THE EFFECT OF INSULIN CONCENTRATION ON HUMAN NEUTROPHILS LUMINOL-DEPENDENT CHEMILUMINESCENCE

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Abstract. We investigated the effect of insulin on reactive oxygen species (ROS) production by human neutrophils stimulated with opsonized zymosan and incubated for 30 minutes with different insulin concentrations. For measuring ROS production, we used luminol-dependent chemiluminescence assay (CLLD). We observed that the CLLD depends on insulin concentration and this supports the hypothesis that insulin may play a role in ROS production by normal human neutrophils.

Key words: human neutrophils, reactive oxygen species, chemiluminescence, insulin, luminol.

INTRODUCTION

Neutrophils play an important role in a variety of physiological and pathological processes, such as host defense, rheumatoid arthritis or post-ischemic reperfusion damages. During phagocytosis of microbial intruders, neutrophils increase their oxygen consumption (the "respiratory burst") by the activity of an NADPH-oxidase that generates superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [3, 5]. The importance of the respiratory burst became obvious when the chronic granulomatous disease syndrome, characterized by predisposition to bacterial and fungal infections, was shown to be associated with decreased oxygen consumptions as well as defective microbial killing [4, 7]. To date, the dormant oxidase has been shown to consist of one membrane bound and four cytosolic components. Upon cell activation, the cytosolic components translocate to the membrane bound component and a functional multi-component electron-transfer system is formed, that catalyzes the reduction of molecular oxygen at the expense of NADPH. The primary products (superoxide anion and hydrogen peroxide) generated by NADPH-oxidase are not reactive enough to account for the bactericidal effects; however, these oxygen species give rise to yet other reactive oxygen species (hydroxyl radicals, singlet oxygen, hypochlorous acid, and, probably, reactive nitrogen species) that are strongly anti-microbial [6].

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The neutrophil respiratory burst can be activated by a number of different soluble and particulate stimuli, including chemoattractants, certain cytokines, phorbol esters, calcium ionophores, different lectins, opsonized as well as various unopsonized microorganisms, drugs, and physical factors [9, 10, 11, 13].

Previous studies [12] showed that the effect of insulin on respiratory burst of mouse neutrophils activated by formyl-methionyl-leucyl-phenyladenine (fMLP) depends on temperature, duration of pretreatment of the neutrophils with insulin and the concentration of combination of insulin and fMLP.

Our aim was to investigate if insulin may influence the respiratory burst of normal human neutrophils activated by opsonized zymosan, at room temperature.

MATERIALS AND METHODS

NEUTROPHIL PREPARATION

To study their specific function in comparison with other types of leukocytes, it is desirable to isolate the PMNL from the whole blood. There are some available techniques for neutrophil isolation [5, 14]. We used a conventional isolation technique based on density differences between the cell populations, as follows.

10 ml blood samples, obtained from healthy adult volunteers, were collected into 15 ml Falcon tubes, each containing 1 ml 1.5% Na_2EDTA in phosphate buffered saline (PBS). PMNLs were separated from whole blood by continuous Percoll gradient centrifugation. This is a fast, one step method that avoids hypotonic lysis of erythrocytes and dextran sedimentation, which may activate neutrophils. The final neutrophil suspension in HBSS (Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+}) has $1.8 \cdot 10^6$ PMNLs per milliliter.

ZYMOSAN PREPARATION

A solution of 50 mg/ml zymosan A (Sigma) in PBS was prepared and kept as stock solution at 4 °C. Daily 1 part of zymosan stock solution was suspended in 9 parts of fresh human serum and rotated slowly for 15 minutes at 37 °C for opsonization. Then the vial was span for 10 minutes at 2000 rpm and the pellet resuspended in HBSS to 1.25 mg/ml.

LUMINOL PREPARATION

A stock solution of 5.5 mM luminol (Sigma, MW 177.16) in DMSO (dimethylsulfoxide, Sigma) was prepared and kept at room temperature, in the dark. Daily a part of stock solution was diluted 1:50 in PBS. 0.2 ml of diluted solution were added per vial, resulting a final concentration of 4μM.

CHEMILUMINESCENCE ASSAY

Several chemiluminescent dyes that are activated by reactive oxygen species have been developed. Among these dyes, luminol (5 amino-2,3-dihydro-1,4 phthalazindione) is the most frequently used in the free radical research field. Luminol is an activity amplifier with a high quantum yield, so that the chemiluminescence assay is very sensitive [2]. The luminol excitation reaction may take place extracellularly and intracellularly. In order that luminol should react with oxidants in the intracellular compartment, it has to cross biological membranes, but very little is known about the diffusion properties of the luminol molecule [5].

For measuring luminol-dependent chemiluminescence of human neutrophils, we used a TD20/20 Turner Design luminometer. Light emission (chemiluminescence), expressed as cpm, was measured for 70 minutes.

To each scintillation vial we added 700 μL HBSS, 36 μL luminol solution (in order to obtain a final concentration of 4 μM), 72 μL opsonized zymosan, and 185 μL PMNLs suspension. Previous aliquots from neutrophil suspension ($1.8 \cdot 10^6$ PMNLs per milliliter) were incubated for 30 minutes, at room temperature, with insulin (ActaRapid Novartis). The insulin concentrations in the final probes were 3, 6, 11, 22, and 44 μU/ml, respectively.

RESULTS

Neutrophils were isolated from human venous blood obtained from 10 normal adult donors (6 women and 4 males).

Two control probes were prepared in each experiment: one contains nonstimulated neutrophils (no opsonized zymosan), and the other one, neutrophils and opsonized zymosan (to induce cell activation), both without insulin. The enhancement of chemiluminescent response of stimulated neutrophils in comparison with nonstimulated neutrophils is a measure of ROS production during the respiratory burst.

In order to study the insulin effect, the probes were incubated with insulin at different concentrations and then activated with opsonized zymosan.

We registered the chemiluminescence kinetics of nonactivated and opsonized zymosan activated PMNLs, at different insulin concentrations (Fig. 1). Each spot of the curves is an average of the values for the 10 normal adult donors.

CLLD depends on insulin concentration. At 22 μU/ml insulin, CLLD values are very close to those of stimulated control probe (opsonized zymosan stimulated neutrophils without insulin). When neutrophils were incubated with the other insulin concentrations (6, 11 and 44 μU/ml), the CLLD values are lower. For a lower insulin concentration than the physiological ones (6 – 26 μU/ml [8]), respectively 3 μU/ml, the CLLD values are very close to those of nonstimulated control probe (nonactivated neutrophils without insulin).

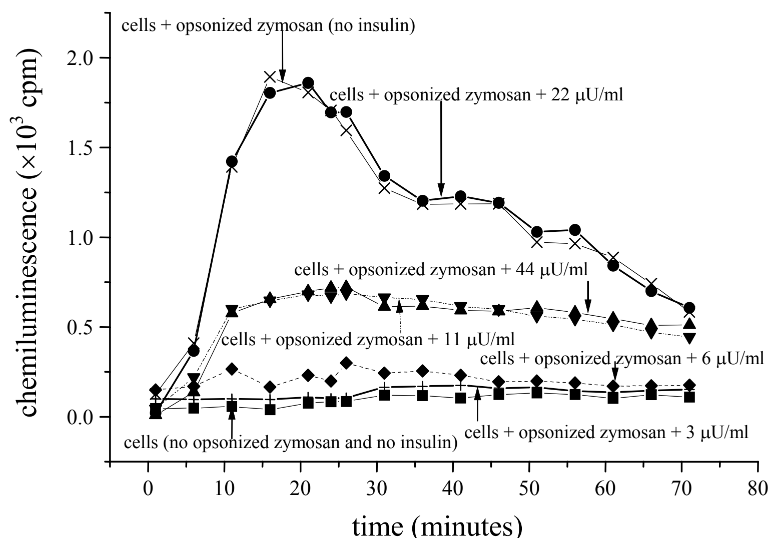

Fig. 1. – Chemiluminescence kinetics of nonactivated and opsonized zymosan activated PMNLs, at different insulin concentrations: 3, 6, 11, 22 and 44 μU/ml, respectively.

In all situations, the maximum chemiluminescent emission appears after about 20 minutes.

DISCUSSION AND CONCLUSIONS

We investigated the effect of insulin concentration on ROS production by human neutrophils using luminol-dependent chemiluminescence assay. For all insulin concentrations, the CLLD values are lower than or equal to those of stimulated control probe (neutrophils with opsonized zymosan but no insulin). At 22 μU/ml insulin, the CLLD values are very close to that of stimulated control probe. So, we may assume that insulin inhibits ROS production. For a lower insulin concentration than the physiological ones, the CLLD values are very close to those of nonstimulated control probe (neutrophils without opsonized zymosan and insulin). That means that in this case the ROS production is strongly repressed.

The CLLD variation upon insulin concentration supports the hypothesis that insulin may play a role in ROS production by normal human neutrophils, but the mechanism is not clear.

Insulin effects at cellular level are mediated by insulin receptors. To date, insulin receptors from erythrocyte membrane are well identified [1], but very little is known about insulin receptors from neutrophil membrane. Further investigations are necessary to characterize the insulin receptors in human neutrophil membrane and its correlation with the NADPH-oxidase activity.

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