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Detection of oxygen activation and determination of the activity of antioxidants towards reactive oxygen species by use of the chemiluminigenic probes luminol and lucigenin*, **

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Abstract. Reactive oxygen metabolites can transfer, via oxygenation, the chemiluminigenic probes luminol and lucigenin to an excited state and thus induce light emission from these probes. This technique has been applied to a study of the effect of common food antioxidants on the availability of reactive oxygen species in aqueous model systems and in microsomal preparations. In all systems tested, propyl gallate and its congener octyl gallate proved to be the most active scavengers of the oxygenating species involved in the chemiluminescence reaction. In aqueous model systems, butylated hydroxyanisole was 10 times less efficient than the gallic acid ester antioxidants. In biological material, the lower scavenger activity of butylated hydroxyanisole is further compromised by its ability to induce a marked increase in production of hydrogen peroxide in the endoplasmic reticulum. Butylated hydroxytoluene is only marginally active in aqueous media and in biological material. It is concluded that gallic acid ester antioxidants may possess a significant protective potential towards toxic and carcinogenic agents due to their ability to remove toxic oxygen metabolites.

Key words: Rat liver microsomes – Hepatocytes – Reactive oxygen species – Chemiluminescence – Antioxidants

Introduction

The stepwise reduction of molecular oxygen in biological material generates a number of potentially harmful oxygen metabolites: the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical. This sequence can take place in various compartments of the cell (Freeman 1984) and may be involved in a number of pathological events connected with lipid peroxidation (Sevanian and Hochstein 1985), organ injury (Tate and Repine 1984), DNA damage and tumor promotion (Troll and Wiesner 1984). An efficient antioxidant defense system directed by the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase is available to the cell which can remove reactive oxygen species or prevent their generation. Low molecular weight compounds with reducing properties are probably also involved in the defense reaction. Among these, vitamin E is the prototype of what is generally called an "antioxidant". The vitamin E-like property is defined by reaction with lipid and lipoperoxide radicals formed during lipid peroxidation, and it is this scavenger action against organic radicals which establishes the technological importance of antioxidants in food preservation and in industrial processes. Antioxidant action on inorganic oxygen radicals has been repeatedly studied with α-tocopherol (Fukuzawa and Gebicki 1983; Tajima et al. 1983; Ozawa and Hanaki 1985), and it has been concluded that chromanoxyl radical formation can be achieved by $OH^{\cdot}$ and by O_2^{-}. Data on the reaction of synthetic antioxidants with inorganic oxygen radicals are only sporadically found in the literature (Simic and Hunter 1983; Bors et al. 1984). Moreover, such reactions have not been studied extensively in biological material. However, the well-known protective effects of antioxidants against a wide variety of carcinogenic, mutagenic and toxic agents (Kahl 1984) may in part be due to such scavenger properties against active oxygen, although antioxidant action on the metabolic activation or inactivation of the noxious agent, on lipid peroxidation initiated by such an agent or on radical species formed from it may also contribute to the beneficial biological action of antioxidants.

Experiments on oxygen activation invariably deal with the problem that the principle of the detection method used is to measure the oxidizing or reducing power of the oxygen metabolite in question; therefore, the method tends to be nonspecific. The generation of light-emitting excited derivatives of the chemiluminigenic probes luminol and lucigenin (Fig. 1) has been extensively employed in inflammation studies to quantify and characterize the secretion of active oxygen by phagocytozing cells, and some evidence has been gathered by the use of specific scavengers and of cells with specific enzyme deficiencies that luminol is exclusively oxygenated by hydrogen peroxide and/or a species derived from it, while lucigenin may rather be oxygenated by the superoxide anion radical (Allen 1982; Aniansson et al. 1984). However, the chemical reactions involved have not yet been elucidated in detail (Allen 1982). These chemiluminigenic probes have been used in the present experiments to study the influence of a number of food antioxidants on processes occurring in aqueous model systems and in microsomal membranes which are assumed to be elicited by reactive oxygen species.

* Dedicated to Professor Dr. med. Herbert Remmer on the occasion of his 65th birthday

** Part of the doctoral theses of A. W. and S. W.

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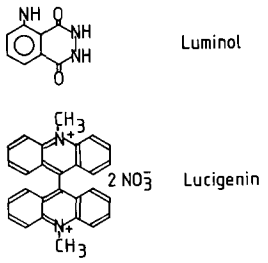

Fig. 1. Chemical structure of luminol and lucigenin

Methods

Conventionally prepared liver and lung microsomes were obtained from male Wistar rats. Chemiluminescence measurements were performed in a "Biolumat LB 9505" (Berthold, Wildbad, FRG) equipped with six photomultipliers in order to allow for simultaneous sample handling. Aqueous model systems consisted of either 0.2 mM hypoxanthine plus 0.5 mg xanthine oxidase/ml or of a solution of 0.6% H_2O_2. In microsome experiments, 2 mg/ml microsomal protein obtained from phenobarbital-pretreated animals were incubated at 37° in 66 mM Tris-HCl buffer pH 7.5 in the presence of an NADPH-generating system and 5% luminol (1 mM) or lucigenin (8 mM). The final incubation volume was 200 µl. The reaction was started by the addition of NADPH (final concentration 500 µM) and followed for 20 and 80 min, respectively. Hydrogen peroxide formation in microsomes was determined by formaldehyde production from methanol via the peroxidatic action of catalase (Hildebrandt et al. 1978). Formaldehyde formation from dimethyl sulfoxide was used to estimate the availability of agents possessing the oxidizing properties of $OH^{\cdot}$ radicals in microsomal preparations (Klein et al. 1981).

Results and discussion

If lucigenin is oxygenated by the superoxide anion radical then it should exhibit chemiluminescence in an aqueous system containing xanthine oxidase and hypoxanthine.

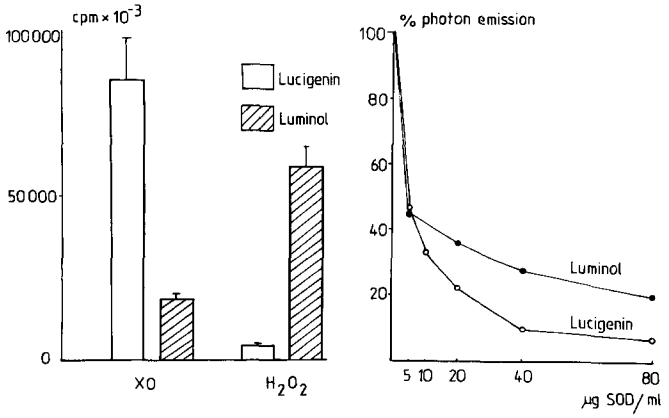

Fig. 2 A, B. Light emission from luminol and lucigenin in aqueous media. **A** Photon yield in the presence of xanthine oxidase (*XO;* 0.5 mg/ml) plus hypoxanthine (0.2 mM) or in the presence of H_2O_2 (0.6%). Data are means ± SE ($n = 4-19$). **B** Inhibition of light emission in the XO system by superoxide dismutase *(SOD).* Data are means of 2–7 experiments

Figure 2 A shows that this is indeed the case. Since H_2O_2 is also present in the xanthine oxidase system, the occurrence of luminol-dependent chemiluminescence in this system is in accordance with the alleged specificity of the probes. However, luminol chemiluminescence is suppressed by SOD to a similar extent to lucigenin chemiluminescence (Fig. 2 B). This would point to a role of superoxide in luminol oxygenation unless the inhibition is attributed to the interruption of a Haber-Weiss reaction in the medium. A solution of H_2O_2 should be expected to give rise to luminol chemiluminescence, as is indeed seen in Fig. 2 A; however, a small but consistent light emission from lucigenin is also obtained in this system which is as effectively removed by catalase as is the H_2O_2-dependent luminol chemiluminescence (data not shown). Thus while these simple experiments in aqueous model systems in general seem to fit the published interpretation of the two probes, unequivocal conclusions on their specificity cannot be drawn from the data.

Antioxidants differ in their ability to suppress light emission in such aqueous systems. Propyl gallate (PG) was the most active inhibitor tested. The concentrations of butylated hydroxyanisole (BHA) required to obtain the same degree of inhibition exceed those of PG by one order of magnitude. Butylated hydroxytoluene (BHT) is inactive in the xanthine oxidase/lucigenin system and only marginally active in the H_2O_2/luminol system (not shown). It should be noted that antioxidant activity in the latter system markedly exceeds that in the former system: almost complete suppression of luminol chemiluminescence induced in a solution of H_2O_2 is obtained with 0.5 µM PG but similar suppression of lucigenin chemiluminescence in the xanthine oxidase system requires 50 µM PG. The addition of catalase to the xanthine oxidase system markedly diminishes antioxidant inhibition, indicating that the superoxide radical is not sensitive to the scavenger activity of the antioxidants tested.

In microsomes, the use of specific scavengers will not be of much help in discriminating the contribution of individual oxygen metabolites to the chemiluminescence reactions because of the dual role of superoxide in the iron-catalyzed Haber-Weiss reaction assumed to occur in biological material (Halliwell and Gutteridge 1985). The addition of NADPH to microsomes containing lucigenin leads to a burst of photon emission peaking around 10 min and then declining (Fig. 3). This chemiluminescence can be inhibited by SOD but high concentrations of the enzyme are required. This may be due to the fact that the enzyme cannot penetrate the membrane; however, it should be noted that lucigenin itself is fairly hydrophilic and that it is not clear whether the reaction takes place within the membrane or rather at a lipid-water interface which should be accessible to the enzyme. Very high concentrations of catalase and of the $OH^{\cdot}$ radical scavenger dimethyl sulfoxide (DMSO) also exert a certain degree of inhibition, and the inhibitory action appears to be somewhat additive with that of SOD. We have observed a similar inhibition pattern with luminol as the chemiluminigenic probe (Weimann et al. 1984).

Figure 4 shows the time course of the chemiluminescence in a typical luminol experiment. Two differences as to the lucigenin experiment in Fig. 3 should be noted. 1) The absolute amount of photons emitted is about 1000 fold lower. No pertinent explanation for this difference can at present be offered. In phagocytes, such marked differences

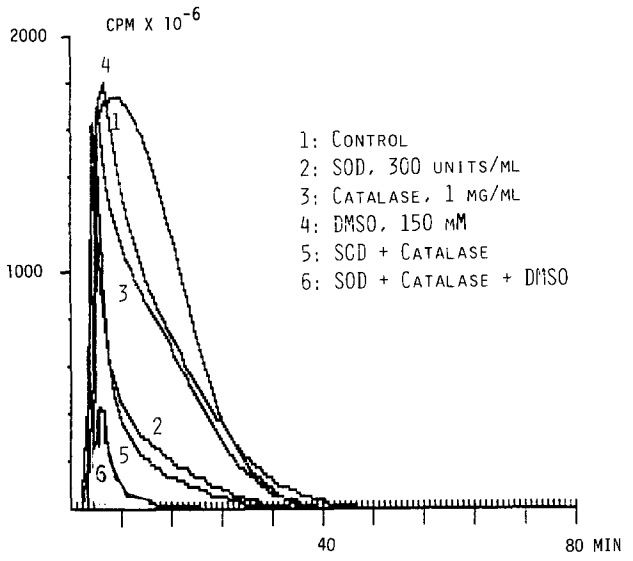

Fig. 3. Scavenger effects on lucigenin-induced light emission in NADPH-consuming rat liver microsomes. Original recording of a single experiment. Start with 500 μM NADPH (final concentration). *SOD:* superoxide dismutase, *DMSO:* dimethyl sulfoxide

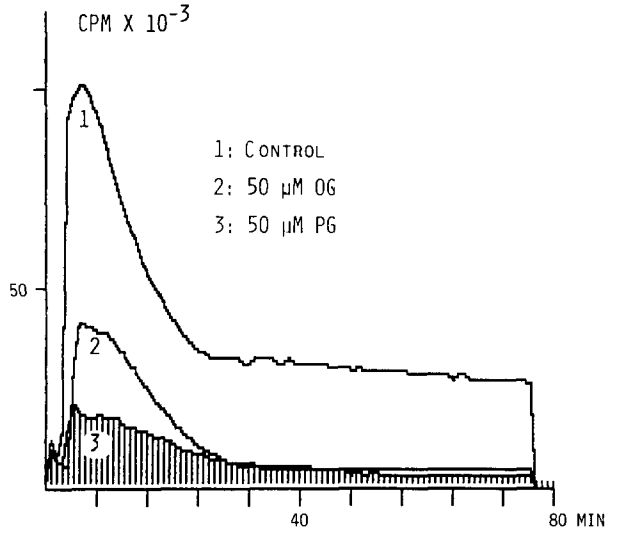

Fig. 4. Influence of two gallic acid ester antioxidants on luminol-induced light emission in NAPDH-consuming rat liver microsomes. Original recording of a single experiment. Start with 500 μM NADPH (final concentration). *OG* octyl gallate, *PG* propyl gallate

between the extent of luminol chemiluminescence and lucigenin chemiluminescence is not observed (Allen 1982; Aniansson et al. 1984). 2) Light emission does not decrease to zero after the initial peak; in fact, a slow rise lasting for another 2 h can be observed in some experiments (not shown). It is conceivable that this variable late phase is due to a product occurring during lipid peroxidation of the aging microsomal suspension; however, in the presence of reduced iron and ADP (a condition facilitating both OH· radical formation and lipid peroxidation) only the initial peak is increased by a factor of about 2 but the late phase is not stimulated (not shown).

Figure 4 also demonstrates the ability of two food antioxidants of the gallic acid ester type to depress luminol

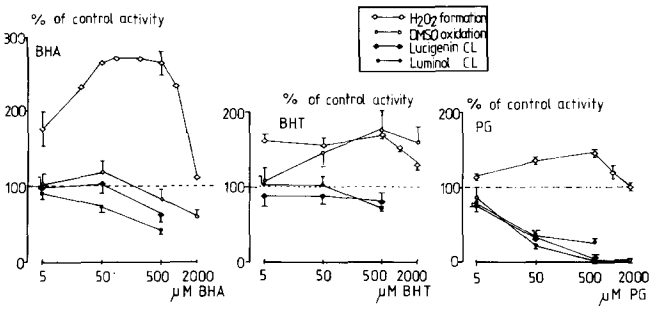

Fig. 5. Differential effects of three antioxidants on various parameters of oxygen metabolism in NADPH-consuming rat liver microsomes. Percentage of respective parameter in the absence of antioxidants is plotted vs antioxidant concentration. Values are means ± SE ($n = 3-6$). Means of control values in the absence of antioxidant were 10.4 + 0.9 nmoles $H_2O_2 \times$ mg protein$^{-1} \times$ min^{-1}, 1.14 + 0.09 nmoles formaldehyde from DMSO × mg protein^{-1} × min^{-1}, 597 + 30 counts × 10^{-3} between 0 and 20 min for luminol CL and 962 + 138 counts × 10^{-6} between 0 and 20 min for lucigenin CL. When absolute values were tested for statistical significance with the Student's *t*-test significant extra production of H_2O_2 was calculated at 5 μM BHA, 5 μM BHT and 500 μM PG. DMSO oxidation was significantly inhibited at 50 μM PG and significantly increased at 500 μM BHT. Significant suppression of luminol CL was obtained at 50 μM BHA, at 500 μM BHT and at 5 μM PG, while lucigenin CL was significantly suppressed at 50 μM PG. *BHA* butylated hydroxyanisole; *BHT* butylated hydroxytoluene; *PG* propyl gallate; *DMSO* dimethyl sulfoxide; *CL* chemiluminescence. From: Kahl R, Hildebrandt AG (1986) Food Chem Toxicol (24,1007).

chemiluminescence in microsomes. A comparison of the inhibitory potency of BHA, BHT and PG is given in Fig. 5 for luminol chemiluminescence and for lucigenin chemiluminescence, as measured during the first 20 min after NADPH addition. Only PG is active in both tests, BHA exhibits some inhibition of luminol-induced light emission at a high dose level (500 μM), while BHT is inactive in both tests. Thus, the ranking of the inhibitory potency of the antioxidants parallels that found in aqueous model systems, indicating that potency differences are not due to differences in solubility of the individual antioxidants in the microsomal membrane. This is further substantiated by the observation that the highly lipophilic gallic acid esters octyl gallate and dodecyl gallate show the same range of inhibitory activity as their fairly hydrophilic congener PG (data not shown). It should, however, be noted that more pronounced effects on luminol chemiluminescence are obtained with the weak inhibitors if light emission is followed over a longer period because BHA and BHT will depress the sustained chemiluminescence observed in the late phase (cf. Fig. 4). Thus, photon emission remaining in the presence of 50 μM BHA is 75 ± 9% during the first 20 min but only 25 ± 6% during an 80-min period; no inhibition is observed in the 20-min interval with 50 μM BHT but only 35 ± 5% of photon emission is left when integrated over the 80-min interval. This means that in the overall process occurring in the microsomal membrane – the components of which are not yet completely understood – BHA and BHT are as potent inhibitors as the gallic acid esters (remaining chemiluminescence at 50 μM PG in the 20-min interval: 36 ± 5%, in the 80-min interval: 31 ± 6%) while they are weakly active or virtually inactive in the initial process. No such differences are of course obtained in

the lucigenin assay because a second slow phase cannot be measured in this case.

Figure 5 also describes antioxidant effects on the production of an agent possessing the oxidizing properties of $OH^{\cdot}$ radicals towards dimethyl sulfoxide and on the production of hydrogen peroxide by microsomes. In the endoplasmic reticulum, these oxygen metabolites are assumed to arise from superoxide anion radicals released during autoxidation of the oxycytochrome P-450 complex (Ullrich and Kuthan 1980). These data may be of some interest in the interpretation of the chemiluminescence results. Synthetic antioxidants induce increased production of hydrogen peroxide by microsomes (Cummings and Prough 1983; Rössing et al. 1985). Any scavenging activity of such antioxidants towards $OH^{\cdot}$ radicals may thus be compromised by increased levels of the $OH^{\cdot}$ radical precursor. BHA is the most potent hydrogen peroxide-producing antioxidant, and its failure to inhibit DMSO oxidation may be related to this large extra production of H_2O_2. PG is only marginally active in producing H_2O_2 in liver microsomes, and any ensuing increase of $OH^{\cdot}$ concentrations might be expected to have little impact on the overall effect of PG in view of its excellent $OH^{\cdot}$-scavenging properties. BHT is also relatively weak as a hydrogen peroxide producer but in contrast to PG will not exhibit significant $OH^{\cdot}$ radical scavenging activity as predicted from the data obtained in aqueous media. Accordingly, increased microsomal oxidation of DMSO results in the presence of BHT.

Assuming that the ability of an antioxidant to suppress the oxidation of DMSO as well as the photon emission elicited by the oxygenation of luminol and/or lucigenin is indicative of a benefical biological action affording protection against reactive inorganic oxygen molecules in addition to their well-known protection against lipid peroxidation, our data suggest that only PG (and its congeners octyl gallate and dodecyl gallate the data of which are very similar to the PG data) is useful in this respect. The moderate oxygen radical scavenging activity of BHA will be compromised in the endoplasmic reticulum by its ability to produce considerable amounts of hydrogen peroxide and thus to act as a prooxidant. This relation may, however, be more favorable in other cell compartments. The oxygen radical scavenger efficiency of BHT is not even sufficient to counteract its prooxidant effect in liver microsomes.

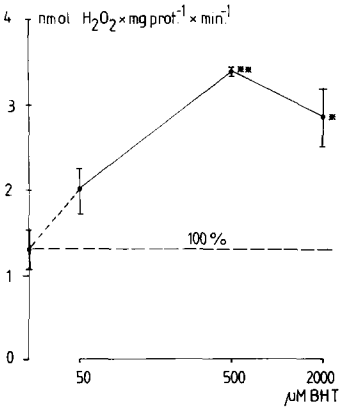

Fig. 6. Extra production of H_2O_2 in NADPH-consuming rat lung microsomes induced by butylated hydroxytoluene. Values are means ± SE ($n=4$). $^{+}p<0.05$, $^{++}p<0.001$. *BHT* butylated hydroxytoluene

This may be even more dramatic in lung endoplasmic reticulum where the hydrogen peroxide producing capacity of BHT exceeds that observed in liver microsomes (Fig. 6).

The present data indicate that a) chemiluminigenic probes can be used to measure oxygen activation in biological material, b) antioxidant activity towards reactive oxygen species can be determined with these probes, and c) gallic acid ester antioxidants far exceed BHA and BHT in their ability to remove such reactive oxygen species. The protective potential of gallic acid ester antioxidants towards toxic, mutagenic and carcinogenic agents has not been studied as extensively as that of BHA and BHT (Kahl 1984). We suggest that it might be promising to further study this potential.

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