction between NO and superoxide. The data shown in Tables 2 and 4 demonstrate that only a partial inhibition is achieved, even at high concentrations of SOD. It is unlikely that SOD enhances a H_2O_2-dependent pathway, since the combination of SOD and catalase was no more effective in inhibiting the degradation of deoxyribose than was SOD alone (Table 2). Alternatively, we suggest that NO can effectively compete with SOD for superoxide under these conditions. We estimate that the rate constant for the reaction between superoxide and NO will be in the order of $10^8\ M^{-1} \cdot s^{-1}$, compared with the rate constant for dismutation of superoxide by SOD of $2 \times 10^9\ M^{-1} \cdot s^{-1}$. Although the possibility of superoxide-independent production of hydroxyl radicals cannot be ruled out, it is clear that the partial inhibition of SIN-1-dependent degradation of deoxyribose by SOD is consistent with a process completely dependent upon the reaction of superoxide and NO to produce hydroxyl radicals.

The data presented here support the hypothesis that hydroxyl radicals are produced by the homolytic fission of peroxynitrite, that is itself formed by the reaction of NO and superoxide. The apparent efficiency of hydroxyl radical production from SIN-1 is low. Fig. 3 shows that the maximum yield of MDA at infinite deoxyribose concentration is about 10 μM, a value equivalent to 3% of the concentration of SIN-1 calculated to have decomposed during the time of incubation. It is possible to conceive of many reactions that would lower the efficiency of hydroxyl radical production. These include non-enzymic dismutation of superoxide, the reaction of NO with oxygen, the internal rearrangement of peroxynitrite to produce nitrate, and perhaps also the reaction of peroxynitrite with deoxyribose. These problems notwithstanding, it is clear that a sustained steady-state concentration of NO and superoxide, such as that formed during the auto-oxidation of SIN-1, can result in the formation of hydroxyl radicals that most probably arise from the degradation of peroxynitrite.

The rates of formation of NO and superoxide under the conditions used for the present study (3.68 μM/min and 7.02 μM/min respectively) are considerably higher than those likely to be expected under normal conditions *in vivo*. For example, it has been determined that the stimulated guinea pig heart releases NO at a rate of 22 nM/min [23]. However, in pathophysiological conditions the production of NO and superoxide by macrophages as part of an inflammatory response may generate a sufficient flux of these oxidants to modify proteins and initiate lipid peroxidation. Although hydroxyl radicals may only represent a minor product of the interaction of superoxide and NO, it is as yet unknown what level of hydroxyl radical generation can be regarded as deleterious. The fact that a strong

oxidant is produced from the simultaneous generation of NO and superoxide in the absence of transition metal ions suggests that peroxynitrite may act as an important mediator of oxidative stress in many pathological conditions.

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