EVALUATION OF THE INTRACELLULAR RADICAL GENERATION BY STIMULATED LEUKOCYTES BY TOTAL LUMINOL CHEMILUMINESCENCE

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ABSTRACT

Luminol-enhanced chemiluminescence (LCL) is a widely used method for quantitative evaluation of reactive oxygen species (ROS) generated by stimulated leukocytes. The chemiluminescent kinetics registered (Total LCL) is an expression of simultaneously running processes of extra- and intracellular radical generation. In literature, model chemiluminescent systems, designed to separate extra- from the intracellular light, have been proposed. These systems, however, change cellular activity. The aim of the present study was to propose an approach for evaluating the intracellular ROS generation by stimulated leukocytes in whole blood. Heparin anti-coagulated blood from healthy volunteers was used. The chemiluminescent kinetics reflecting leukocyte activity to produce ROS were registered by a computerized chemiluminometer. To distinguish extra- from the intracellularly generated light by the cells, some model chemiluminescent systems were used. It was found that the intracellular light generated by stimulated leukocytes was a substantial part of Total LCL. The proportion between the extra- and intracellular light within the Total response depended on the concentration of the particulate stimulant. At a low zymosan concentration (0.25 mg/ml) Total LCL response was found to be intracellular. On the basis of the results obtained we conclude that Total LCL at a low stimulant concentration is of an intracellular origin, which could be used to study the effect of different compounds on the intracellular oxidative leukocyte activity. In the course of some pathological processes accompanied by suppurative foci formation, bacteriemia, etc., the proportion between the number of bacteria and leukocytes considerably changes. A change in the stimulant concentration allows to model these pathological states and to evaluate the extra- and intracellular radical generation by stimulated leukocytes.

Keywords: chemiluminescence, reactive oxygen species, intracellular, leukocytes

INTRODUCTION

Luminol-enhanced chemiluminescence (LCL) is a widely used method for quantitative evaluation of reactive oxygen species (ROS) generated by stimulated leukocytes (isolated and in whole blood). The LCL kinetics registered by zymosan-stimulated whole blood is a smooth curve with no visible phases. In fact, however, it is a result of simultaneously running processes of extra and intracellular ROS generation. The extra and intracellular light may have different relative contribution to the chemiluminescent response depending on the pathophysiological state of individuals and experimental conditions (1). In literature, a number of chemiluminescent systems designed to produce predominantly extra- or intracellular light have been pro-

posed (2,3). These systems require additional influences to be applied on the cells, which may change their activity and make the study of the effect of drugs and toxic compounds on them difficult. The specificity of such influences has been questioned and hence, it is preferable for the conclusions to be made on the basis of pure systems without using agents-modifiers.

The aim of the present work was to find the experimental conditions, under which the Total LCL is a measure of the intracellular ROS production by zymosan-stimulated leukocytes in whole blood without using agents-modifiers.

MATERIAL AND METHODS

Blood from healthy volunteers anti-coagulated with heparin (10 U/ml) was used. The chemiluminescent kinetics reflecting leukocyte activity to produce ROS were registered by computerized chemiluminometer working in a photon-counting mode (4).

The following model systems were used to evaluate the extra- and intracellular light produced by the cells:

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1. *Standard system* containing 0.1 ml whole blood (1:10), 0.2 ml luminol (0.1 mmol/l), zymosan (4 mg/ml or 0.25 mg/ml) and Krebs-Ringer phosphate buffer (KRP) in a total volume of 2 ml. LCL kinetics registered from this system was denoted as Total LCL.
2. *Intracellular system – Ist type*, according to Walan et al (2) containing besides the components of the standard system also 150 U/ml superoxide dismutase and 1500 U/ml catalase.
3. *Intracellular system – IInd type*, according to Briheim et al (3) containing besides the components of the standard system also 1% human serum albumin (HSA). LCL kinetics registered from these system were denoted as Intra LCL.
4. *Extracellular system* containing 0.1 ml whole blood (1:10), zymosan (4.0 mg/ml or 0.25 mg/ml) and KRP in a total volume of 2 ml. The cells were preliminary incubated with 0.03 mmol/l luminol for 30 min.

RESULTS AND DISCUSSION

It is considered that at a high concentration of stimulating agent, leukocytes attempt to ingest a great number of particles. As a result, a significant quantity of ROS produced are secreted into the extracellular space since the phagocytic vacuoles fail to close effectively (5). Hence, a change in the stimulant concentration may change the proportion between the extra- and intracellularly generated ROS within the Total response.

I. Dependence of Total LCL on zymosan concentration

The time development of Total LCL response in dependence of zymosan concentration (0.25, 2 and 4 mg/ml) is shown in Fig.1. With the increase of stimulant concentration the maximum LCL intensity increases and the time to the peak decreases.

Fig.1. Dependence of Total LCL on zymosan concentration.

The extra- and intracellular light, however, are indistinguishable in these curves. To study in what way the intracellularly generated light depends on the stimulant concentration, some model chemiluminescent systems were used.

II. Dependence of Intracellular (Intra) LCL on zymosan concentration

a) intracellular system - Ist type

Superoxide dismutase and catalase were used to remove the extracellularly produced superoxide and hydrogen peroxide. The total and intracellular responses at a high and low zymosan concentrations are given in Fig. 2 and Fig.3, respectively.

Fig.2. Total and Intra LCL registered simultaneously at 4.0 mg/ml zymosan.

Fig.3. Total and Intra LCL registered simultaneously at 0.25 mg/ml zymosan.

- **High zymosan concentration:** The Total LCL intensity was significantly higher than the intracellular one and reached its maximum value earlier.
- **Low zymosan concentration:** The intracellular LCL response did not depend on zymosan concentration. It was even slightly increased as compared to the total one, a result which is in accordance with literature (3).

b) Intracellular system – IInd type

The system was realized by using human serum albumin (HSA) – a scavenger of the extracellularly generated ROS. The total and intracellular LCL responses in dependence of stimulant concentrations are given in Fig.4. The results were found identical to those obtained by the Ist type of intracellular system.

Fig.4. Effect of HSA on LCL at different zymosan concentrations. Total LCL - continuous lines; Intra LCL - dotted lines.

III. Dependence of Extracellular LCL on zymosan concentration

To visualize only the extracellular response, different LCL systems have been proposed in literature. In the present work we propose a system for extracellular LCL reflecting the initial steps of cellular stimulation.

It is known that luminol is a lipophilic compound and added to the cells becomes membrane-bound (6). That is why, the cells were preliminary incubated with luminol. Luminol was not added to the reaction mixture for not being included into phagosomes during phagosome formation. Thus, the registered kinetics reflects the extracellular radicals released near the leukocyte membranes. Since the quantity of the immobilized luminol is not great, it does not visualize the whole cellular response but only some part of it - during the initial steps of the activation cascade. Using such a system it was found that the level of the extracellular ROS secreted at a low zymosan concentration was negligible (data not shown).

IV. Application of the approach proposed to study piroxicam effect on ROS generation by leukocytes in whole blood

It is known that piroxicam, a non-steroidal anti-inflammatory agent significantly reduces the extracellular ROS generation by stimulated leukocytes (7). This property of the drug is considered to have a major contribution to the reduced joint destruction in inflammatory arthritis. On the other hand, it has been established that piroxicam does not reduce the phagocytic ability of the cells and their intracellular ROS production.

Piroxicam effect on the intra- and extracellular ROS production by stimulated leukocytes was demonstrated by Total LCL registered at a low and high zymosan concentration (Fig.5). It was found that piroxicam reduced Total LCL at a high zymosan concentration i.e. extracellular ROS production, a result confirmed by some other methods (for instance spectrophotometric ones).

Fig.5. Piroxicam effect on Total LCL at different zymosan concentrations.

On the other hand, the agent did not reduce Total LCL intensity at a low zymosan concentration, respectively the intracellular ROS generation.

CONCLUSION

On the basis of the results obtained we conclude that:

1. The intracellular light generated by stimulated leukocytes is a substantial part of the Total LCL response.
2. The proportion between the extra- and intracellular light within the total response strongly depends on the concentration of particulate stimulant. At a low zymosan concentration (0.25 mg/ml) Total LCL is mainly due to intracellular radical generation.
3. Total LCL at a low zymosan concentration (0.25 mg/ml) could be used to study the effect of different compounds on the intracellular radical generation without using agents-modifiers.
4. In the course of certain pathological processes accompanied by suppurative foci formation, bacteriemia, ect., the proportion between the number of bacteria and leukocytes considerably changes. A

change in the stimulant concentration allows to model these pathological states and to evaluate the extra- and intracellular radical generation by stimulated leukocytes.

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