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DETECTION OF ACTIVE OXYGEN IN RAT HEPATOCYTE SUSPENSIONS WITH THE CHEMILUMINIGENIC PROBE LUCIGENIN

Regine Kahl, Andreas Weimann and Alfred G.Hildebrandt

Max von Pettenkofer Institute, Federal Health Office
Postfach 33 00 13, D-1000 Berlin 33, F.R.G.

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SUMMARY - The chemiluminigenic probe lucigenin has been employed to detect the production of active oxygen species in suspensions of intact rat hepatocytes. Light emission from lucigenin arises from oxygenation by superoxide anion; hydrogen peroxide or a species derived from it may contribute to the reaction. The inhibitory action of antioxidants on the availability of active oxygen species produced by hepatocytes was tested. Propyl gallate was the most potent inhibitor, butylated hydroxyanisole and butylated hydroxytoluene were less active. The latter compounds cause an alteration of the the cell membrane at high concentrations.

Oxygen consumption is accompanied by the production of the potentially harmful oxygen metabolites, the superoxide anion radical, hydrogen peroxide and the hydroxyl radical. In phagocytozing cells, oxygen activation is essential for the specific defense function of the cell (1) while in other cell types it is assumed to be involved in a number of pathological events connected with lipid peroxidation (2), organ injury (3), DNA damage and tumor promotion (4). The cell possesses a cooperative antioxidant defense system consisting of the enzymes superoxide dismutase, catalase and glutathione peroxidase and of low molecular radical scavengers, among them most notably vitamin E. It may be assumed that this defense line will keep oxygen radical concentrations low unless it is overwhelmed by some kind of oxidative stress. Therefore, the detection of active oxygen species will be more difficult in nonphagocytozing cells than in subcellular fractions derived from these cells. However, hydrogen peroxide formation can, by indirect means, be followed in intact hepatocytes, and

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its intracellular stimulation by drug substrates has been demonstrated (5). Superoxide anion is assumed not to be released into the medium by the hepatocyte, but easily detectable release can be induced by the uptake of quinones undergoing redox cycling (6). In phagocytes, photon emission in the presence of chemiluminigenic probes has been employed to measure oxygen activation during the respiratory burst (7,8). In the present study, we describe the detection of active oxygen species in a suspension of hepatocytes by use of the chemiluminigenic probe lucigenin and the removal of these oxygen metabolites by some synthetic antioxidants.

METHODS

Untreated male Wistar rats (250-350 g) were anesthetized with approximately 70 mg pentobarbital/kg body weight i.p. and received 1000 I.U. heparin / kg body weight. The livers were inserted into a recirculating perfusion system operated with Ca^{++}-fortified Hepes buffer pH 7.6 containing 500 mg/l collagenase IV (obtained from Sigma Chemicals, Munich) at 37°C for 12-15 min. The cells were then transferred to a washing solution and washing was repeated three times. Viability was determined by Trypan blue exclusion. For chemiluminescence measurements, a total of 400 000 cells were incubated in a final volume of 200 µl Hepes buffer pH 7.6. The reaction was started by the addition of 10 µl lucigenin (8 mM in aq.bidest.) and followed for 20 min. Measurements were performed in a "Biolumat LB 9505" (Berthold, Wildbad, F.R.G.) equipped with six photomultipliers in order to allow for simultaneous sample handling. Antioxidant addition was performed in 10 µl acetone. 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.

RESULTS AND DISCUSSION

Specificity of the lucigenin test - It is assumed that lucigenin is oxygenated to a light-emitting species by the superoxide anion (8,9), Accordingly, lucigenin chemiluminescence is obtained in a xanthine oxidase system (Fig.1a) and can be inhibited by superoxide dismutase (Fig.1b). However, a small but consistent lucigenin chemiluminescence is also observed in a solution of H_2O_2 (Fig.1a), and the light emission from the xanthine oxidase system is partially suppressed by catalase though very high concentra-

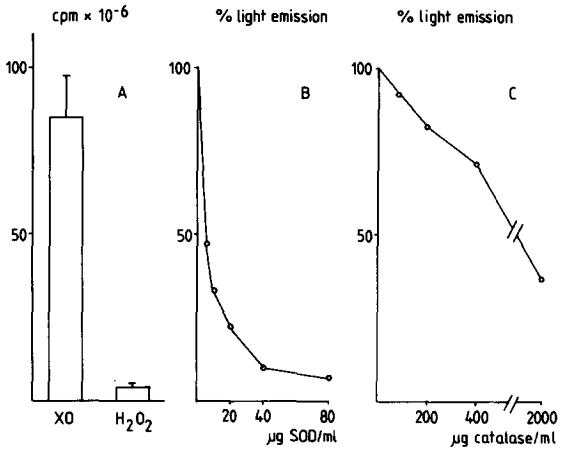
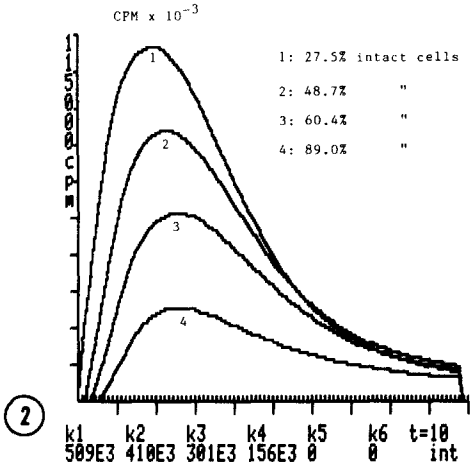


Fig.1 Lucigenin chemiluminescence in aqueous model systems.
A: Photon yield in a xanthine oxidase (XO) system (0.2 mM hypoxanthine, 0.5 mg/ml xanthine oxidase, Tris buffer pH 7.4) and in 0.6% H_2O_2. Values are means ± S.E.M. (n=4-19).
B: Inhibition of light emission in the xanthine oxidase system by superoxide dismutase (SOD). Values are means of 2-7 experiments.
C. Inhibition of light emission in the xanthine oxidase system by catalase. Values are means of 2-5 experiments.

tions of the enzyme must be used (Fig.1c). It appears possible from these findings that H_2O_2 or a species derived from it, the hydroxyl radical, contribute to the overall chemiluminigenic reaction.

Lucigenin chemiluminescence in hepatocyte suspensions - In preliminary experiments using hepatocytes not subjected to an extensive washing procedure, we observed that the chemiluminescence obtained by the addition of lucigenin to the medium did not correspond to the percentage of intact cells. We concluded that oxygen activation in cellular fragments induced by NADPH released from damaged cells superimposes the chemiluminescence response from intact cells. This was verified by experiments in which NADPH was added to hepatocyte suspensions containing increasing fractions of damaged cells. Under these conditions, an inverse relationship between light emission and the percentage of intact cells exists (Fig.2). A preparation containing 100% of intact



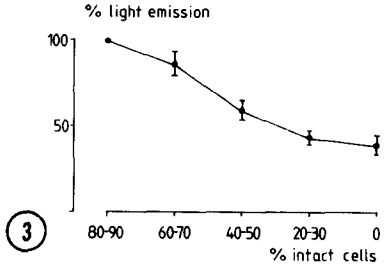


Fig.2 Inverse relation between lucigenin chemiluminescence and percentage of intact cells in the presence of NADPH. Original recording.

Fig.3 Dependence of lucigenin chemiluminescence on the percentage of intact cells in suspensions of extensively washed hepatocytes. Light emission at 80-90% of intact cells was considered as 100%. Values are means ± S.E.M. (n=8-9).

cells cannot, by our procedure, be obtained. Since lucigenin chemiluminescence in hepatic microsomes (10) exceeds that measured in hepatocytes by 2-3 orders of magnitude, the contribution from damaged cells to the chemiluminescence can be large though the percentage of damaged cells may be small. Therefore, we introduced a protocol of extensive washing of the cell suspension before use in order to remove NADPH released from damaged cells. The positive relationship between lucigenin chemiluminescence and the percentage of intact cells observed under these conditions is shown in Fig.3. It should be noted that a background chemiluminescence from damaged cells still exists. We assume that at 90% of intact cells up to 10% of the overall chemiluminescence response can be due to reactions occurring in cell fragments.

It is not clear from the present experiments if the lucigenin test measures intracellular oxygen metabolites, oxygen metabolites produced at the outside of the plasma membrane or oxygen metabolites released into the medium. Lucigenin uptake studies

have not been successful due to the lack of radioactive substance. Lucigenin is fairly hydrophil , and it is not very likely that it penetrates the cell membrane. It has been claimed that in phagocytes lucigenin - in contrast to luminol - only exerts an extracellular effect (11). It is questionable to what extent superoxide anion radical will leave the unstimulated hepatocyte, and it remains to be elucidated to what extent H_2O_2 or $OH^{\cdot}$ radicals contribute to the light emission measured in hepatocyte suspensions.

Influence of synthetic antioxidants on chemiluminescence - We have previously shown (10) that gallic acid ester antioxidants efficiently reduce luminol and lucigenin chemiluminescence in NADPH-fortified liver microsomes while butylated hydroxyanisole and butylated hydroxytoluene are only marginally active ore even inactive in these tests. This may be partially due to the ability of the latter antioxidants to produce large amounts of hydrogen peroxide in the endoplasmic reticulum (10). Fig.4 demonstrates that 30% suppression of chemiluminescence by synthetic antioxi-

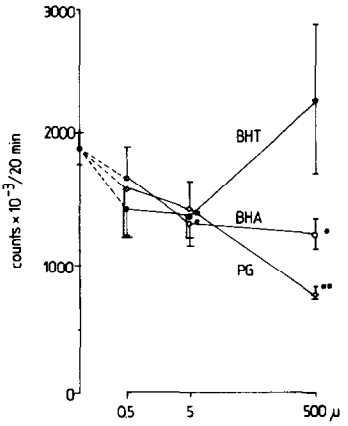


Fig.4 Effect of synthetic antioxidants on lucigenin chemiluminescence in hepatocytes. Values are means ± S.E.M. (n=4-5). * $p < 0.05$; ** $p < 0.01$. BHT: butylated hydroxytoluene, BHA: butylated hydroxyanisole, PG: propyl gallate.

dants can be obtained at relatively low concentrations (5 μM) which are close to those present in human tissues at contemporary dietary habits (12). At a very high concentration (500 μM), inhibition by propyl gallate is increased to approximately 60%; inhibition by butylated hydroxyanisole is not increased, possibly because at that concentration significant extra production of hydrogen peroxide in the endoplasmic reticulum by this antioxidant interfers with its radical scavenger properties. Data obtained at 500 μM butylated hydroxytolene are highly variable. This may be due to the vesicular alteration of the cell membrane caused by high concentrations of butylated hydroxytoluene, but also of butylated hydroxyanisole (Fig.5). The nature of this lesion has not been further elucidated in the present study. It has been reported (13) that butylated hydroxytoluene and to a lesser degree also butylated hydroxyanisole cause a destruction of the plasma membrane of rat erythrocytes.

It has been stated above that H_2O_2 or $OH^{\cdot}$ radical may participate in the chemiluminescence reaction. The suppressive action of

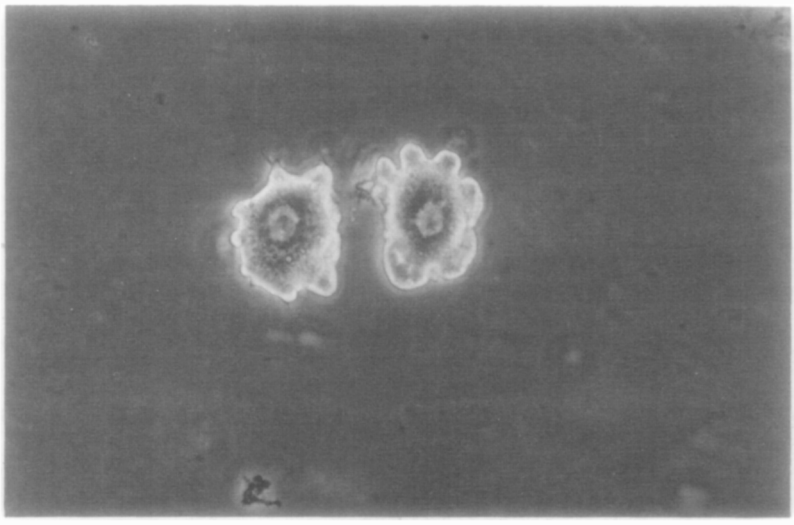


Fig.5 Alteration of the cell membrane of hepatocytes following incubation with 500 μM butylated hydroxyanisole. Native cells, magnification 1:40.

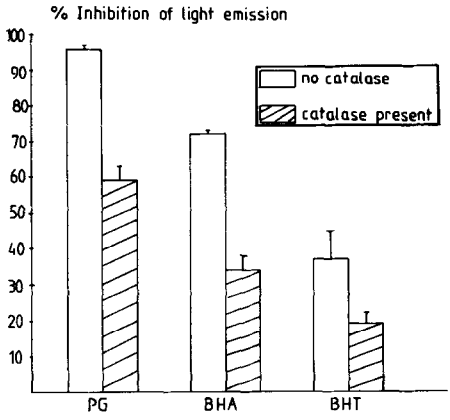


Fig.6 Influence of catalase on the inhibitory effect of synthetic antioxidants on lucigenin chemiluminescence in the xanthine oxidase system. Antioxidants 50 µM, catalase 2 mg/ml. Values are means ± S.E.M. (n=5). PG: propyl gallate; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene.

antioxidants may thus be directed to superoxide anion, provided it is released into the medium, or to OH· radicals produced by a Fenton reaction at the cell membrane or in the medium. Model studies in the xanthine oxidase system demonstrate that both species may be affected (Fig.6): Antioxidant inhibition is markedly higher in the absence of catalase but some inhibition remains if H_2O_2 and OH· are removed by the addition of catalase. Further studies are necessary to finally identify the target molecule of the antioxidants in hepatocyte suspensions.

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