OH-Radical-Type Reactive Oxygen Species Derived from Superoxide and Nitric Oxide: A Sensitive Method for their Determination and Differentiation

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Z. Naturforsch. **52 c,** 564–570 (1997); received April 30/June 6, 1997

Reactive Oxygen Species, Peroxynitrite, Fenton-Type Oxidants

Reactive oxygen species are involved in many diseases where the radical species $OH^{\cdot}$, peroxynitrite and the non-radical, hypochlorous acid, play an outstanding role. The formation of $OH^{\cdot}$-type oxidants is essentially confined to a few types of reactions. The most prominent ones are the one-electron reduction of hydrogen peroxide by Fe^{2+} or Cu^{+}- ions (Fenton-type reactions), reaction of hypochlorite with superoxide and finally formation and decay of peroxynitrite (ONOOH), formed from superoxide and NO. In this communication we wish to report on a simple model system allowing to differentiate between these ROS: ethene formation from ACC is only detectable in the presence of hypochlorite (v. Kruedener *et al.*, 1995) and not detectable with Fenton-type oxidants or SIN-1 (3-morpholinosydnonimine, a peroxynitrite generator by releasing sequentially superoxide and NO) at 10 μM concentrations. On the other hand, ethene formation from KMB is negligible in the presence of hypochlorite but proceeds rapidly with Fenton-type oxidants (4 μM H_2O_2; 4 μM Fe^{2+}) as well as with 1 μM SIN-1. Stimulation of Fenton-type oxidants and not of SIN-1 by EDTA and characteristic patterns of inhibition by SOD, catalase, hemoglobin and uric acid allow a differentiation between these two potential precursors of OH-radicals. Synthetic ONOOH shows different reaction kinetics as compared to SIN-1. Inhibition of ONOOH-dependent ethene formation by different compounds occurs more or less "random" indicating an unspecific influence of proteins and also small molecules. Comparism of the individual inhibition types of several selected compounds allows a differential analysis as to the generation pathway of the final oxidants, OH- radical or peroxynitrite.

Introduction

Reactive oxygen species (ROS) are involved in most diseases. Oxygen acitvation forming (ROS) is biologically necessary for all aerobic cells (Halliwell and Gutteridge, 1989; Elstner, 1990, 1993). ROS formation concerning signal transfer is well under control. On the other side certain types of ROS inevitably lead to diseases and destructions via diffusion-controlled reactions based on site-specific escape of strong oxidants of the $OH^{\cdot}$-type from their "solvent cages" (Pryor und Squadrito, 1995; Elstner *et al.*, 1987; Bors *et al.*, 1984; Youngman and Elstner, 1981). These species operate in subcellular areas of only a few nanometers since "site-specific" production and destruction of neighbouring molecules occur in the same local environment. It is hard, if not impossible, to differentiate between the extremely fast and almost randomly reacting ROS. Therefore the term „OH-radical-type-oxidants" is often used.

The most prominent ways of the formation of OH-radical and its equivalents have very recently been discussed by Hippeli and Elstner (1997).

Formation of OH-radicals by Fenton-type or Haber-Weiss chemistry has been known for years whereas decay of ONOOH producing $OH^{\cdot}$ is quite new (for review see Pryor and Squadrito, (1995)). ONOOH is formed from Nitrogen monoxide (NO) and superoxide in an extremely fast reaction (k = 6.7 x 10^9). NO in turn is formed in all types of leukocytes and in endothelial cells from L-arginine catalyzed by the enzyme NO-synthase (NOS). The product of interaction between NO and $O_2^{\cdot -}$, ONOOH, has to be seen as "solvent

Abbreviations: ACC, 1-amino-cyclopropane-1-carboxylic acid; BSA, bovine serum albumine; EDTA, ethylenediaminetetraacetic acid; KMB, α-keto-γ-methylthiobutyric acid; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine, $C_6H_{10}N_4O_2 \cdot HCl$; SOD, superoxide dismutase.

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cage", {HO-NOO},decaying homolytically into OH· and NO_2 (Pryor and Squadrito, 1995):

$$ONOOH \rightarrow \{ONO\cdot\cdot OH\} \rightarrow NO_2\cdot + OH\cdot. \quad (1)$$

For this type of oxidants the term "crypto-OH" has been introduced (Youngman and Elstner, 1981).

However, the percentage of free OH-radical seems to be very small (1–4% of total ONOOH) (Pou *et al.,* 1995). It asks the question after the nature of the extremely destructive potential of ONOOH. There are several indications, that ONOOH reacts by its own – and not as a precursor of OH-radical (Beckman *et al.,* 1994; Gow *et al.,* 1996; Sakuma *et al.,* 1997).

In this communication we compare reactivities of OH-radical-type oxidants in several indicator systems. These oxidants were generated

a. in a Fenton-type reaction (iron catalyzed H_2O_2-reduction);
b. via the decay of 3-morpholinosydnonimine (SIN-1, releases spontaneously superoxide and NO, forming ONOOH) and
c. via the synthesis of ONOOH from H_2O_2, KNO_2 and NaOH, according to Beckman *et al.* (1994).

The question was asked whether Fenton-type oxidants yield identical reactivities in indicator reactions as compared to SIN-1- and ONOOH-derived oxidants. If not, we would be able to differentiate between these oxidants on the basis of simple model reactions.

Materials and Methods

Reagents

SIN-1, 3-morpholinosydnonimine, $C_6H_{10}N_4O_2\cdot$ HCl, was a gift from Dr. R. Grewe, Fa. Hoechst AG, Frankfurt. SIN-1 is a white solid with a molecularе weight of 206.7. It is soluble in DMSO, ethanol and water and it spontaneously decomposes to yield superoxide radical anion and NO. ACC, BSA, desferrioxamine mesylate (desferal), Fe_2SO_4, hemoglobin, KMB, KNO_2 and SOD were obtained from Sigma, Munich; EDTA, H_2O_2, D-mannitol, formate and uric acid were from Merck, Darmstadt; catalase was purchased from Boehringer, Mannheim. All other chemicals were of the highest grade of purity available (Merck). The gases for gas chromatography were obtained from Messer Griesheim, Mannheim; the carrier gas was N_2 (type 5.0) 25 ml/min; the burning gases were H_2 (type 5.0; 25 ml/min) and synth. air (250 ml/min); ethylene calibration gas: 1 ml = 235.15 pmol, 1 bar.

Peroxynitrite-synthesis

Peroxynitrite (ONOOH) was synthezised according to Beckmann *et al.* (1994) as follows: 0.7 M hydrogen peroxide solution in 5 ml 0.6 M HCl was mixed with 5 ml 0.6 M KNO_2 in an ice bath. One second after mixing, 5 ml ice cold 1.2 M NaOH was added. This mixture was stored over night at −20 °C. The top layer was collected and stored as stock solution. The concentration was determined photometrically at 302 nm based on an absorption coefficient $E_{302} = 1670\ M^{-1}cm^{-1}$.

Indicator systems

The formation of reactive oxygen species was detected as ethylene release from either α-keto-γ-methiol-butyric acid (KMB) or 1-amino-cyclopropane-1-carboxylic acid (ACC). The KMB-reaction is more or less specific for Fenton-type oxidants, whereas ACC-fragmentation is relatively specific for oxidants like hypochloric acid or organic chloramines (v. Kruedener *et al.,* 1995). The oxidative potentials of oxidants derived from a) iron-catalyzed H_2O_2-reduction; b) SIN-1 and c) synthetic ONOOH were determined in the presence of KMB and ACC respectively. The type of reacting oxidant was characterized by using the following inhibitors: SOD, detoxifying $O_2^{\cdot-}$; catalase, detoxifying H_2O_2; hemoglobin, reaction with NO; RSA, unspecific reaction with ROS; uric acid, reaction with ONOOH; desferal, inactivation of Fe^{3+}; EDTA, iron chelator, enhancing Fenton-reactions; mannitol and formate as OH-radical scavengers.

The ethylene formation from KMB or ACC was analyzed by gas chromatography as described previously (v. Kruedener *et al.* 1995). The values for ethylene production in the figures refer to picomoles per total reaction and were calculated with the aid of an ethylene calibration gas: 1 ml = 235.15 pmol, 1 bar. A detailed description of reaction conditions and composition of the reaction mixtures are given in the individual figures.

Results

SIN-1-dependent ethene release from KMB as compared to ACC

As shown previously (v. Kruedener *et al.*, 1995) ethene formation from ACC by Fenton-type oxidants at physiological relevant concentrations (1–10 μM) is negligible. Therefore we tested ethene formation with SIN-1 as ONOOH-donor and found that there was no ethene release from ACC in the mentioned concentration range. With 1 mM SIN-1 ca. 500 pmol ethene were formed in 30 min from ACC (Fig. 1a). We conclude that the nature of oxidants formed by SIN-1 is more likely close to the OH^--type and not like HOCl. In contrast, KMB reacted sensitively with SIN-1 similar to Fenton-type oxidants. Approximately 500 pmol ethene are formed from KMB in the presence of 1 μM SIN-1. Thus, KMB reacts more sensitive with SIN-1 by a factor of 1000 as compared to ACC.

Optimal pH of the KMB reaction is above 7.0 and practically constant up to 9.5 (data not shown). At pH 6.5 only approximately 20% ethene is formed as compared to pH 7.4. For the further experiments concentrations of 10 μM SIN-1 and 0.1 M phosphate buffer pH 7.4 were selected.

Fenton-type oxidant-dependent ethene release from KMB as compared to ACC

OH-radicals were generated from iron-catalyzed H_2O_2-reduction. Ethene release from KMB increases linearly with equimolar H_2O_2/Fe^{2+} concentrations up to 200 μM; at 4 μM concentrations approximately 1700 pmol ethene are formed in 30 min.; with 200 μM concentrations ca. 33000 pmol ethene can be measured (Fig. 1b). In order to keep as close as possible to physiological conditions 4 μM H_2O_2/Fe^{2+} were chosen for further experiments. At this concentrations ethene formation from ACC was not detectable (data not shown).

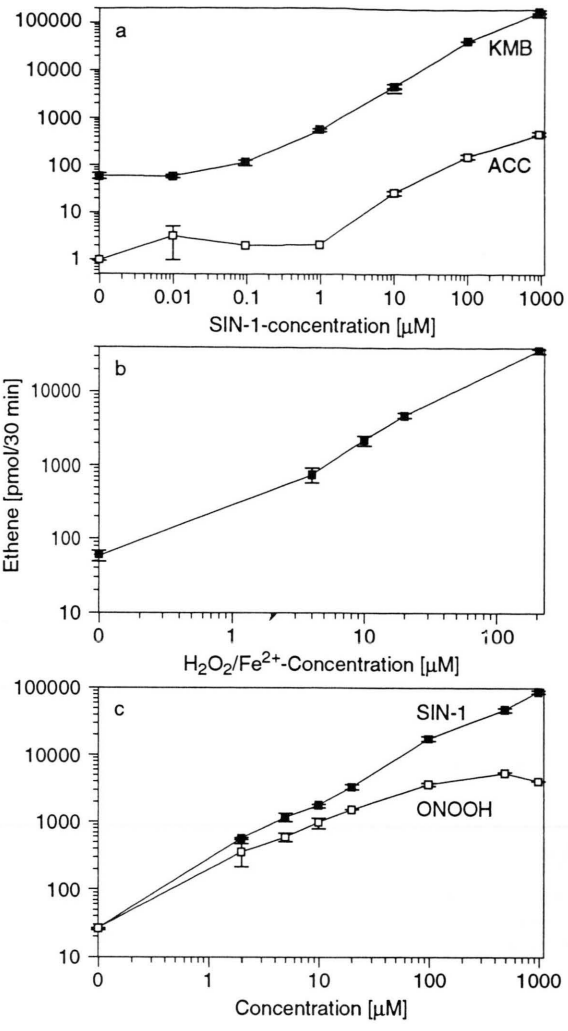

Fig. 1a-c. Ethene release from KMB or ACC depending on SIN-1-, Fenton-type oxidant- and ONOOH-concentration.
Reaction mixtures contained in a total volume of 2 ml 0.1 M phosphate buffer, pH 7.4; 10 nM-1 mM SIN-1; 1.5 mM KMB and ACC respectively (Fig. 1); 4–200 μM H_2O_2; 4-200 μM Fe^{2+}; 1.5 mM KMB (Fig. 1b); 5–100 μM SIN-1 and ONOOH respectively; 1.5 mM KMB (Fig. 1c); reactions were done at 37 °C, incubation time was 30 min. Standard deviation represents $n = 4$.

Combined Fenton/SIN-1-system: effects on ethene release from KMB

In order to test whether the SIN-1 and Fenton-type oxidants interfere as driving species in ethene release from KMB the Fenton-system was tested in the presence of increasing amounts of SIN-1 in a concentration range between 0.1 and 100 μM. As shown in Table I merely an additive effect and neither synergistic nor inhibitory trends could be observed. Thus, the oxidants derived from SIN-1 and the Fenton-system behave as individual species in KMB oxidation apparently without interaction.

Table I. Influence of SIN-1 on the Fenton-type oxidant-induced ethene release from KMB.
Reaction mixtures contained in a total volume of 2 ml 0.1 M phosphate buffer, pH 7.4; 4 μM H_2O_2; 4 μM Fe^{2+}; 0.1–100 μM SIN-1; 1.5 mM KMB; reaction conditions were as described for Fig. 1. Standard deviation represents $n = 4$.

SIN-1-Concentration [μM]	Ethene release from KMB (α-keto-γ-methylthiobutyric acid) [pmol/30 min]	Ethene release from KMB (α-keto-γ-methylthiobutyric acid) + H_2O_2/Fe^{2+} [pmol/30 min]
0	20 ± 5	2117 ± 174
0.1	27 ± 2	1891 ± 156
1	198 ± 20	1922 ± 186
10	2058 ± 118	3722 ± 302
100	19928 ± 620	19590 ± 449

ONOOH-dependent effects on ethene release from KMB

Similar to SIN-1, synthetic ONOOH is able to initiate ethene release from KMB in a concentration-dependent manner (Fig. 1c). As compared to SIN-1, the yield on a molar basis is somewhat lower. This may be due to impurities of nitrate in the preparation absorbing at the same wavelength as ONOOH, thus increasing the absorbance without exhibiting a corresponding KMB-oxidizing activity (data not shown).

As shown in Fig. 2, ethene release from KMB will exhibit different patterns of time courses if SIN-1 and synthetic ONOOH are compared: while the SIN-1-driven reaction follows a sigmoidal increase with time and is scarcely visible after 5 min of reaction, the ONOOH-driven reaction follows a "saturation-type" line with a rapid start where approximately 30% of the total amount of ethene after one hour is formed already after 5 min.

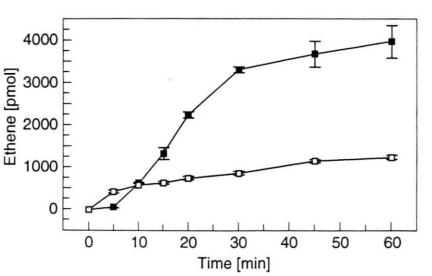

Fig. 2. Time-course of KMB-fragmentation by SIN-1 and ONOOH.
Reaction mixtures and conditions were as described for Fig. 1, containing 10 μM SIN-1 and 20 μM ONOOH respectively; reactions were observed over a period of 60 min.; -■- ethene release induced by SIN-1, -□- ethene release induced by ONOOH.

Effects of various inhibitors on ethene release from KMB

SIN-1 as oxidant

SIN-1-driven ethene release from KMB is inhibited to different degrees by SOD, hemoglobin, BSA, uric acid, desferal, mannitol and formate, where hemoglobin is the best inhibitor by blocking the reaction by more than 95% (Fig. 3a). Catalase and EDTA are essentially without influence. The strong inhibition by desferal is more than surprising: this chelator is estimated as specific inhibitor for reactions including Fe^{2+}/Fe^{3+}-redox cycling and has been used as such for years by numerous workers and groups.

The Fenton-system as oxidant

Fig. 3b shows that EDTA is an excellent stimulator of ethene release from KMB induced by Fenton-reaction. All other added substances inhibite ethene formation to different degrees where the influence of SOD is almost negligible and desferal (as here expected) inhibits by close to 90%. As compared to the SIN-1-system, the effects of SOD and catalase are reversed.

The combined Fenton/SIN-1-system

The results presented in Fig. 3c reflect both features characteristic for either one system: inhibition by SOD and catalase exhibit characteristics of the SIN-1-system, also less expressed. Hemoglobin

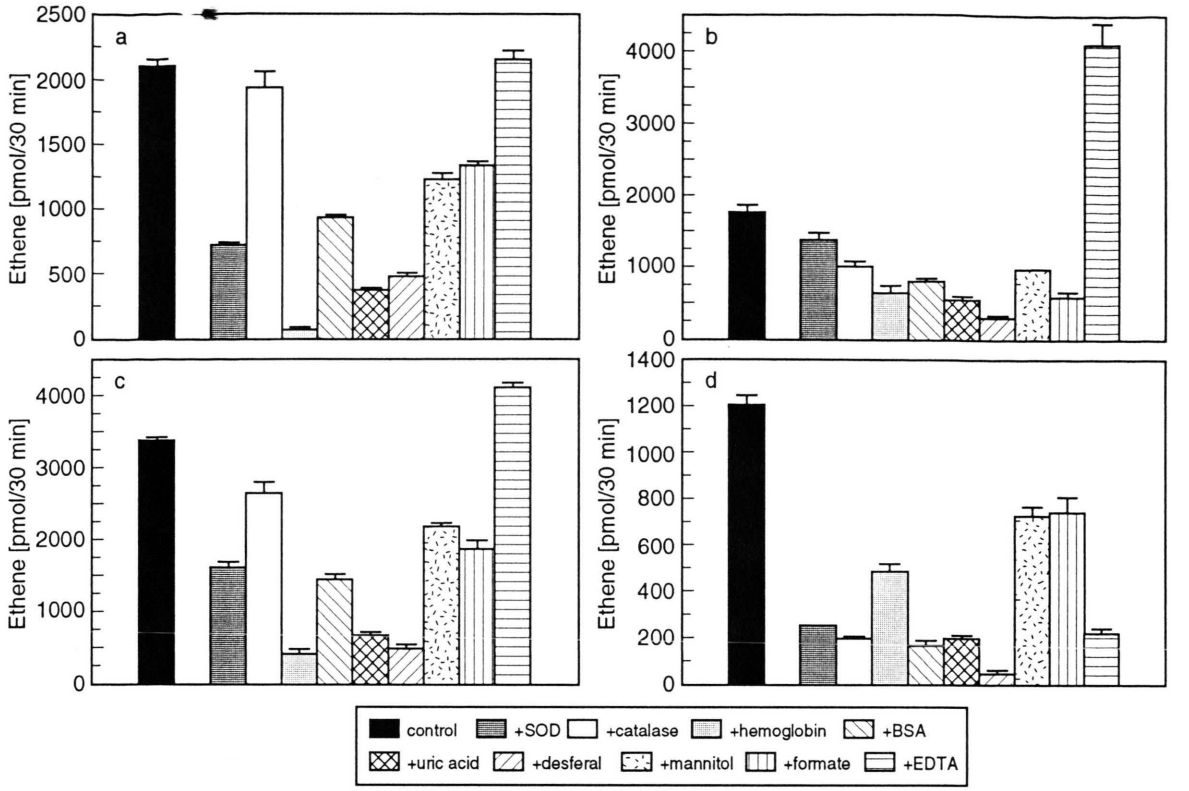

Fig. 3a-d. Effect of Inhibitors on KMB-fragmentation by SIN-1, Fenton-type oxidant and ONOOH. Reaction mixtures contained in a total volume of 2 ml 0.1 M phosphate buffer, pH 7.4; 10 μM SIN-1 (Fig. 3a) or 4 μM H_2O_2/4 μM Fe^{2+} (Fig. 3b) or 10 μM SIN-1/4 μM H_2O_2/4 μM Fe^{2+} (Fig. 3c) or 20 μM ONOOH (Fig. 3d); 1.5 mM KMB; 100 U SOD; 100 U catalase; 50 μM = 1.29 mg hemoglobin; 1.29 mg BSA; 1 mM uric acid; 1 mM desferal; 5 mM mannitol; 5 mM formate; 0.2 mM EDTA; reaction conditions were as described for Fig. 1. Standard deviation represents $n = 4$.

and desferal are the best inhibitors and EDTA acts as a poor stimulant. The overall picture expressed by comparism of all tested additions shows more or less the features of the SIN-1-system, slightly corrected towards the properties in the pure Fenton-system.

Synthetic ONOOH as oxidant

As shown in Fig. 3d, ethene formation from KMB is inhibited by all tested substances to different extent. The best inhibitors are desferal, uric acid and BSA, followed by catalase, EDTA and SOD while formate and mannitol show less effects.

Discussion

Strong oxidants are both responsible for unspecific immunological responses as well as for host-cell and tissue damage. Besides hypochlorous acid, which is a strong oxidant but not a free radical, several radical-type oxidants are under discussion as final destructive species responsible for oxidative damage. As recently summarized (Hippeli and Elstner, 1997), the OH-radical is supposed to be derived from a series of reactions and complex interactions including superoxide, hydrogen peroxide, nitrogen monoxide, transition-metal catalysis and hypochlorous acid. This makes it extremely difficult, if not impossible, to differentiate between these individual species with simple indicator systems. In this communication we wish to report on the comparism of OH-radical-type strong oxidants

deriving either from a Fenton-system, iron catalyzed hydrogen peroxide reduction or from superoxide reacting with nitrogen monoxide thus producing peroxynitrite. The indicators for strong oxidants KBM and ACC releasing ethene after oxidative attack allow to differentiate between the non-radical hypochlorite and Fenton-type oxidants or the ONOOH-pathway. ACC reacts exclusively with HOCl and not with radical-type oxidants or ONOOH. In contrast, KMB exhibits negligible ethene release in the presence of HOCl but is a good indicator for the other types of oxidants. As demonstrated in Table I neither synergistic nor interfering effects will be observed if both oxidants, Fenton and SIN-1 are present in the reaction mixture.

Our intention was the differentiation between the Fenton- and the ONOOH-pathway. The ONOOH-pathway induced by SIN-1 includes superoxide and NO. Therefor, a strong inhibition by SOD and hemoglobin seems indicated, where a drastic effect of catalase or EDTA should be absent. Exactly this result is obtained with SIN-1 as shown in Fig. 3a. The inhibition by uric acid, mannitol and formate indicates their general activities as radical scavengers while the inhibition by desferal seems to announce a "false-positive" involvement of iron redox cycling (see below). On the other side, the Fenton-way includes hydrogen peroxide and iron-catalysis. Therefore, an inhibition by catalase, OH-scavengers and desferal and a strong stimulation by EDTA are logic (Fig. 3b). In the presence ot both Fenton- and ONOOH-pathway, a mixed inhibitory pattern is visible where the effect of EDTA is diminished and the strong inhibition by hemoglobin, uric acid and desferal are preserved (Fig. 3c).

KMB-oxidation by synthetic ONOOH should be comparable with KMB oxidation by SIN-1 on a molar basis if impurities of nitrate in ONOOH are drawn into calculation (Fig. 1c). However, clear differences in the kinetics of KMB-oxidation by either synthetic ONOOH or SIN-1 can be observed. The activities with ONOOH exhibit saturation-type kinetics (Fig. 2) typical for second order reactions where one partner is present in excess (1.5 mM KMB as compared to 0.02 mM ONOOH;); correspondingly, sigmoidal reaction kinetics with SIN-1 indicate cooperative initiating processes (formation of ONOOH from NO and $O_2^{\cdot-}$?) until a quasi-linear oxidation process is observed (between 5 and 30 min reaction time) where formation of the final oxidant, ONOOH, and its reaction with KMB are in equilibrium. The reaction after slowing down of the SIN-1-KMB reaction after 30 min indicates increasing consumption of SIN-1 during the linear reaction process.

The almost unpredictable activities of different "inhibitors" are typical for "random" reactivities of ONOOH with all classes of reaction partners (Fig. 3d). The strong inhibition by EDTA as well as by desferal allows a differentiation from Fenton-oxidants, where only desferal is a strong inhibitor and EDTA acts as a stimulator (c.f. Fig. 3b). Since ONOOH-driven KMB-oxidation is not dependent on the catalysis by transition-metals such as iron or copper, the inhibitory effects of both EDTA and desferal, like the SOD-effect, have to be evaluated as unspecific. The inhibition by BSA is sincerely due to the presence of reactive sulfhydryl groups within the molecule. Alltogether, the utilization of either ACC or KMB as reaction partner for strong oxidants on the one hand, and the effects of a series of more or less specific inhibitors such as SOD, catalase, hemoglobin, uric acid, desferal and EDTA on the other hand, allow an approximative differentiation of oxidants such as HOCl, Fenton-type oxidants and ONOOH. Since ONOOH does not exist biologically as such, random reactions as indicated in Fig. 3d are not likely to be of physiological significance. The formation of ONOOH from NO and O_2.- as experimentally achieved with SIN-1 is of great importance. Therefore the differentiation patterns obtained by the comparison of Fenton-oxidants and SIN-1-driven reactions are of major interest and might be useful for corresponding experimental approaches *in vivo,* with certain tissues or on a cellular level.

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