Chemiluminescence Measurement of the Generation of Reactive Oxygen Species

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Introduction

Phagocytosis is one of the oxygen depending processes in organisms. During the unspecific immunological defense, the activity of pentosephosphate cyclus is dramatically increased forming NADPH. NADPH is needed to reduce the oxygen which is bonded to membrane-based cytochromes. Therefore, the oxygen demand is strongly increased (respiratory burst).
During this process, oxygen is converted into superoxide anions, hydrogen peroxide, monomolecular oxygen and hydroxylradicals by means of serveral kinds of phagocytic cells (e.g. neutrophil, eosinophil and basophil leucocytes, macrophages).
These extracellular highly reactive oxygen species (ROS) cause many biological effects such as destruction of bacterial cells, parasites and tumor cells, promoting inflammation and modulating the immune reaction (Fig. 1).

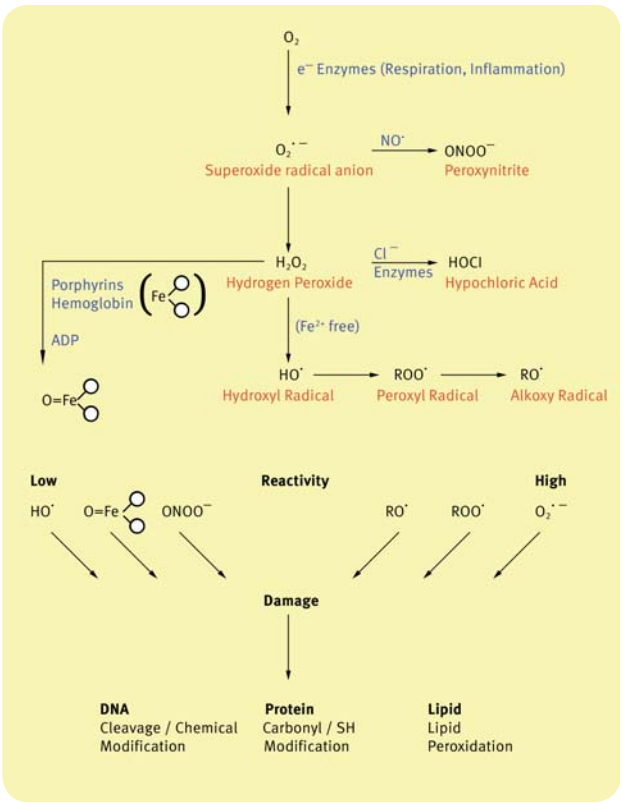

Fig. 1: Reactive oxygen species and oxidative damage

Reactive oxygen species are formed by phagocytic cells under various conditions:

- Most of the cells are able to form ROS by themselves without any stimulation
- During the phagocytosis of foreign matter
- During cell proliferation and/or cell cytotoxicity processes
- By stimulation with soluble stimuli (e.g. immuno complexes, anaphylatoxins C3a/C5a, chemotactic peptides, ionophores, lectines and phorbolester)

The generation processes of reactive oxygen species can be monitored using luminescence analysis. Measurements of chemiluminescence are highly sensitive and specific, owing the possibility to investigate the different kinds of reactive oxygen species simultaneously ($HO^{\cdot}$, $O_2^{\cdot -}$, H_2O_2, $^1O_2$). Because of the very weak native luminescence phenomena, luminol or lucigenin dependent chemiluminescence have been used frequently for the detection of superoxide radical anions in biological systems. Luminol reacts in its univalently oxidized form and lucigenin reacts in its univalently reduced form with $O_2^{\cdot -}$. In both cases, light production depends on the formation of an unstable endoperoxide or dioxetane, which decomposes to an electronically excited product. This product releases a photon as it falls to the ground state. In the case of luminol, hydrogen peroxide is more reactive than $O_2^{\cdot -}$, but the superoxide radicalanions were detected faster by lucigenin than hydrogen peroxide (Fig. 2).

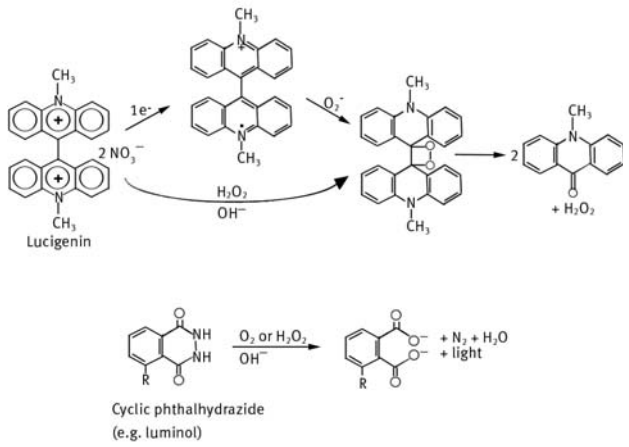

Fig. 2: Luminescence phenomena caused by the reaction with ROS

Materials and Methods

The following strains of Candida were used for the measurement of cellular luminescence:
C.albicans, C.tropicalis, C.guilliermondii, C.glabrata, C.parapsilosis.

- The strains of fungi were cultured 24 h on Sabouraud-Glucose-Agar in the presence of penicillin and gentamicin.
- Yeast suspensions were prepared in RPMI-1640 medium with concentrations of 10^7 to 10^{11} blastospores/mL or in isotonic NaCl-solution with the same concentration.
- The cell counts were assessed either with a counting chamber or a CASY 1 device (Schärfe-System, Germany).
- 200 µL of several yeast suspensions were used.
- 10 µL of the Lucigenin solution (10^{-4} mol/L) was added into each well at 30°C or 37°C by an injection pump.

The chemiluminescence measurements were carried out by means of a BMG LABTECH microplate reader, such as LUMIstar, NOVOstar, POLARstar, or FLUOstar (Fig. 3).

Fig. 3: Microplate reader FLUOstar OPTIMA

For the measurement, the slow kinetic method was used. The results are mean values of accumulated single readings over a period of 24 minutes. The statistics of the chemiluminescence counts and the calculations of correlation coefficients were performed by means of Microsoft Excel and the dedicated BMG LABTECH evaluation software. P ‹ 0.05 was considered statistically significant.

Results and Discussions

The results of lucigenin dependent chemiluminescence measured in suspensions of *Candida albicans, Candida guilliermondii, Candida parapsilosis, Candida tropicalis, Candida glabrata* and *Trichosporon capitatum* are shown in Table 1.

Table 1: Comparison of different yeast species

	Candida albicans	*Candida guilliermondii*	*Candida parapsilosis*	*Candida tropicalis*	*Candida glabrata*	*Trichosporon capitatum*
cells	RLU	RLU	RLU	RLU	RLU	RLU
E+7/mL	0	0	2	0	0	19
E+8/mL	0	0	4	0	7	94
E+9/mL	2	12	19	25	35	101
E+10/mL	30	48	61	90	126	124
E+11/mL	64	122	156	102	189	324
	r = 0.91 p = 0.02	r = 0.96 p = 0.01	r = 0.960 p = 0.009	r = 0.74 p = 0.10	r = 0.84 p = 0.07	r = 0.970 p = 0.004

(RPMI-medium, T=30°C, Lucigenin-solution 100 µL)

It was notable that the generation of reactive oxygen species in *C. albicans* could not be detected at concentrations of 10^7 to 10^9 blastospores/mL. Detectable chemiluminescence values were measured at ≥ 10^{10} blastospores/mL. The reproducibility of the results could be demonstrated by inter assay variation as well as linear correlation (r=0.91 resp. 0.98) of the concentration of blastospores and the relative light units (RLU). The linear and direct proportion of the ROS generation and blastospore concentration could be confirmed due to the calculated correlation coefficients. The reproducibility of the results has been demonstrated by inter assay variation on serveral days. To eliminate potential effects of the RPMI medium, ROS have been additionally measured in NaCl solution. There could be even higher values found in some cases (Table 2).

Table 2: Comparison of different yeast species

	Candida albicans	*Candida glabrata*	*Trichosporon capitatum*
cells	RLU	RLU	RLU
E+5/mL	60	53	36
E+6/mL	67	33	53
E+7/mL	81	93	60
E+8 /mL	93	75	138
E+9/mL	306	148	455
E+10/mL	415	248	623
	r = 0.84 p = 0.07	r = 0.90 p = 0.03	r = 0.98 p = 0.002

(NaCl-solution, T=30°C, Lucigenin-solution 100 µL)

These experiments show that the RPMI medium is not able to stimulate the ROS generation, but more likely to inhibit because of its antioxidizing constituents (vitamines, glutathione).

Conclusion

The ability of various candida yeasts and blastomyces to generate ROS can be monitored by means of lucigenin-dependent chemiluminescence.
The chemiluminescence method is sensitive enough to detect very low ROS concentrations produced by several fungi without any stimulation. It was notable that detectable chemiluminescence signals can be found in candida species at concentrations of ›10^8 blastospores /mL.
A linear and direct proportion between ROS levels and blastospore concentrations could be found. ROS production may contribute to the inflammatory reaction in the initial phase of Candida infections and may cause tissue damage to the host (induction of lipid peroxidation and formation of leukotrienes).
The activated oxygen species are aggressive and toxic depending on their concentration.

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