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J. CHOROSTOWSKA-WYNIMKO[1], J. KUS[2], E. SKOPIŃSKA-RÓŻEWSKA[3]

TEOPHYLLINE INHIBITS FREE OXYGEN RADICALS PRODUCTION BY HUMAN MONOCYTES VIA PHOSPHODIESTERASE INHIBITION

[1]Laboratory of Molecular Diagnostics and [2]Department of Lung Diseases, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; [3]Department of Pathology, Biostructure Center, Medical University, Warsaw, Poland

Recent evidence suggests that theophylline, apart from bronchodilatation, derives its therapeutic activity in asthma from anti-inflammatory effects. Free oxygen radicals play important role in the perpetuation of inflammatory processes underlying bronchoconstriction and airway hyperresponsiveness in asthmatics. In our previous studies, we have analyzed the immunomodulatory effects of theophylline on human monocyte metabolic activity and showed that theophylline in doses of 5-20 μg/ml inhibited the process of zymosan-induced activation, decreasing total and maximum O_2^- production. The aim of present study was to analyze the mechanism of theophylline action on human monocytes. Therefore, the effects of papaverine - a phosphodiesterase inhibitor, LAS 31025 - selective phosphodiesterase IV inhibitor, 8-phenyltheophylline - A_1 and A_2 adenosine receptors antagonist, and 8-cyclopenthyl-1, 3 dipropylxanthine - selective A_1 receptor antagonist on O_2^- release were assessed. Adenosine receptor antagonist exerted no influence, while papaverine and LAS 31025 suppressed spontaneous, zymosan-induced total and maximum O_2^- production. The suppressant effect was concentration-dependent. We conclude that theophylline in therapeutic and subtherapeutic concentrations suppresses human monocyte metabolic activity *via* phosphodiesterase inhibition.

Key words: *adenosine receptors, monocytes, phoshodiesterase, superoxide anion, theophylline*

INTRODUCTION

Theophylline has been utilized in the treatment of respiratory diseases since the 1930s and until today it is one of the most prescribed drugs. The direct bronchodilatory effect on the bronchi smooth muscles has been, for many years

considered, its main mechanism of action. However, in the 1990s considerable amount of evidence has been published showing that theophylline also affects immune cells activity and its ability to inhibit allergic bronchitis in asthmatics might be even more important than the above mentioned smooth muscle relaxation in the bronchi (1). Accordingly, a significantly decreased number of immune cells, mostly T lymphocytes and eosinophils, the key cellular populations maintaining allergic inflammation in the respiratory system, have been demonstrated in moderate (7, 17, 18, 21) and severe (14) asthma patients persistently treated with theophylline. Similarly, the quality of life scores, ventilation parameters, and short-acting beta-agonist consumption have improved in theophylline-treated groups. However, though clinical effects of theophylline are rather well documented, the mechanism of its immunomodulatory action has not yet been satisfactory explained. Also, most experimental studies analyzing these processes have evaluated theophylline concentrations that are considerably higher than clinically applicable ones.

Our previous studies have clearly demonstrated a significant inhibitory effect of therapeutic and subtherapeutic concentrations of theophylline on oxygen free radicals but not on the early phase of TNF-α production and release by human monocytes (2, 3). Importantly, an immunosuppressive effect of theophylline has been dose-dependent and has also been exerted by its subtherapeutic concentrations (<5μg/ml) that are free of side effect risks (4).

As the continuation of the above mentioned studies, the present analysis was aimed at the mechanisms of theophylline inhibitory effect on reactive oxygen species (ROS) production by human monocytes. The two known pathways utilized by theophylline on the cellular level, non-selective inhibition of phosphodiesterase isoenzymes (PDE) activity and adenosine A_1 and A_2 receptors blocking have been evaluated in the present study using chemiluminescence method that allows both qualititative and quantitative analysis of ROS production. Several well characterized inhibitors of these pathways have been evaluated: papaverine (PAP), a strong nonselective phosphodiesterase inhibitor, LAS 31025, a selective inhibitor of the phosphodiesterase type IV isoenzyme prevalent in the monocyte cellular compartment, 8-phenylteophylline (8-PT), A_1 and A_2 adenosine receptor antagonist, and 8-cyclopenthyl-1, 3-dipropylxantine (DPCPX), a selective A_1 adenosine receptor antagonist.

To retain any possibile resemblance to the *in vivo* environment it was choosen to analyze the monocytes suspended in the peripheral blood mononuclears (PBMC), as their specific isolation procedures do affect intracellular activation and metabolism. Also, lymphocytes prevailing in PBMC are not chemiluminescently active in the zymosan-induced setup and, therefore, do not interfere with the measurement of ROS production by monocytes, which has been demonstrated in our preliminary experiments, and also by other authors (5). It is unlikely that lymphocytes might affect monocyte activity indirectly via cytokine

induction following preincubation with theophylline, as the experimental procedure utilized in present study was too short, not longer than 30 min (5).

MATERIAL AND METHODS

Isolation of cells

PBMC were isolated from heparinized blood obtained from healthy volunteers using a modified Boyum method (6), washed in the 0.9% NaCl solution and counted. The percentage of monocytes in the PBMC suspension was evaluated by a neutral red staining. Afterward, cells were suspended in PBS with 1% glucose (Polfa, Poland) and 1% albumin (Sigma, USA) in a concentration relevant to 1 x 10^6 monocytes/ml.

Chemiluminescence test

Monocyte chemiluminescence was evaluated according to the Easmon and Cole method (7). Shortly, cells were incubated for 30 min in room temperature and darkness with PBS as a control or in one of the analyzed substances:

- PAP (Sigma, USA): 1.9 μg/ml, 7.6 μg/ml, 30.4 μg/ml;
- LAS 31025 (Almirall, Spain): 0.0304 μg/ml, 0.304 μg/ml, 3.04 μg/ml, 30.4 μg/ml;
- 8-PT (Sigma, USA): 0.0625 μg/ml, 0.250 μg/ml, 1.0 μg/ml;
- DPCPX (Sigma, USA): 10^{-5} μg/ml, 10^{-3} μg/ml, 10^{-1} μg/ml.

Afterward, lucigenin (Sigma, USA) was added (5 x 10^{-6} M) and opsonized zymosan (Sigma, USA) in a final concentration of 1 mg/ml. Basic chemiluminescence activity of monocytes was evaluated in the zymosan-free control setup (with PBS). All measurements were conducted using the LKB-Wallac 1251 luminometer (Bio-Orbit Oy, Finland) in 36.6°C, every 71 s for 30 min. Monocyte chemiluminescent activity was evaluated by a light emission peak (milivolt, mV) and light emission in time (mV/s) representing respectively peak and total production of superoxide anion (O_2^-) by monocytes.

Statistical analysis

Student *t*-test for independent variables and analysis of variances were implemented, together with regression and correlation analysis. $P<0.05$ was considered significant.

RESULTS

It was demonstrated that both adenosine receptor antagonist, 8-PT active towards A_1 and A_2 and DPCPX selective towards A_1, in all examined concentrations did not influence the basic or zymosan-induced chemoluminescent activity of human monocytes and, therefore, the superoxide anion production.

On the other hand, phosphodiesterase inhibitors acting via other mechanisms significantly affected monocyte metabolic activity, both basic and stimulated. Papaverine, a nonselective phosphodiesterase inhibitor, incubated with monocytes in its highest concentration of 30.4 μg/ml, significantly inhibited the total ($P<0.01$) and peak ($P<0.05$) O_2^- production (*Fig. 1* and *Fig. 2*). Also, in zymosan-induced monocytes, both total ($P<0.01$) and peak metabolic activity

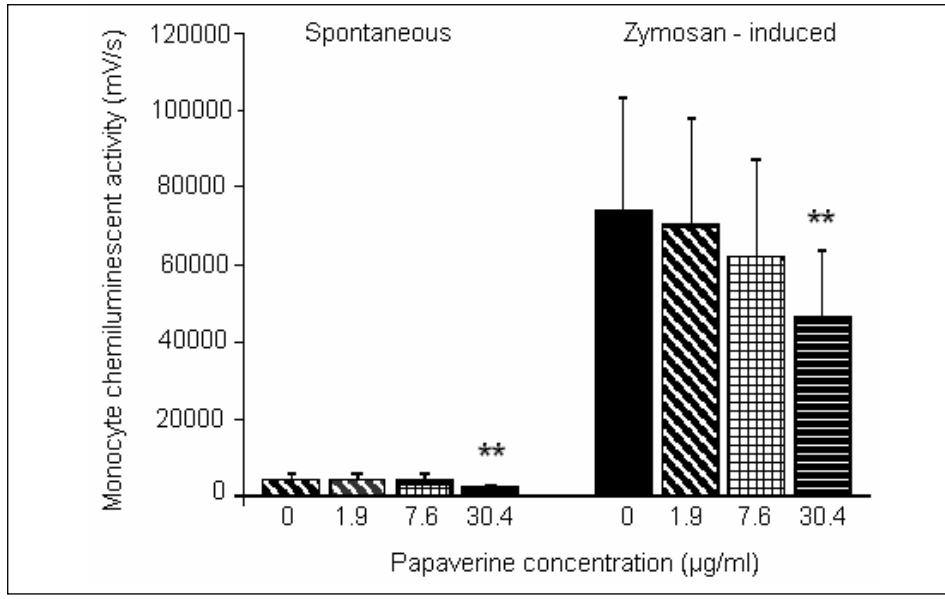

Fig. 1. Effects of papaverine on peak release of superoxide anion (O_2^-); **P<0.01 *vs*. control

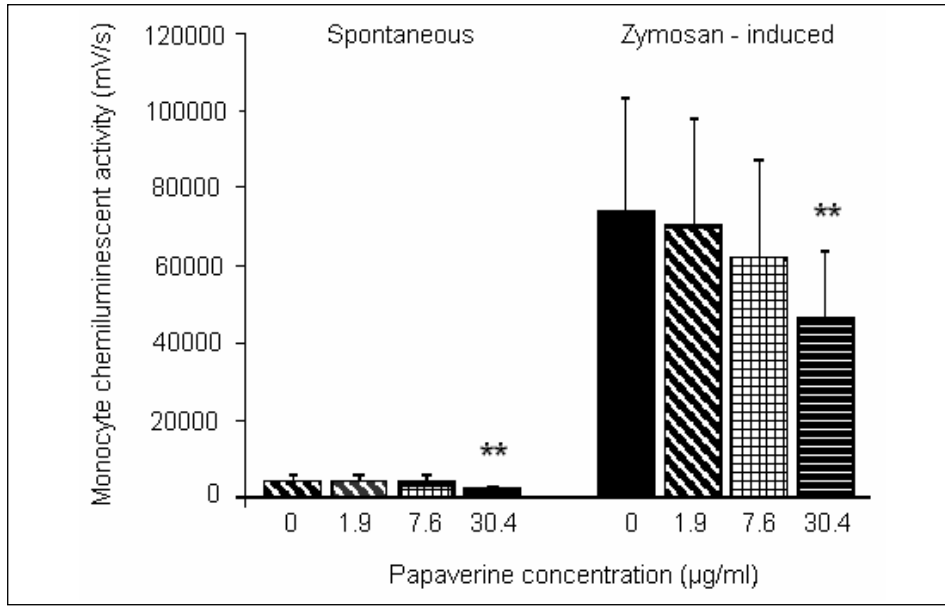

Fig. 2. Effects of papaverine on total release of superoxide anion (O_2^-); **P<0.01 *vs*. control.

(P<0.01) were suppressed by this highest papaverine concentration (*Fig. 1, Fig. 2,* and *Fig. 5*). A strong correlation was demonstrated between papaverine

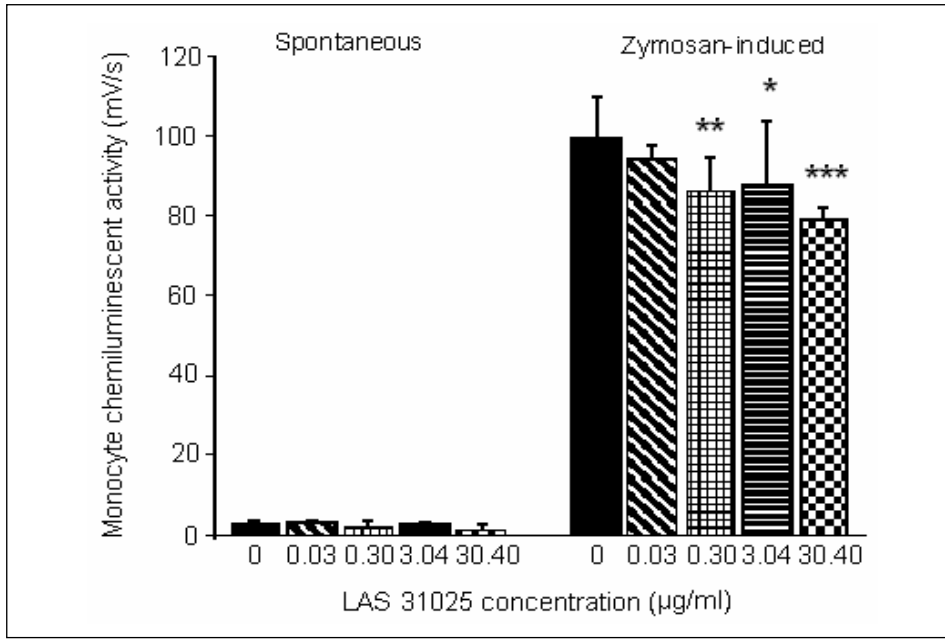

Fig. 3. Effects of LAS 31025 on peak release of superoxide anion (O_2^-); *P<0.05, **P<0.01, and ***P<0.001 vs. control.

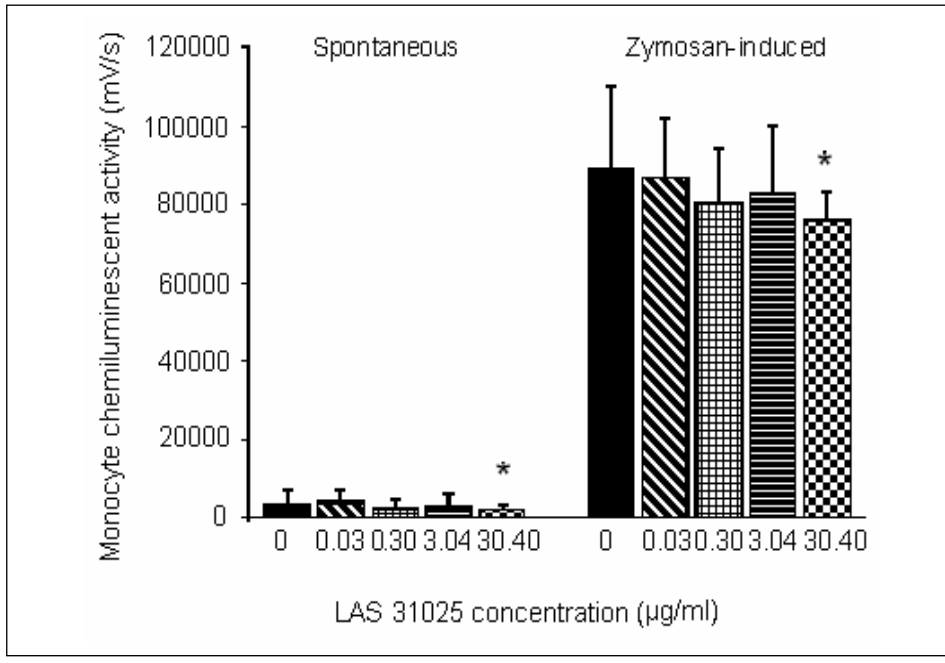

Fig. 4. Effects of LAS 31025 on total release of superoxide anion (O_2^-); *P<0.05 *vs*. control.

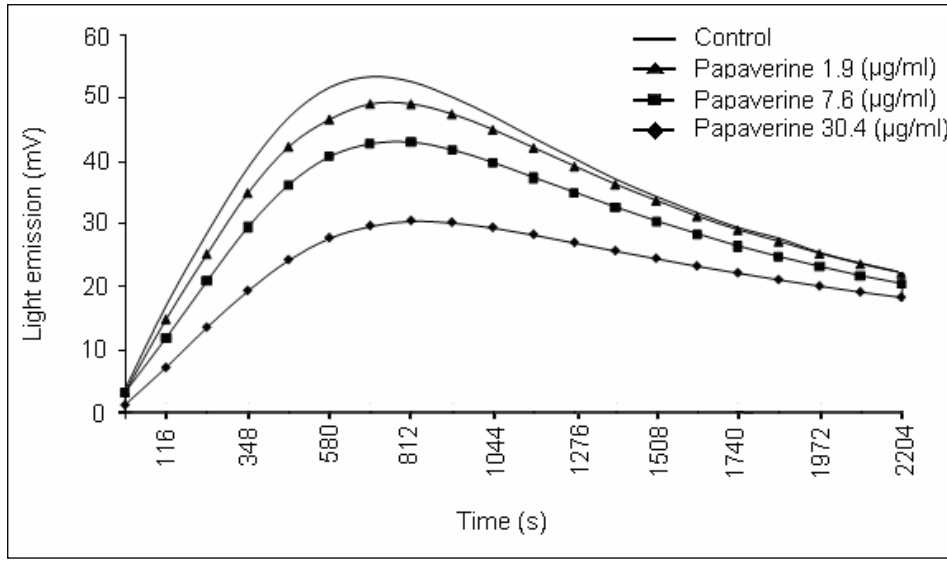

Fig. 5. Dynamics of superoxide anion release by zymosan-induced human monocytes preincubated with papaverine.

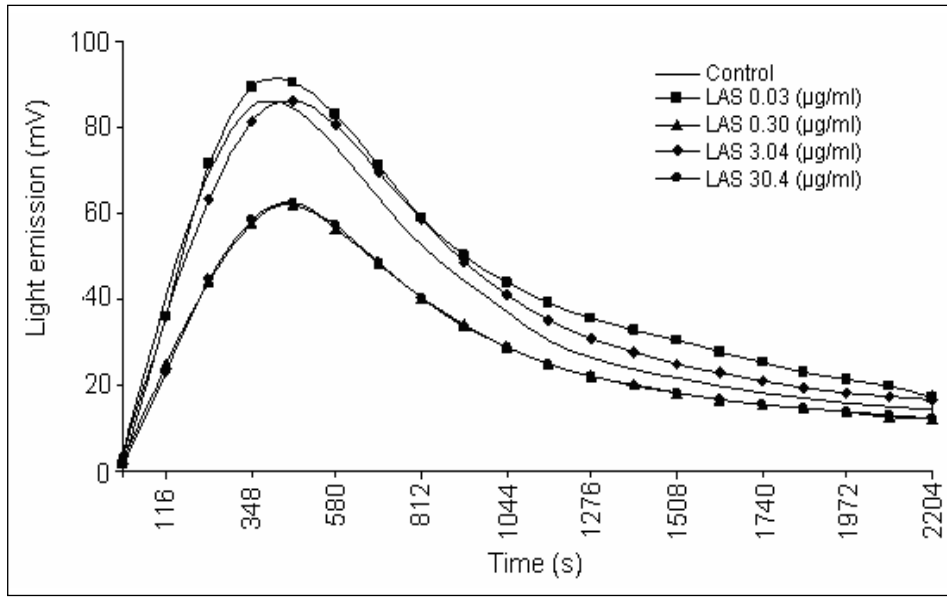

Fig. 6. Dynamics of superoxide anion release by zymosan-induced human monocytes preincubated with LAS 31025.

concentration and a decrease in both parameters assessed (total and peak O_2^- release) ($P<0.006$, $P<0.002$, respectively).

Similarly to papaverine, LAS 31025, a phosphodiesterase inhibitor selective toward IV isoenzyme, did not exert any significant inhibitory effect on the monocyte spontaneous chemiluminescent activity, except in its highest concentration 30.4 μg/ml ($P<0.05$) (*Fig. 3* and *Fig. 4*). However, maximum light emission, representing the peak O_2^- release by zymosan-induced cells, was significantly suppressed by all examined LAS 31025 concentrations: 0.304 μg/ml ($P<0.01$), 3.4 μg/ml ($p<0.05$), 30.4 μg/ml ($P<0.001$), while the total O_2^- production was inhibited by the highest dose of 30.4 μg/ml ($P<0.05$) only (*Fig. 3, Fig. 4*, and *Fig. 6*). Significant correlations have been demonstrated between LAS 31025 concentration and suppression of the peak O_2^- production by zymosan-induced monocytes ($P<0.0004$).

DISCUSSION

The ability to generate and release free oxygen radicals is one of the most important biological mechanisms characteristic for human monocytes. It serves as a vital process in phagocytosis, both related to non-specific cellular responses and to specific cell activation following antigen-IgE complexing with monocyte IgεRII (CD23). ROS are well known for their destructive effects on the integrity of cellular membranes (lipid peroxidation and arachidonic acid cascade activation), cytoplasm proteins, and nuclear DNA structure. ROS play an important role in the pathomechanism of asthma, as they directly stimulate bronchial hyperresponsiveness and bronchial smooth muscle contraction (8). Moreover, it has been demonstrated that monocytes isolated from the asthmatics are characterized by increased ability to produce ROS and monocyte chemiluminescent activity correlates well with disease activity (9). Therefore, theopylline inhibitory effect on the superoxide anion demonstrated in our previous studies fits well with the current opinion on asthma pathomechanism and recommended treatment.

As mentioned, data concerning theophylline effect on the metabolic activity of monocytes and alveolar macrophages are scarce, although do confirm to its anti-inflammatory activity. Consequently, the present study was focused on the key molecular pathways utilized by theophylline in its interactions with monocytes. Superoxide anion (O_2^-) was chosen for measurements in this particular experimental setup, as it is the first of several oxygen radicals produced by the monocyte following its activation.

The present study clearly demonstrates that phosphodiesterase inhibitors - non-selective (papaverin) and selective towards isoenzyme IV (LAS31025) were able to suppress O_2^- generation. Accordingly, Dent et al (10) have demonstrated a strong correlation between theophylline suppressive effect on the alveolar macrophage metabolic activity and on phosphodiesterase inhibition in cellular homogenates, with a concomitant increase in intracellular cAMP. Interestingly,

our experiments showed that a non-selective papaverine demonstrated a considerably stronger inhibitory effect on the O_2^- production, both total and peak, than the LAS 31025 selective towards phosphodiesterase isoenzyme IV. It might be due to their different affinity to several genes encoding type IV isoenzyme family and to the fact that other phosphodiesterase isoenzymes might participate in the processes regulating "oxygen burst" (1). Apart from the type IV family prevailing in the monocytes, activity of other phosphodiesterase isoenzymes (type I) was suggested by some authors (11, 12). In addition, Brown et al (12) have recently underscored the possible involvement of calcium channels in the oxidative stress in macrophages (12).

In contrast to phoshodiesterase inhibitors, adenosine receptor antagonists - 8-PT and DPCPX did not affect any of the evaluated parameters characterizing monocyte metabolic activity, both basic and stimulated. Therefore, it might be concluded that the inhibitory effect exerted by theophylline on O_2^- production by monocytes does not depend on its interplay with adenosine receptors. Importantly, it has previously been observed that in granulocytes, both neutrophils and eosinophils, O_2^- generation was inhibited by theophylline via A_2 adenosine receptors (13, 14).

In conclusion, this study shows that theophylline directly inhibits ROS production in human monocytes and, consequently, their metabolic activity, without the need for cooperation with other cells, cytokines, or mediators. Moreover, a suppressant effect was exerted by clinically relevant concentrations of theophylline, comparable with those observed in the serum of treated asthmatics, which links these *in vitro* experiments with the *in vivo* environment.

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Author's addres: Joanna Chorostowska-Wynimko, Laboratory of Molecular Diagnostics, National Institute of Tuberculosis and Lung Diseases, 01-138 Warsaw, Plocka 26 St., e-mail: j.chorostowska@igichp.edu.pl