Superoxide-Dependent and -Independent Nitrite Formation from Hydroxylamine: Inhibition by Plant Extracts*

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Reactive oxygen species such as OH·, peroxynitrite and the non-radical, hypochlorous acid, play outstanding roles in many diseases. The formation of OH· (Fenton-)-type radicals is catalyzed by enzymes such as xanthine oxidase (XOD) via one-electron reduction of molecular oxygen producing superoxide radical anions ($O_2\cdot^-$). Subsequent transfer of one electron to hydrogen peroxide by Fe^{2+} or Cu^+- ions yields OH-radicals measurable as ethene release from 1-keto-4 methylthiobutyrate (KMB). Xanthine oxidase or activated neutrophils are prominent sources of this strong oxidant produced at inflammatory sites.

Many natural compounds such as salicylates or flavonoids interfere either with the production of these activated oxygen species or function as radical scavengers and thus as antioxidants. Extracts from willow-bark (*Salix spec.*) and also other species such as ash-tree (*Fraxinus spec.)* or poplar (*Populus spec.)* have been used as antiinflammatory drugs since a long time. In this communication we wish to report on model reactions to demonstrate

a) the radical scavenging activities of such plant extracts inhibiting ethene release from KMB induced by Fenton-type oxidants and

b) the inhibition of the formation of nitrogen monoxide (NO) from hydroxylamine including XOD either in the presence or absence of myoglobin (MYO) measurable as nitrite formation: In the absence of MYO, superoxide dismutase is an excellent inhibitor of nitrite formation but is inactive in its presence. Extracts from the willow-bark or the drug Phytodolor^R, however, are inhibitory both in the presence and absence of MYO. As active principle, the flavonoid rutin included in these extracts is likely to function as one inhibitor of the XOD-mediated reaction.

Introduction

Oxygen acitvation forming ROS is biologically necessary and can be measured in all aerobic cells and tissues (Halliwell and Gutteridge, 1989; Elstner, 1990). Reactive oxygen species (ROS), however, are involved in inflammatory diseases, arteriosclerosis, cancer, diabetes, cataract, and many more. Major principles for biological generation of ROS such as OH-radical (OH·) include xanthine oxidase (XO), Fenton-type oxidants, activated leukocytes, different NAD(P)H oxidases, decay of peroxynitrite (ONOOH) and the reaction of hypochlorite with superoxide (Candeias *et al.,* 1993). These most prominent ways of the formation of OH·-equivalents have very recently been discussed by Hippeli and Elstner (1997). OH·-production by XO or by Haber-Weiss chemistry has been known for years whereas the decay of ONOOH producing OH· is quite new (see review by Pryor and Squadrito, 1995). ONOOH is formed from NO and superoxide in an extremely fast reaction (k= 6.7 x 10^9). Nitrogen monoxide (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 cage" ({HO-NOO}; Pryor and Squadrito, 1995), decaying very slowly and homolytically into OH· and $NO_2\cdot$. The simultaneous

Abbreviations: CAT, catalase; FO, Fenton-type oxidant; KMB, 1-**k**eto-4 **m**ethylthio **b**utyrate; MYO, myoglobin; ROS, reactive oxygen species; SA, salicylic acid; SE, *salix* extracts; PD, dry matter of the plant drug, Phytodolor^R; SE-h, PD-h, derivatized extracts; SOD, superoxide dismutase; X, xanthine; XOD, xanthine oxidase.

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production of both NO and $O_2\cdot^-$ at inflammatory sites thus is very likely to induce the production of the destructive oxidant, ONOOH.

The formation of the precursor NO from arginine seems to proceed in a two-step reaction including i) the P_{450}- enzyme, NO-synthase, oxidatively releasing hydroxylamine from arginine and ii) production of NO· from NH_2OH in a reaction driven by superoxide (Vetrovsky *et al.*, 1996; Modolell *et al.*, 1997). The first stable product in this reaction is nitrite (NO_2^-). This reaction has been introduced as early as 1970 (Elstner *et al.*, 1970) and later on described as a simple test for SOD (Elstner and Heupel, 1976) since the XOD-driven nitrite formation is completely inhibited by SOD. A modification of this test system included the presence of MYO which strongly stimulated nitrite formation and completely annihilated the inhibitory activity of SOD; the flavonoids quercetin or rutin, however, still retained their inhibitory functions by completely excluding nitrite formation from hydroxylamine via the inhibition of the enzymic XOD-activity in a concentration range between 10^{-5} to 10^{-3}M (Elstner *et al.*, 1987; Elstner, 1990). Since these compounds inhibit both NO· and $O_2\cdot^-$ production their benefical functions as antioxidants may well concern this type of reaction during inflammatory processes. In this communication we report on tests as to the well known anti-inflammatory and analgetic activities of extracts of the bark of willow (*Salix* spec) as well as Phytodolor^R (PD). We recently reported on the antioxidative properties of the individual plant extracts combined in PD, namely *Fraxinus*, *Populus* and *Solidago* in various test systems (Meyer *et al.*, 1995) and especially on myeloperoxidase-catalyzed oxidations (v. Kruedener *et al.*, 1996).

We show that in comparism to the inactive, pure SA or its pure derivatives, salicin or saligenin as main ingredients present in SE as well as in PD, these plant extracts inhibit nitrite formation from hydroxylamine both in the absence or presence of MYO. This activity may be due to rutin also present in SE. In contrast, KMB fragmentation by Fenton-type oxidant (Fe^{2+}-H_2O_2) is strongly inhibited by all the above mentioned extracts, further enhanced by their derivatization.

Materials and Methods

Materials

SE and PD were produced and partial HPLC-analysis was supplied by Steigerwald-Arzneimittelwerk GmbH (Darmstadt). SE and PD represent different extracts from *Salix* bark or a mixture of dry matters from *Populus*, *Solidago* and *Fraxinus* (PD, commercially available as drug under the name "Phytodolor^R" Steigerwald-Arzneimittelwerk GmbH). 1 g dry weight of SE contained (partial analysis by HPLC): 107–124 mg salicin and 1,14–1,19 mg salicylic alcohol. The aqueous extract of PD contained in 1 g dry matter (partial analysis): 1.38 mg isofraxidine, 0.94 mg rutin, 9.74 mg salicin and 0.44 mg salicylic alcohol. Derivatized extracts were obtained by a special treatment of SE or PD (patents pending or in preparation).

KMB and XOD (xanthine oxidase, E. C. 1.2.3.2.) were from SIGMA-Deisenhofen; all other chemicals were either purchased from SIGMA, Boehringer-Mannheim or Merck-Darmstadt. The enzyme activity (units) is defined according to the details given by the distributer.

Methods

As recently reported the production of different reactive oxygen species (ROS) in biochemical model reactions or by activated neutrophils can be differentiated by ethene production as quantified gaschromatographically by the headspace technique (v. Kruedener *et al.*, 1995; Hippeli *et al.*, 1997).

Experimental conditions

KMB fragmentation forming ethene

The reaction mixtures contained in 2 ml:

XOD-system: 0.1 M phosphate buffer pH 7.4; 0.5 mM xanthine(X); 0.08 U XOD; 1.5 mM KMB and 100 U SOD, 100 U CAT, 5 μM MYO, 2 mM SA or 0.1 mg/ml SE, where indicated.

FO-system: 0.1 M phosphate buffer pH 7.4; 1.5 mM KMB; 10 μM Fe^{2+}; 10 μM H_2O_2; the reaction was conducted for 30 min at 37 °C as described (v. Kruedener *et al.*, 1995; Hippeli *et al.*, 1997).

Hydroxylamine oxidation

Nitrite formation from hydroxylamine was determined photometrically by the addition of sulfanilamide and alpha-naphthylethylenediamine after incubation of the corresponding reaction mixtures as described (Elstner and Heupel, 1976).

Further experimental details are given in the individual Figures and Tables.

All experiments were repeated twice and run with 4 parallels where standard deviations are given.

Results

As mentioned above, the most prominent and generally toxic ROS produced after infections and during inflammations are represented by the XOD-reaction producing OH-radicals and Fenton-type oxidants (FO) via superoxide and hydrogen peroxide, especially represented by Fe^{2+}-H_2O_2; NO and superoxide, both simultanously derived from activated leukocytes or endothelial cells finally yield peroxynitrite, ONOOH. These species have been tested as to their destructive potential towards specific indicators for ROS (Hippeli *et al.*, 1997).

We here report on mechanisms of the protection against these toxic species by SE and PD containing small antioxidative molecules such as salicylic acid derivatives and flavonoids such as rutin or isofraxidine.

For the documentation of health-promoting effects during inflammatory processes and chronic infections we use KMB and hydroxylamine as indicators for ROS which are known to play crucial roles in these processes: the methionine derivative 1-keto-4-methylthio butyrate (KMB) is a sensitive indicator for FO and hydroxylamine oxidation by superoxide yielding nitrite is an indicator for the formation of NO and thus potential ONOOH-production.

Fenton-type oxidant (FO) and ethene release from KMB

Destructive reactions by FO can be easily observed by following ethene release from KMB (see above). As shown in Fig. 1a KMB fragmentation is inhibited in a concentration-dependent manner by salicylic acid as well as by aqueous extracts SE or PD containing the salicylic acid derivatives saligenin and salicin as well as several flavonoids: it is clearly visible that in this test system a concentration of approximately 0.01 mg/ml derivatized SE corresponds to approximately 2 mM salicylic acid rendering this test system suitable for standardizing this plant extracts as an antioxidative drug. Effects of derivatization of SE or PD is only evident with the lowest concentrations (0.01 mg/ml) tested.

Xanthine oxidase-(XOD-) dependent ethene release from KMB

As shown in Table I ethene release from KMB driven by X/XOD is completely blocked by SOD and inhibited by 87% by CAT and by 65% by MYO. 2 mM Salicylic acid inhibit KMB fragmentation by roughly 50% and SE or PD by between 70 and 80%. CAT- or MYO- inhibition is further enhanced by salicylic acid, SE or PD. PE-h shows the strongest inhibition in all systems.

Xanthine oxidase-dependent nitrite formation from hydroxylamine

Next we tested the most important enzymic system producing superoxide and FO, xanthine oxidase (XOD), as to the effects of SE on hydroxylamine oxidation. KMB fragmentation by XOD is by more than 90% inhibited by 100 U SOD or catalase (Table I; c.f also Elstner *et al.*, 1986). Nitrite formation from hydroxylamine driven by XOD is also completely inhibited by SOD and has been taken as a test system for SOD activities in biological materials (Elstner and Heupel, 1976).

The results shown in Fig. 1b demonstrate that, at concentrations of 0.1 mg/ml reaction volume, there is more than 50% inhibition by SE. Derivatization has no effect. Just as with 2 mM salicylic acid, almost no effect of PD on the XOD-driven nitrite formation from hydroxylamine can be seen which is, however, inhibited by approximately 90% by 100 U SOD. This is in contrast to the Fenton-experiment (see Fig. 1a) where salicylic acid and both SE and PD strongly inhibit the FO-driven ethene release from KMB.

Modulation of XOD-dependent oxidative mechanism by myoglobin

As mentioned above the strong oxidant, ONOOH, is produced from NO and superoxide.

Figure 1 a-c

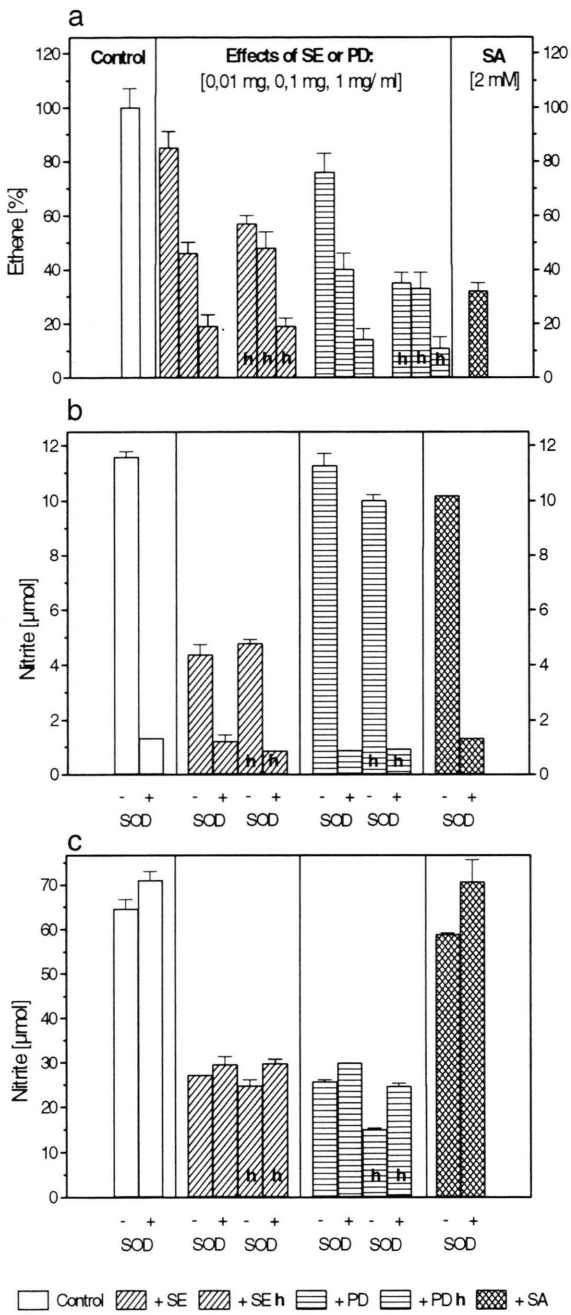

Fig. 1. Influence of aqueous extracts from the bark of Salix spec (SE) or Phytodolor^{R} (PD) on:
a. Ethene formation from 2-keto-4-methylthiobutyrate (KMB) by Fenton-type oxidants:
Experimental conditions: The reaction mixture contained in 2 ml: 0.1 M phosphate buffer pH 7.4; 1.5 mM KMB; 10 µM Fe^{2+}; 10 µM H_2O_2; the reaction was conducted for 30 min and ethene was quantified from the head space of the reaction vessels as described (v. Kruedener *et al.*, 1995). Substances tested: SA (2 mM) and the aqueous extracts SE and PD as shown in the Figure.
100% reaction correspond to 7650 pmol ethene formed after 30 min incubation of the reaction mixture at 37 °C.
In all reported experiments the standard deviations were less than 10% and eight parallel experiments are given as a mean value.
b. Hydroxylamine oxidation by the xanthineoxidase reaction.
Experimental conditions: The reaction mixture contained in 2 ml: 0.1 M phosphate buffer pH 7.4; 0.5 mM NH_2OH; 0.08 U XOD; 0.5 mM xanthine; 0.1 mg SE, SE-h and PD, PD-h were present as indicated; 2 mM SA was tested as comparism; addition of 100 U SOD when indicated.
The reaction was conducted for 30 min at 37 °C and nitrite was quantified as described under materials and methods.
In all reported experiments the standard deviations were less than 10% and eight parallel experiments are given as a mean value.
c. Hydroxylamine oxidation by the xanthine oxidase reaction in the presence of myoglobin.
Experimental conditions were as outlined in b but in the presence of 5 µM MYO.

Thus, under most prooxidative conditions, NO (measured as nitrite, NO_2^-) is produced from hydroxylamine (Elstner and Heupel, 1976). This reaction was completely inhibited by SOD and strongly stimulated by myoglobin (Elstner *et al.*, 1987). We showed that, in the presence of XOD, xanthine and hydroxylamine, NO_2^- formation as an indicator for intermediary NO is completely

Table I. Inhibition by *Salix* extracts and dry matter of the plant drug Phytodolor^{R} of KMB fragmentation by the xanthine oxidase-reaction in the absence or presence of myoglobin.
Experimental conditions: The reaction mixture contained in 2 ml: 0.1 M phosphate buffer pH 7.4; 1.5 mM KMB; 0.08 U XOD; 0.5 mM xanthine; the reaction was conducted for 30 min and ethene was quantified from the head space of the reaction vessels as described (v. Kruedener *et al.*, 1995). Tested substances: 5 μM MYO; 0.1 mg SE, SE-h, PD or PD-h were present as indicated; 2 mM SA was tested in comparism; 100 U SOD or CAT were added as indicated.
100% reaction corresponds to 4862 pmol ethene formed within 30 min incubation of the reaction mixture at 37 °C.
In all reported experiments the standard deviations were less than 10% and eight parallel experiments are given as a mean value.
Systems:
I= controls; II= with SA; III= with SE; IV= with SE-h; V= with PD; VI= with PD-h.

Test system	I	II	III	IV	V	VI
XOD/X	100	46	28	25	23	20
+SOD	2	1	2	1	2	1
+CAT	13	7	7	6	7	6
+MYO	34	20	6	6	3	4
+SOD+MYO	2	2	2	2	1	1
+CAT+MYO	25	12	9	7	6	1

abolished by 100 U SOD. Addition of 10^{-5} M myoglobin instead of 100 U SOD strongly enhances nitrite formation from hydroxylamine. In the presence of both myoglobin and SOD, nitrite formation was unaffected by SOD. Thus, myoglobin abolished the protecting effect of SOD. Both the flavonoids, quercetin and rutin, however, inhibited nitrite formation by the XOD system similar to SOD even in the presence of myoglobin. This function is not due to dismutation of, or reaction with, superoxide, but more likely due to inhibition of the enzymic activity of XOD. Likewise, MPO-activity is inhibited by quercetin or by rutin. A review on inhibitory activities of flavonoids is given in Elstner (1990). KMB fragmentation by XOD is strongly inhibited by SOD, CAT and also by MYO. Addition of MYO to the test systems containing SOD has no effect on the inhibition by SOD of KMB fragmentation (Table I). In contrast, nitrite formation from hydroxylamine by the XOD system is stimulated by 5 μM MYO by approximately sixfold from 11 μmol/30 min to 65 μmol/30 min as shown in Fig. 1c as compared to Fig. 1b. This reaction is not influenced by salicylic acid or by SOD but inhibited by more than 50% by SE and PD both with or without derivatization. If we compare the pure substances present as "leading components" in SE, the following results are obtained (Fig. 2a): Saligenine and salicine are inactive even at the highest concentrations tested (100 μM) whereas rutin and quercetin exhibit an I_{50} at approximately 5–10 μM. In neither of this tested systems SOD has inhibitory effects; in contrast a slight (ca.10%) stimulation by SOD can be observed (Fig. 2b).

Discussion

The antiinflammatory activity of willow (*Salix* spec.)-bark extracts has been rediscovered in 1763 (Morton and Meisinger, 1977). Salicylic acid derivatives ("salicylates") still are widely in use as anti-inflammatory drugs with little side effects. The corresponding reaction mechanisms concern the modulation of enzymic activities such as myeloperoxidase (Pekoe *et al.*, 1982), free radical scavenging (Ahnfelt-Ronne and Nielsen, 1987; Betts *et al.*, 1985) and inhibition of arachidonic acid-induced platelet aggregation (Kagawa *et al.*, 1992) to mention just a few.

On the other hand, the reaction of salicylic acid or its derivatives with OH-radical type oxidants (see Fig. 1a) yields hydroxylation products such as 2,5- and 2,3- dihydroxy benzoic acids which have frequently been used as an indicator (Elstner, 1990) for the existence of such radical-producing reactions in different tissues such as brain slices (Oubidar *et al.*, 1996), brain mitochondria after ischemia-reperfusion (Piantadosi and Zhang, 1996), diabetes (Ghiselli *et al.*, 1992), diaphragm fatigue during strenuous work (Diaz *et al.*, 1993) or in activated neutrophils (Davis *et al.*, 1989). "A cautionary note" concerning this test system as to the nature of the strong oxidant, OH·, has been brought up again, however, very recently by Halliwell and Kaur (1997). They stress the importance of not only measuring one hydroxylation product but in addition to both 2,3- and 2,5-dihydroxybenzoate also 5-nitrosalicylate in order to evaluate the contribution of ONOOH in addition to "real" OH-radicals.

In this context the question may be asked which biologically "original" role salicylates might play in higher plants: salicylic acid i) seems to act as a

Figure 2 a-b

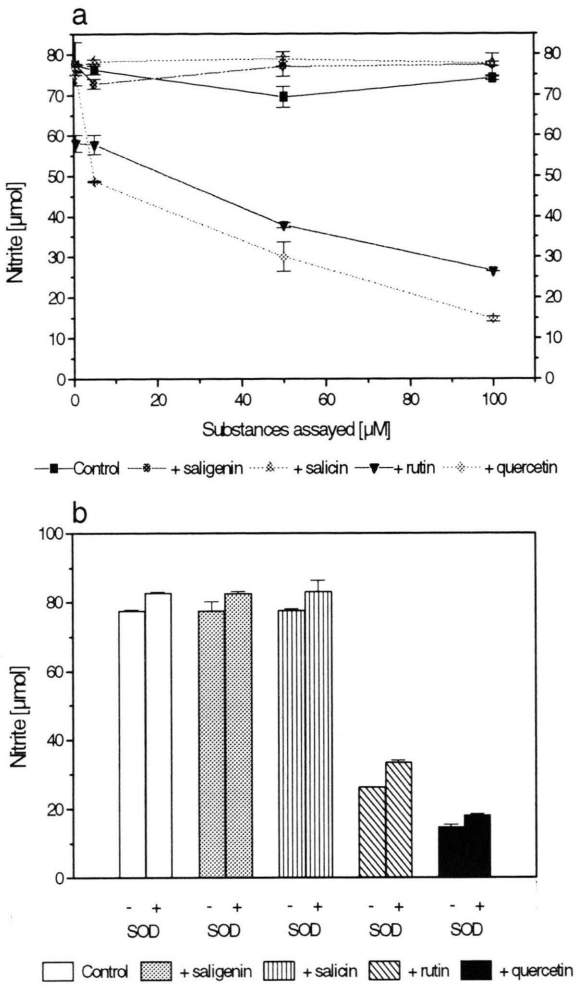

Fig. 2. Influence of saligenin, salicin, rutin and quercetin on:
a. Xanthine oxidase-myoglobin -driven hydroxylamine oxidation.
Experimental conditions were as outlined in Fig. 1b. The tested substances were added in the micromolar concentrations of 0.5; 5; 50 and 100.
b. Xanthine oxidase-myoglobin -driven hydroxylamine oxidation in the presence or absence of superoxide dismutase.
Experimental conditions were as outlined for Fig. 1c.

modulator of catalases (Durner and Klessig, 1996), ii) function as "reducing substrate" for plant ascorbate peroxidase (Kvaratskhelia *et al.*, 1997) and iii) seems to play an important role in plant resistance towards pathogens (Durner *et al.*, 1997). Alltogether, in phytopathology salicylates seem to play a role in oxidative processes connected with plant stress and pathogen-resistance. In principle, this also holds for animal systems.

In this communication we compare the antioxidative properties of differently prepared and derivatized extracts from the barks of willow trees (SE, *Salix* spec.) and PD. We chose two different test systems namely FO and XOD, measuring ethene release from KMB and nitrite formation from hydroxylamine in the presence or absence of MYO.

Fenton-type oxidant

As shown in Fig. 1a KMB fragmentation by FO (Fe/H_2O_2) is inhibited by both SE either native or derivatized where at lower concentrations (0.1 mg/ml) both SE-h and PD-h are more active than the untreated extracts. In this reactions 0.1–0.2 mg/ml SE correspond to approximately 2 mM salicylic acid. Inhibition of KMB fragmentation most probably is due to competition for FO between compounds in SE or PD with KMB as indicator molecule. Excellent radical scavenging properties of a vast amount of phenolics has been reported in the past (Bors and Saran, 1987; Bors *et al.*, 1992; Elstner and Kleber, 1990; Stadler *et al.*, 1995; Halliwell, 1996). In the case of salicylic acid or its derivatives, hydroxylation products have been identified and used as indicators for the formation or the presence of OH-radicals or FO (Ghiselli *et al.*, 1992; Diaz *et al.*, 1993; Oubidar *et al.*, 1996). One characteristic feature of low molecular natural antioxidants is their cooperative function (Buettner, 1993). In this respect we tested the functions of SE and PD in a reaction normally completely inhibited by SOD but rendered SOD-insensitive in the presence of the heme-containing molecule, MYO.

XOD-driven oxidations: KMB fragmentation and nitrite formation from hydroxylamine

KMB fragmentation

XOD-catalyzed hydroxylation of salicylic acid forming 2,5- and 2,3- dihydroxybenzoic acid in a ratio of 5:1 has been used as an indicator for the protective effects of natural products such as phytic acid against free radical attack during disease development (Owen *et al.*, 1996). XOD-catalyzed

KMB fragmentation is strongly blocked by SOD and CAT and partially inhibited by MYO, salicylic acid and SE or PD (Table I). The strong influence of SOD on this reaction, in contrast to hydroxylamine oxidation (see below), is not changed by MYO indicating that different types of oxidants must be driving KMB- fragmentation and hydroxylamine oxidation: the KMB reaction is driven by an SOD-sensitive formation of an OH-radical-type oxidant according to the Fenton-Haber-Weiss chemistry whereas in the presence of MYO, hydroxylamine oxidation occurs at the expence of an SOD-insensitive (eventually via SOD-stimulation of hydrogenperoxide production) hydrogen peroxide-heme reaction product often addressed as "compound I" (Elstner, 1990).

Hydroxylamine oxidation

NO together with superoxide forms the aggressive peroxynitrite, ONOOH, in a very fast, diffusion-limited process (Pryor and Squadrito, 1995). ONOOH possesses oxidative properties similar to FO or OH-radicals (Hippeli and Elstner, 1997; Hippeli *et al.,* 1997). Superoxide sincerely is in the center of oxygen activation and an ubiquitous metabolite in aerobic metabolism (Sies, 1985; 1991; Halliwell and Gutteridge, 1989; Elstner *et al.* 1987; 1990; 1991; 1991a; 1993). It is produced by monovalent reduction of atmospheric oxygen. At sites of inflammation activated neutrophils, mitochondrial electron transport and enzymes such as XOD are involved in its production. Via hydrogen peroxide and transition metal catalysis FO and OH-type strong oxidants finally may be reponsible for tissue damage. NO, on the other hand, is formed at the same inflammatory centers by different types of NO-synthases. Because NO synthesis seems to proceed via a two-step mechanism (Modolell *et al.,* 1997) involving superoxide as a driving oxidant in the conversion of hydroxylamine into NO, ONOOH is likely to be formed and involved in oxidative damage at these inflammatory sites. Since NO in the blood vessel system is produced by endothelial cells and functions as an important vasodilator, also exhibiting antioxidative features (Hippeli and Elstner, 1997) its continuous production from arginine is a prerequisite for vessel tonus-homeostasis. We showed earlier that XOD both catalyzes ethene release from KMB (Elstner *et al.,* 1986) and the production of nitrite from hydroxylamine (Elstner and Heupel, 1976) where both reactions are completely SOD-inhibitable. The formation of nitric oxides from hydroxylamine is rendered SOD-insensitive by MYO, however, which might represent the "true" physiological condition since NO-synthases are heme-proteins by themselves. Thus *in vivo,* NO synthesis may well be SOD-insensitive.

ONOOH toxicity after overstimulation of NO production together with monovalent oxygen reduction at inflammatory sites may thus well be brought under control by the inhibition of superoxide generators such as XOD by inhibitors such as flavonoids (Elstner *et al.,* 1987). ONOOH-mediated oxidations on the other hand are sincerely to a certain extent under the control of glutathion peroxidase which has very recently been described to exhibit also the activity of a peroxynitrite reductase (Sies *et al.,* 1997).

We showed earlier (Elstner *et al.,* 1987) that the flavonoids rutin and quercetin inhibit both SOD-sensitive as well as -insensitive nitrite formation from hydroxylamine. As shown in Figure 1 the extracts SE and PD differ in their activities: although equally active in the FO-system (Fig. 1a) only SE is inhibitory in the SOD-sensitive XOD system (Fig. 1b). In the presence of MYO, however, both SE and PD are inhibitory either with or without SOD whereas salicylic acid has no effect. Neither saligenin nor salicin can be addressed as inhibitors since, in contrast to rutin or quercetin, a concentration-dependent decrease of nitrite formation from hydroxylamine can not be shown (Fig. 2a). SOD has no effect on these reactions (Fig. 2b). Alltogether SE and PD have the properties to both sensitively scavenge FO and inhibit XOD-dependent KMB-fragmentation and nitrite formation from hydroxylamine as an indication for a physiological role to prevent both OH-radical- or FO-type- and ONOOH-toxicity.

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