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Reactive Oxygen Metabolite Production is Inhibited by Histamine and H_1-antagonist Dithiaden in Human PMN Leukocytes

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The study evaluated the distinction between extracellular and intracellular production of reactive oxygen metabolites (ROM) in isolated polymorphonuclear leukocytes (PMNL) stimulated with opsonised zymosan (OZ) and investigated its modulation by the endogenous mediator histamine (0.1–100 μmol/l) and by the H_1-antagonist dithiaden (1–100 μmol/l). For this observation, a modified luminol and an isoluminol amplified chemiluminescence (CL) technique were used. Our results showed that PMNL activated with OZ responded with a respiratory burst accompanied by both extra- and intracellular generation of ROM. Histamine and dithiaden significantly decreased both the extra- and intracellular component of chemilumiescence stimulated with OZ. While dithiaden decreased both the extra- and intracellular part of CL with the same potency, histamine decreased preferentially the extracellular part of CL. The fact that histamine as well as the H_1-antagonist dithiaden decreased the respiratory burst indicates that not only histamine receptors but also non-receptor mechanisms could be involved in the reduction of CL. Interaction with enzymes (NADPH-oxidase, myeloperoxidase, phospholipase A_2) or interference with PMNL membrane structure may well result in reduction of the chemiluminescence signal.

Keywords: Histamine; Dithiaden; Opsonised zymosan; Luminol enhanced chemiluminescence; Isoluminol enhanced chemiluminescence

INTRODUCTION

Polymorphonuclear leukocytes (PMNL) undergo a respiratory burst in response to infection or inflammation and the reactive oxygen metabolites (ROM) produced are toxic to microbes and surrounding tissue. Although PMNL are effector cells, they may interact reciprocally with other cells through either direct cell-to-cell contact or through their mediators and thus regulate immune or inflammatory responses.[1] Histamine represents one of the important endogenous mediators and regulators of inflammation and allergic responses. It is released from tissue mast cells and blood basophils and was demonstrated to be capable of altering ROM generation in PMNL.[2–4] Moreover, recently reported data indicated the role of histamine as an autocrine regulator of ROM production in leukemic granulocytes,[5] and the putative clinical benefit of histamine administration as supplementation to cancer immunotherapy.[6] ROM generation might be affected by antihistamine drugs used in therapy of allergic processes, in which PMNL are involved. Although the competitive antagonism with histamine at cellular receptors (particularly H_1-receptors) is considered to be the mechanism of action of antihistamine drugs, they have been shown to have side non-receptor mediated effects.[7] In addition, H_1-antagonists inhibited generation of ROM in PMNL,[8,9] which on the one hand might minimalise tissue damage but on the other it might decrease microbial killing.

In the present work, the influence of histamine and dithiaden, the H_1-histamine receptor antagonist on opsonised zymosan (OZ) induced generation of

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ROM, was studied in isolated PMNL. To investigate the effect of histamine and dithiaden on OZ induced intracellular (resulting mainly in destruction of ingested microorganisms and other particles) and extracellular ROM production (causing cellular and tissue damage) in PMNL, a modified luminol- and isoluminol-amplified chemiluminescence (CL) technique was used.[10–13]

MATERIALS AND METHODS

Materials

Zymosan (zymosan A from *Saccharomyces cerevisiae*), dextran (average mol. wt. 464 kDa), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), superoxide dismutase (SOD) (from bovine erythrocytes) were from Sigma–Aldrich Chemie (Germany), horseradish peroxidase (HRP) and catalase (CAT) (from beef liver) from Merck (Germany), Lymphoprep (density 1.077 g/ml) from Nycomed Pharma (Norway), dithiaden^{R}-4(3-dimethylamino (propylidiene)-4,9-dihydrothieno-(2,3b) benzo[e]) thiepin from Léčiva (Czech Republic), histamine dihydrochloride from Calbiochem (Switzerland).

Blood Collection and Isolation of PMNL

Blood was collected at the blood bank from healthy volunteers (men, aged 20–50 years) by antecubital venepuncture and was immediately mixed with 3.8% v/w trisodium citrate, in the ratio of 36 ml of blood to 4 ml of citrate, in polypropylene centrifugation tubes. Erythrocytes were allowed to sediment in 3% dextran solution (1*g*, 25 min, 22°C) and PMNL were separated by gradient centrifugation on Lymphoprep (500*g*, 30 min, 22°C). After hypotonic lysis of contaminating erythrocytes, PMNL were washed and resuspended in calcium and magnesium free phosphate buffered saline (PBS; in mmol/l: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4; pH = 7.4) to a final concentration of 10^7 PMNLs/ml. The obtained cell suspension contained more than 96% viable cells as evaluated by trypan blue exclusion.

Opsonisation of Zymosan A

Zymosan (10 mg/ml) was boiled in PBS for 30 min followed by centrifugation (10 min, 1300*g*) and washed twice in PBS (10 min, 1300*g*). Washed zymosan was incubated as 10 mg/ml of human serum (pooled from 12 healthy volunteers) at 37°C for 30 min followed by centrifugation at 1300*g* for 10 min. OZ was then washed three times in PBS and finally resuspended in PBS (20 mg/ml), and stored frozen in aliquots.

Chemiluminescence Assay

CL was studied as previously described by Jančinová *et al.*[13] Briefly, CL was measured in 1.0 ml samples containing 200 μl of PMNL (10^6 cells for luminol-enhanced CL or 5×10^5 cells for isoluminol-enhanced CL), 135 μl PBS, 50 μl 18 mmol/l $CaCl_2$, 25 μl 10 mmol/l $MgCl_2$ and 500 μl Tyrode solution (in mmol/l: 137 NaCl, 2.7 KCl, 12 $NaHCO_3$, 0.4 NaH_2PO_4, 1 $MgCl_2$, 5.4 EDTA, 5.6 dextrose, pH = 6.9). In some experiments, (HRP, 4 U/ml), (SOD, 100 U/ml) and (CAT, 2000 U/ml) were added to the reaction mixture in volumes of 20 μmol/l. The samples were preincubated at 37°C for 2 min under stirring conditions (1000 rpm) with 20 μl of dithiaden (final conc. 1, 10, 50 and 100 μmol/l) or 20 μl histamine (final conc. 0.1, 1, 10, 100 μmol/l), before addition of 20 μl of luminol or isoluminol (final conc. 5 μmol/l). After incubation for further 3 min at 37°C the system was activated with 50 μl OZ (final conc. 0.1 mg/ml). CL responses were measured in a lumi-aggregometer model 500 (Chrono-log, Havertown, USA) at an appropriate sensitivity setting and chart speed 10 mm/min, and were evaluated as the peak of the CL curve expressed in mV.

Statistical Analysis

All values are given as means ± SEM. Statistical significance of differences between means was established by Student's *t*-test and *p* values below 0.05 were considered statistically significant.

RESULTS

Since luminol can cross biological membranes and be indicative of ROM generation both outside and inside PMNL, the effects of histamine and dithiaden were studied by systems which detect: (i) extracellularly produced ROM (isoluminol), and (ii) generation of ROM inside human PMNL (luminol in the presence of SOD/CAT).

To estimate the effect of OZ on the extracellular part of CL, the experiments were performed in the assay system with isoluminol, HRP (a limiting factor for intensity of CL) and SOD/CAT (selective elimination of extracellularly released ROM) added to the system before activation[11,13] (Fig. 1). Administration of SOD/CAT inhibited the isoluminol response induced by OZ from 283.6 ± 25.0 to 5.7 ± 0.5 mV, yielding a more than 95% inhibition. It is evident that under our experimental conditions isoluminol-enhanced CL stimulated by OZ represented the extracellular part of CL since it was almost

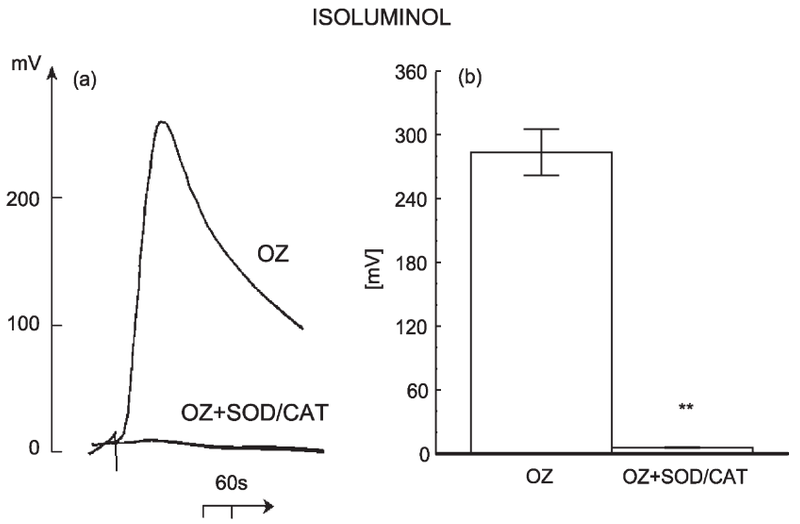

FIGURE 1 Effect of SOD and CAT on isoluminol enhanced CL in OZ stimulated human PMNL in the presence of HRP. (a) Representative CL curves. (b) Each value represents the mean from 6 peaks of CL (mV) ± SEM. (**) show statistically significant differences ($p < 0.01$) between values.

completely suppressed by SOD/CAT. The generation of ROM inside PMNL was defined in the assay system using luminol and SOD/CAT.[13,14] Figure 2 shows that the luminol-enhanced CL in PMNL stimulated with OZ was inhibited by SOD/CAT from 164.1 ± 8.7 to 77.6 ± 2.7 mV. Exclusion of the extracellular part of CL by SOD/CAT indicates that about 50% of ROMs were formed intracellularly. The effect of histamine on extra- and intracellular CL stimulated with OZ is shown in Fig. 3. Isoluminol-enhanced CL stimulated with OZ was significantly decreased by histamine in the concentrations of 1, 10 and 100 μmol/l to 79.5 ± 3.6, 71.8 ± 1.8 and 46.3 ± 6.1%, respectively. The CL signal in PMNL stimulated with OZ in a system of luminol and SOD/CAT was significantly decreased with histamine in the concentrations of 10 and 100 μmol/l to 86.8 ± 2.9 and 77.1 ± 2.5%, respectively. Histamine significantly decreased both the extra- and intracellular component of CL stimulated with OZ, however, the inhibitory effect of histamine on isoluminol enhanced CL was more intensive than that on luminol enhanced CL (Fig. 3).

Figure 4 shows the effect of dithiaden on extra- and intracellular CL induced with OZ. Dithiaden in the concentration of 10 μmol/l significantly reduced

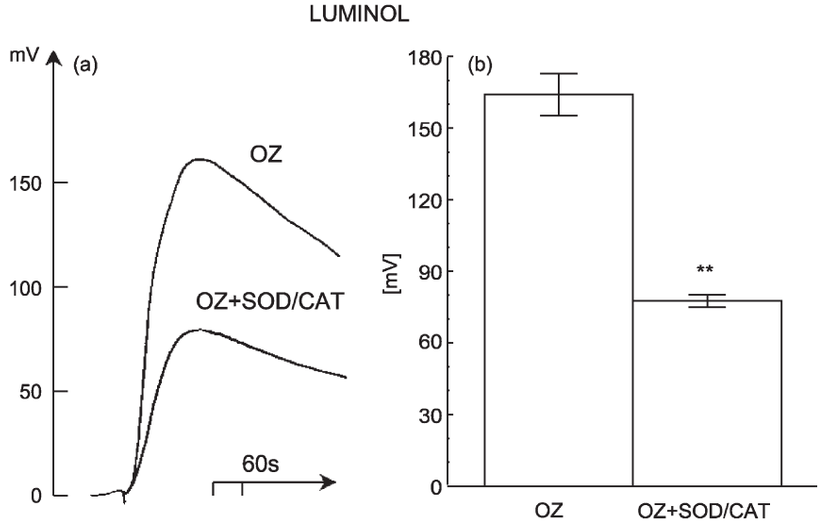

FIGURE 2 Effect of SOD and CAT on luminol enhanced CL in OZ stimulated PMNL. (a) Representative CL curves. (b) Each value represents the mean from 6 peaks of CL (mV) ± SEM. (**) show statistically significant differences ($p < 0.01$) between values.

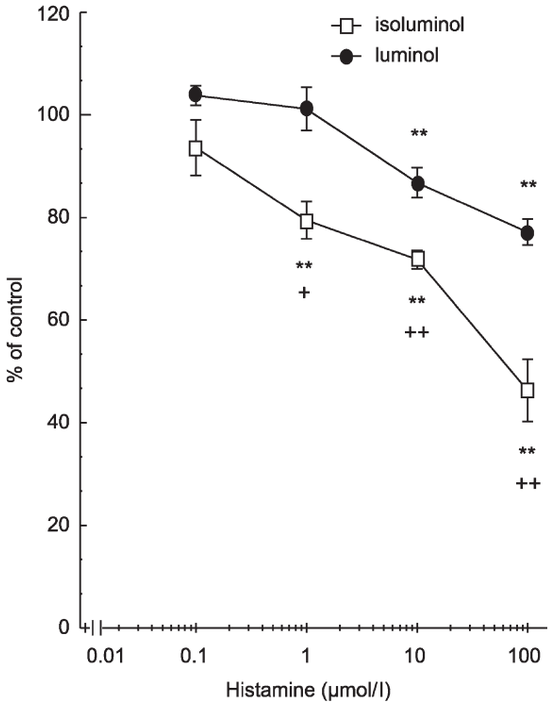

FIGURE 3 Effect of histamine on isoluminol and luminol enhanced CL of PMNL stimulated with OZ. Values represent the mean from 6 peaks of CL ± SEM and are expressed as percentage of control values: 222.7 ± 27.8 mV (isoluminol, HRP), 59.4 ± 5.0 mV (luminol, SOD, CAT). (**) show statistically significant differences ($p < 0.01$) compared to control values (without the drug tested), (+) show statistically significant differences ($p < 0.05$) and (++) show statistically significant differences ($p < 0.01$) between luminol and isoluminol enhanced CL of PMNL in the presence of the drug tested.

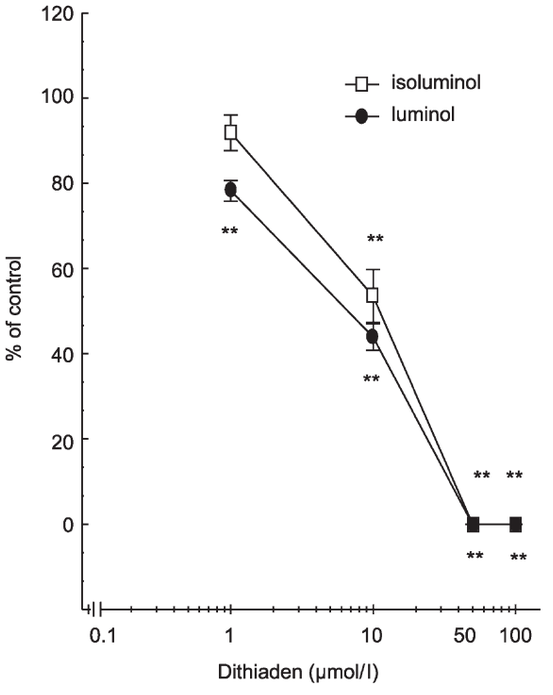

FIGURE 4 Effect of dithiaden on isoluminol and luminol enhanced CL of PMNL stimulated with OZ. Values represent the mean from 6 peaks of CL ± SEM and are expressed as percentage of control values: 283.1 ± 23.4 mV (isoluminol, HRP), 76.8 ± 5.1 mV (luminol, SOD, CAT). (**) show statistically significant differences ($p < 0.01$) compared to control values (without the drug tested).

isoluminol-enhanced CL to 53.5 ± 6.2%. In higher concentrations (50 and 100 μmol/l) dithiaden inhibited this type of CL totally. Luminol-enhanced CL in the presence of SOD and CAT was significantly decreased by dithiaden in the concentrations of 1 and 10 μmol/l to 78.3 ± 2.4 and 43.9 ± 3.1%, respectively. This type of CL was completely inhibited by dithiaden in the concentrations of 50 and 100 μmol/l.

DISCUSSION

The production of ROM in neutrophils as a result of respiratory burst activation is an essential step in host defense against microorganisms and it may also function as a basis for modulation of phagocytic function of neutrophils.[15] When neutrophils are activated by ingestion of foreign materials into phagosomes (phagocytosis), they initiate a respiratory burst by consuming molecular oxygen resulting in the formation of superoxide anion via the action of plasma membrane NADPH-oxidase. Superoxide anion is further metabolized to other ROM, such as hydrogen peroxide, singlet oxygen, hydroxyl radical, or hypochlorus acid.[16] Phagocytic cells emit light while ingesting microorganisms and other particles and CL is often used to measure their ROM generating capacity.[17,18] We differentiated between extracellular and intracellular ROM production by PMNL stimulated with OZ and investigated its modulation by histamine and dithiaden. For this observation we used a modified luminol and isoluminol amplified CL technique.[13] Our results showed that PMNL activated with OZ responded by a respiratory burst composed of both the extra- and intracellular component of CL, as also demonstrated by Saniabadi and Nakano.[19] On the other hand, Dahlgren and Karlsson[12] and Kopprasch *et al.*[20] showed that the predominant part of ROM production in PMNL stimulated by OZ was localized intracellularly. Histamine and dithiaden significantly decreased both the extra- and intracellular component of CL stimulated with OZ, however, the inhibitory effect of dithiaden was more intensive than that of histamine. While dithiaden decreased both the extra- and intracellular part of CL with the same potency, histamine decreased preferentially isoluminol enhanced CL. The decrease of isoluminol enhanced CL by histamine and dithiaden suggests their interference with superoxide generation and/or its liberation outside PMNL. The availability of myeloperoxidase is essential for isoluminol and

luminol-enhanced CL,[11,18,21] and thus inhibition of activity or liberation of myeloperoxidase from azurophilic granules of PMNL might be also involved in histamine and dithiaden modulation of CL. Moreover, our preliminary results showed that dithiaden (1–100 μmol/l) significantly decreased myeloperoxidase liberation from PMNL stimulated with OZ (unpublished observation). This effect of dithiaden could prove beneficial due to the decreased formation of aggressive HCLO.

Besides the well-known effect mediated via H_2 receptors,[22–24] the inhibitory effect of histamine on ROM generation may involved also inhibition of activation of NADPH-oxidase by decreasing expression of complement and immunoglobulin receptors on OZ activated cells. A similar mechanism of histamine action was observed in PMNL activated with FMLP.[25]

Dithiaden exhibited a strong inhibitory effect on both the extra- and intracellular part of CL. Dithiaden represents a cationic amphiphilic drug and its effects might depend on physicochemical properties of the drug molecule.[26] Corroborating this suggestion, the amine group in the structure of histamine H_1-receptor blocking drugs rather than the inhibition of histamine receptors has been indicated to play an important role in the inhibition of the responses of human neutrophils.[27] Moreover, the inhibitory effect of dithiaden on stimulated platelets has been suggested to result from nonreceptor interaction with cytosolic phospholipase A_2.[28,29] Inhibition of PMNL CL due to dithiaden might be brought on by inhibition of phospholipase A_2, since phospholipase A_2 was demonstrated to regulate the transduction pathway of NADPH-oxidase in OZ stimulated granulocytes.[30] Activation of cytosolic phospholipase A_2 by OZ causes a release of arachidonic acid, which might activate NADPH-oxidase in human PMNL.[31,32] We assume that dithiaden, like other cationic amphiphilic drugs, may pass through plasma membrane of PMNL and interact with ROM generation also intracellularly at the arachidonic acid pathway.

The scavenging effect does not seem likely to be involved in inhibition of ROM generation by histamine or dithiaden,[9,33] since neither histamine (0.1–5 mmol/l) nor dithiaden (0.1–5 mmol/l) reduced the concentration of peroxyl radical (data not shown) formed by thermal decomposition of 2,2-azo-bis-2-amidinopropane hydrochloride (for method see Lojek *et al.*).[34,35]

Our results showed that PMNL activated by OZ responded with a respiratory burst accompanied by both extra- and intracellular ROM production. Both histamine and dithiaden decreased extra- and intracellular CL but different sites of their action were indicated. While histamine decreased preferentially extracellular production of ROM, dithiaden inhibited ROM generation also inside PMNL. The fact that histamine as well as the H_1-antagonist dithiaden decreased the respiratory burst indicate that not only inhibition of histamine receptors but also non-receptor mechanisms could be involved in the reduction of chemiluminescemnce by histamine and dithiaden. Interaction with enzymes (NADPH-oxidase, myeloperoxidase, phospholipase A_2) or interference with PMNL membrane structure could result in the reduction of the CL signal.

In conclusion, dithiaden depressed extracellular generation of ROM and subsequently may have decreased the extent of neutrophil-mediated tissue injury. The inhibition of intracellular oxidant production by dithiaden might however diminish the physiological response of PMNL. The inhibitory effect of histamine suggests its potential regulatory mechanism in the control of the oxidative burst reaction in human PMNL.

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