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SPECIFICITY OF OXYGEN RADICAL SCAVENGERS AND ASSESSMENT OF FREE RADICAL SCAVENGER EFFICIENCY USING LUMINOL ENHANCED CHEMILUMINESCENCE

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SUMMARY: The selective scavenging capacities of 19 important oxygen radical scavengers were determined by adding them individually to each of the four oxy radical standards, (superoxide, hydroxy, alkoxy and hydroperoxy, and singlet O_2), calculating the percent chemiluminescence inhibited, and extrapolating O_2 equivalents neutralized from baseline. The sensitivity (0.01nm/ml) and selectivity of this method not only allows identification of individual oxygen free radical species but also quantitates the efficiency of free radical scavengers. © 1988 Academic Press, Inc.

We have directly assessed the specificity and efficiency of 19 important oxygen radical scavengers based upon the principle that all oxygen radicals produce a baseline chemiluminescence which may be luminol enhanced (1,2,3). The intensity of this luminescence varies with the concentration of the radical species and provides a simple, sensitive and specific quantitation of O_2 metabolites. Inhibition of this enhanced chemiluminescence by the addition of various scavengers to the free radical solution provides an accurate assessment of the efficiency of various scavengers against each individual radical species.

METHODS AND MATERIALS

The O_2 metabolites were produced as follows: Superoxide radical (O_2^-) from hypoxanthine (100uM, Sigma) xanthine oxidase (0.13uM, Calbiochem); hydroxy radical using ferrous perchlorate (200uM, Alfa), and H_2O_2 (60uM, Sigma) in the presence of DPTA (200uM, Sigma); alkoxy and peroxy radicals from t-butyl hydroperoxide (Sigma) in 20% t-butanol (Sigma). Singlet $O_2(^1O_2)$ was generated by irradiation of an O_2 saturated solution of methylene blue (89uM) in deuterated water (with phosphate buffer pH 7.4) using an Osram 100 watt Krypton lamp. Methyl orange (0.1M) was used as a cut off filter (600nm).

The luminol enhanced chemiluminescence (CL) was produced in a polystyrene cuvette containing 1ml of 0.01M phosphate buffer (pH 7.4), 200 ul luminol

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(500uM, pH 12.0) plus the oxidizing radical. The chemiluminescence and its rate of decay were measured in a LKB 1250 luminometer at ambient temperature.

The O_2^- produced from hypoxanthine and xanthine oxidase was quantitated independently using the ferricytochrome C method (4). The $^1O_2$ concentrations were quantitated independently by adding 35uM diphenylisobenzofuran (DPBF) to the irradiated O_2 solution and monitoring the formation of dibenzoyl benzene (DBB) with absorbance at 420nm in a kinetic spectrophotometer (5). The efficiency of a number of oxygen free radical scavengers as well as the dose response curves for SOD, DMSO, glutathione peroxidase, and DPBF were studied.

RESULTS AND DISCUSSION

The O_2^- measured was directly proportional to the xanthine oxidase concentration (Fig. I) used in its production (0.05 - 0.025uM). The O_2^- produced, as measured by the ferricytochrome C method (2.5 - 20nm/ml) corresponded to 100 - 900mV of luminescence (inset, Fig. I). Initially 19 important oxygen free radical scavengers were evaluated at concentrations producing maximal luminometer signals (Table 1). From these, four specific scavengers (SOD, DMSO, DPBF, glutathione peroxidase) were selected for dose-response studies. The luminol chemiluminescence of O_2^- was quenched rapidly by SOD, reaching 90% inhibition at 4.0×10^{-7} M (inset, Fig. I).

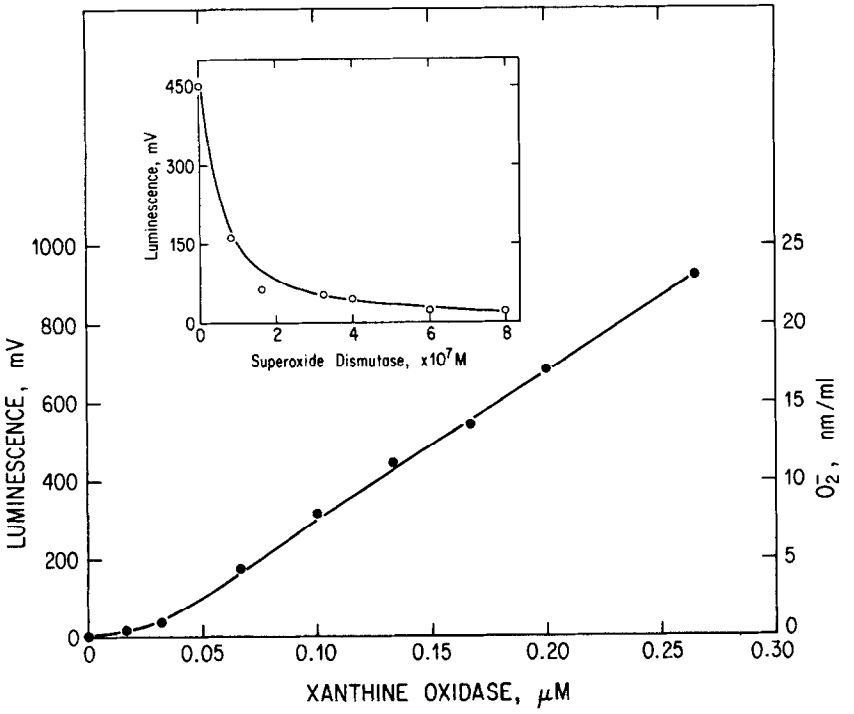

Fig. 1 Superoxide radical production as a function of luminol chemiluminescence, (HX100uM), luminol 200uM, phosphate buffer, Final pH 10.3 Insert: Quenching of CL by SOD.

TABLE 1

Specificity and Selectivity of Oxygen Radical Scavengers

Scavenger	$[O_2]^+$ nm/ml	[OH] nm/ml	[OOH] nm/ml	$[^1O_2]^\#$ nm/ml
None (Standards)	12.0	0.48	0.15	0.20
SOD (400 nM)	0.7	0.24	0.05	0.10
Ascorbate (100 nM)	0.7	0.03	0.15	0.16
Catalase (200 nM)	11.8	0.48	0.15	0.18
DMSO (150 mM)	11.6	0.03	0.15	0.19
Alpha tocopherol (5 nM)	11.6	0.04	0.03	0.19
B-carotene (5 nM)	11.6	0.12	0.15	0.19
Diphenyl-p-phenylene diamine(1 mM)	11.9	0.17	0.15	0.20
Dimethyl-p-nitroso aniline (1 mM)	12.0	0.06	0.15	0.20
NN' dimethyl thiourea (5 mM)	11.6	0.08	0.15	0.20
Methyl glyoxal (1 mM)	12.0	0.18	0.15	0.20
Thiourea (1 mM)	9.3	0.34	0.15	0.20
Mannitol (15 mM)	11.6	0.19	0.15	0.20
Potassium thiocyanate (5 mM)	11.8	0.24	0.15	0.20
L-methionine	11.8	0.09	0.15	0.12
2.4-dinitro fluorobenzene(50 uM)	12.0	0.48	0.15	0.03
DPBF (50 uM)	12.0	0.48	0.15	0.02
Na selenite (2 ppm)	11.9	0.46	0.03	0.19
Glutathione peroxidase (20 nM)	11.9	0.42	0.02	0.19
p-Hydroxy mercury benzoate (2 mM)	11.8	0.44	0.12	0.20

+ Mean of 6 determinations; $[O_2^-]$ quantitated by ferricytochrome C reduction method.

\# Measured initially as um dibenzoyl benzene formed from DPBF and then converted to equivalents in O_2^- radical production.

Hydroxy radical production from Fenton's reaction:

$$Fe^{2+} + H_2O_2 \text{ ------} \rightarrow Fe^{3+} + OH^- + OH$$

increased linearly with H_2O_2 in the range of 20 - 40uM (Fig. 2) and plateaued thereafter. The hydroxy radical enhanced luminol chemiluminescence signal reached a plateau at 50±4.6mV, equivalent to 1.3±0.2 nm/ml of O_2^- concentration. DMSO, a specific OH scavenger, quenched the radical production by 90% at 125mM (inset, Fig.2).

The alkoxy and hydroperoxy radical production from t-butyl hydroperoxide was linear in the range 0.5 - 2.5 mM (Fig. 3). The O_2^- equivalent radical production was, however, small (0.15nm/ml). Alkoxy radicals produced were quenched by reaction with 2mM p-mercury benzoate and yielded a very small signal (0.03nm/ml, O_2^- equivalents). Glutathione peroxidase (200nM) quantitatively quenched the hydroperoxy radical chemiluminescence by greater than 90% (inset, Fig. 3).

Singlet O_2 production was quantitatively monitored (Fig. 4) using this technique. Its production was linear in the first 45 minutes of illumination,

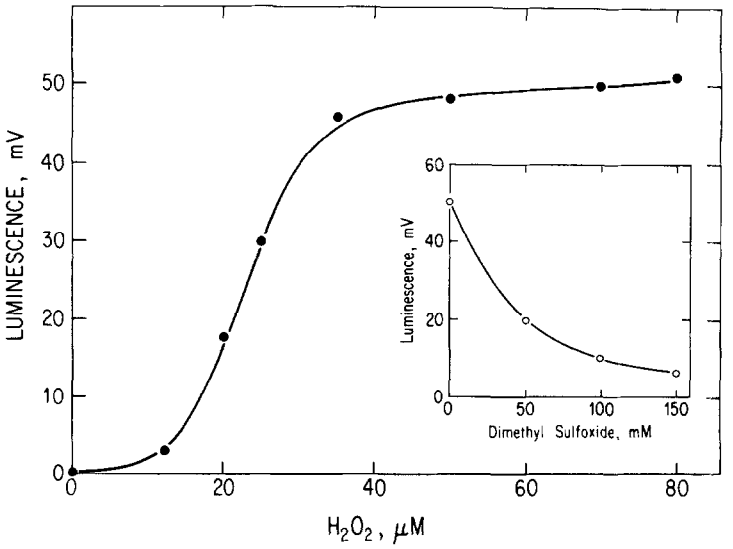

Fig. 2 Hydroxy radical production as a function of chemiluminescence by luminol. Fenton's reaction (Ferrous Perchlorate 200uM, H_2O_2 60uM, DTPA 200uM, phosphate buffer pH 7.4). Insert: Quenching of CL by DMSO.

after which it reached a plateau (Fig. 5), corresponding to 9mV (0.24 nm/ml O_2^-). DPBF (35 uM) quenched the signal by 93% with a concurrent rise in absorbance at 420nm corresponding to production of its product, dibenzoyl benzene (26um DBB produced after 1 hour irradiation).

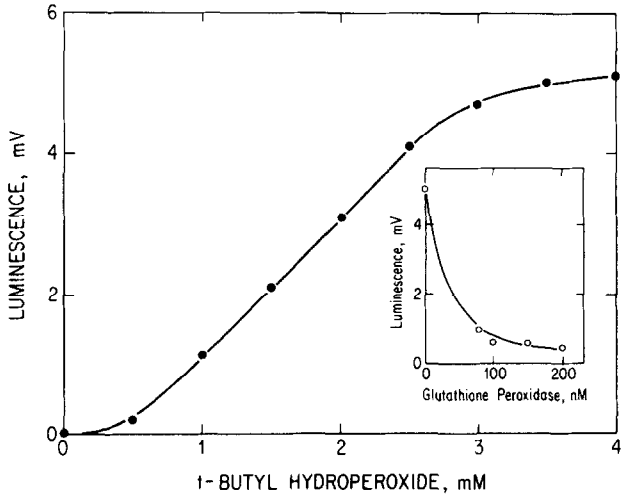

Fig. 3 Hydroperoxy radical formation as a function of luminol chemiluminesence (t-butyl hydroperoxide 0-4mM, Buffer pH10). Insert: Quenching of CL by glutathione peroxidase.

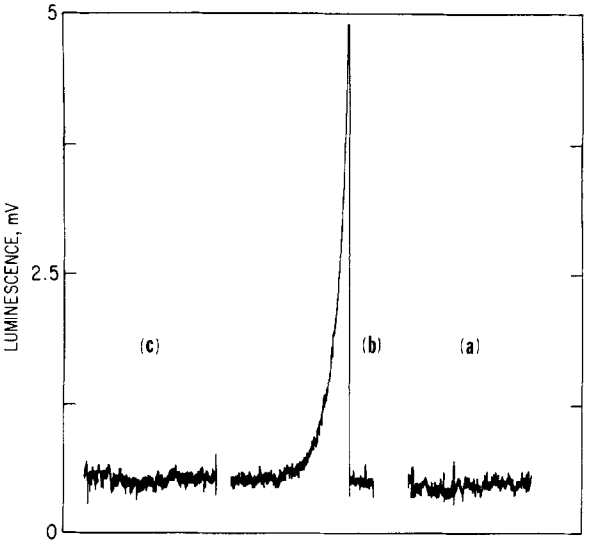

Fig. 4 Singlet O_2 production monitored by luminol chemiluminescence tracings (a) without irradiation, (b) with one hour Kryton lamp irradiation and (c) with one hour of irradiation and DPBF added.

The mechanism of luminol enhanced free radical chemiluminescence is as follows (6,7):

$$LH^- + O_2^- \longrightarrow LH^{\cdot} \xrightarrow{\cdot O_2} LOOH^- \longrightarrow N_2 + AP^{-*} \longrightarrow h\nu + AP^-$$

OH
OOH
$^1O_2$
Fe^{3+}

LH^-, luminol monoanion; $LH^{\cdot}$, luminol radical; $LOOH^-$, luminol dioxetane; AP^{-*}, electronically excited aminophthalate and AP^-, ground state aminophthalate.

The chemiluminescent decay of the superoxide and hydroxy radicals is independent of the concentration of O_2^- and OH since their rate constants (K) are identical, 0.96×10^9 and 0.91×10^9 m^{-1} sec^{-1} respectively, indicating a similar mechanism of luminol chemiluminescent decay.

This data clearly demonstrates the validity of chemiluminescence for *in vitro* identification and quantitation of O_2 metabolites and its usefulness in testing the efficiency, specificity and selectivity of free radical scavengers. A role for O_2^- is indicated if addition of SOD significantly reduces luminol luminescence. If addition of catalase has an inhibitory effect a role for H_2O_2 is implied. If both SOD and CAT are necessary to inhibit luminescence, a role for OH is implied, which can be confirmed by use of DMSO. A role for singlet O_2 is indicated if L-methionine, an OH and $^1O_2$ scavenger, further inhibits the luminescence, confirmed by addition of DPBF.

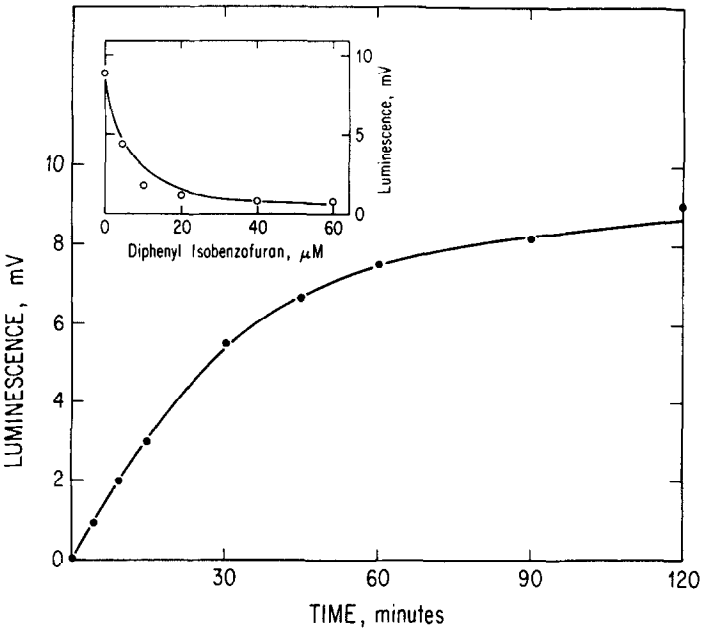

Fig. 5 Luminol chemiluminescence as a function of time of irradiation and production of $^1O_2$ (Deuterated water pH 7.4, O_2 saturated; methylene blue 89uM, 200uM luminol. Methyl orange cut off filter. Insert: Quenching of CL by DPBF.

The production and enhancement of light, using luminol amplification, to detect trace amounts of O_2 metabolites is useful as a general test for the presence of oxidizing radicals. The application of the concept of selective scavenging may provide the basis for specific indentification of these radical species. Caution, however, should be used when applying these techniques to biological systems since a variety of reagents commonly found in vivo such as uric acid, ammonium and ferric salts alter the intensity of the luminescence signal, and therefore, affect interpretation of the data.

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