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Effect of Stobadine on Opsonized Zymosan Stimulated Generation of Reactive Oxygen Species in Human Blood Cells

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Summary

To predict more precisely the effect of stobadine, a pyridoindole antioxidant agent, in the whole organism, we studied its effect on opsonized zymosan-stimulated free radical generation in whole blood, on superoxide generation in the mixture of PMNL : platelets (1:50), as well as on superoxide generation and myeloperoxidase release in isolated PMNL. Without stimulation, stobadine had no effect on reactive oxygen species (ROS) generation and myeloperoxidase release. Stobadine in a concentration of 10 or 100 μmol/l significantly decreased luminol-enhanced chemiluminescence in opsonized zymosan-stimulated whole blood. In concentrations of 10 and 100 μmol/l, it reduced myeloperoxidase release from isolated neutrophils. Stobadine significantly decreased superoxide generation in isolated neutrophils in 100 μmol/l concentration. Its effect was much less pronounced in the mixture of neutrophils and platelets in the ratio close to physiological conditions (1:50). Our results suggest that stobadine might exert a beneficial effect in diseases or states where superfluous ROS generation could be deleterious.

Key words
Human blood cells • Opsonized zymosan • Stobadine • Reactive oxygen species

Introduction

Generation of superoxide ($O_2^{\cdot-}$) by the NADPH-dependent oxidase of polymorphonuclear leukocytes (PMNL) is an essential component of the innate immune response to invading microorganisms. Serum-treated or opsonized zymosan (OZ), a particulate material that can be phagocytozed by PMNL, activates the superoxide-generating respiratory burst in these cells. The superoxide anion, produced by the membrane-bound enzyme NADPH-oxidase (Babior 1999), gives rise to the generation of a large number of other toxic agents produced by free radical reactions, which besides their defending effects may cause undue inflammation (Cohen 1994) and contribute to severe clinical consequences. Thus the modulation of neutrophil function by exogenous means could be therapeutically beneficial to some patients.

Stobadine, a non-toxic compound with antioxidative activity (Horáková and Štolc 1998), might be effective in the prevention or treatment of serious and frequently occurring diseases, such as atherosclerosis, myocardial infarction, stroke and neurodegenerative diseases, or in tissues exposed to ischemia/reperfusion or hypoxia/reoxygenation.

The hallmark of inflammation is the infiltration of specific leukocyte subsets from the blood into the affected tissue (Ben-Baruch *et al.* 1995). Because of

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mutual interaction of blood cells *in vivo* at the site of inflammation (Faint 1992), we studied the *in vitro* effect of stobadine on OZ-stimulated respiratory burst of human whole blood, myeloperoxidase release and on superoxide generation in isolated human blood cells from healthy volunteers.

Methods

Stobadine dihydrochloride (cis (–)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1H-pyrido(4,3-b) indole) was developed at the Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovak Republic, Dextran T500 (Pharmacia Fine Chemicals), Lymphoprep (Nycomed Pharma AS), phorbol-12-myristate-13-acetate (PMA, Sigma), cytochrome c (Merck), and zymosan A (Sigma) were used. All other chemicals used were of analytical grade.

Isolation of platelets

Blood samples were obtained at a blood bank from healthy male donors (20-50 years) and anticoagulated with 3.8 % trisodium citrate (9:1). After centrifugation (260 x *g* for 15 min) platelet-rich plasma was removed, mixed with a solution containing 4.5 % citric acid and 6.6 % glucose (50 µl/ml PRP), and centrifuged at 1070 x g for 10 min. Platelets were resuspended in an equal volume of Tyrode's solution (136.9 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l NaH_2PO_4, 1 mmol/l $MgCl_2$, and 5.6 mmol/l glucose) containing 5.4 mmol/l EDTA, pH 6.5. After 10 min stabilization, the suspension was centrifuged for 6 min at 1070 x *g*. Platelets were resuspended in the same buffer without EDTA (pH 7.4) to obtain $4x10^8$ platelets per 1 µl (Jančinová *et al.* 2001).

Isolation of neutrophils

After removal of platelet-rich plasma, the blood volume was reconstituted with 0.9 % of NaCl. Dextrane (3 % in 0.9 % NaCl) sedimentation (blood:dextran ratio was 2:1) and centrifugation on Lymphoprep was performed by the modified Boyum's method (Drábiková *et al.* 2002). Erythrocytes were removed by hypotonic lysis and PMNL were resuspended in phosphate buffer saline (PBS) solution (137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4), pH 7.4 and washed once with PBS. For individual assays PMNL were diluted as described below. The purity of isolated PMNL was > 96 % (light microscopy).

Zymosan

Zymosan A from *Sacccharomyces cerevisiae* was opsonized according to Lojek *et al.* (2002) in pooled serum, washed three times and frozen at –28 ºC. Immediately before use it was thawed, diluted and added to the cell suspension to a final concentration of 0.5 mg/ml.

Superoxide determination

Superoxide formation was measured in isolated human PMNL as superoxide dismutase inhibitable reduction of cytochrome c (Babior *et al.* 1973). Suspension of PMNL (10^6 cells/1.5 ml PBS with 0.9 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$) or mixture of PMNL and platelets (1:50) was preincubated for 5 min at 37 °C with stobadine (0.1-100 µmol/l) and subsequently stimulated with opsonized zymosan (0.5 mg/ml) for 60 min. Controls for effect of OZ and stobadine on cytochrome c reduction were included. The absorbance at 550 nm was measured using a spectrophotometer Hewlet Packard 8452A.

Chemiluminescence assay

Respiratory burst of blood cells was analyzed in small volumes of whole blood activated by opsonized zymosan by luminometric analysis (Lojek *et al.* 2002) in a luminometer Immunotech LM-01 (Immunotech, Czech Republic). Samples contained 1 µl of whole blood and appropriate amounts of stobadine or Hank's balanced salt solution, pH 7.4 without phenol red for control samples. Final concentration of luminol was 1 mmol/l and of opsonized zymosan 0.5 mg/ml. Controls for effect of stobadine on chemiluminescence were included. The assays were run for 60 min in triplicates. Peak of chemiluminescence emission of each sample was expressed as relative light units (RLU).

Enzyme determination

PMNL of $2x10^6$/sample were preincubated for 5 min at 37 °C with stobadine and stimulated for 60 min at 37 °C by opsonized zymosan.

Myeloperoxidase was assayed by determining the oxidation of o-dianisidine in the presence of hydrogen peroxide in a spectrophotometer Hewlet Packard 8452A at 463 nm (Somersalo *et al.* 1990). Controls for direct interaction of stobadine and MPO were included.

Statistical evaluation

All values are given as means ± S.E.M. and the results were statistically processed by Student's t-test.

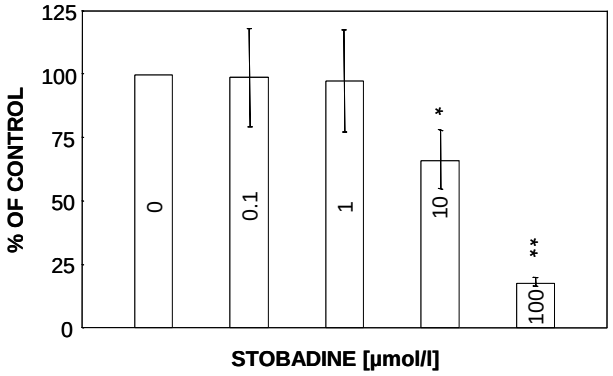

Fig. 1. Effect of stobadine on luminol enhanced chemiluminescence (CL) of whole blood induced with opsonized zymosan. Results are means ± S.E.M. of 6 peaks of CL expressed as percentage of control values, **p<0.01, *p<0.05. Absolute average control value was 256 ± 66.2 RLU.

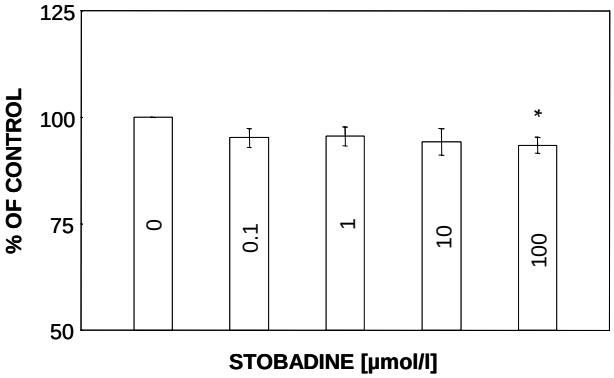

Fig. 2. Effect of stobadine on superoxide generation in the mixture of PMNL and blood platelets (PL) stimulated by opsonized zymosan (OZ) (0.5 mg/ml, 60 min). Values are means ± S.E.M. expressed as percentage of control value (N:PL) obtained after stimulation with OZ, n=6, **p<0.01, *p<0.05. Absolute average control value was 16.03 ± 0.45 nmols/106 PMNL: 50 x 106 PL/min.

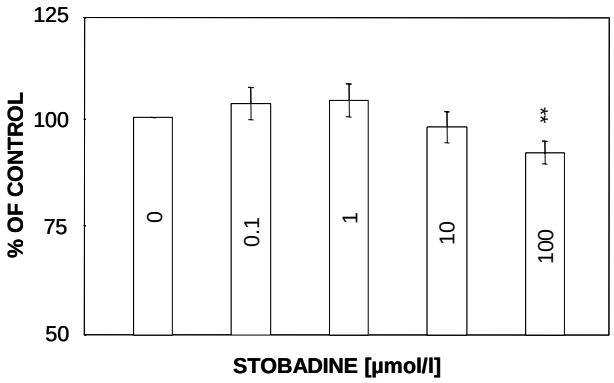

Fig. 3. Effect of stobadine on opsonized zymosan (0.5 mg/ml, 60 min/37 ºC) stimulated superoxide generation versus control without the drug. Results are mean ± S.E.M., n=6, **p<0.01, *p<0.05. Absolute average control value of superoxide generation was 13.1±2.1 nmol /106 PMNL/min.

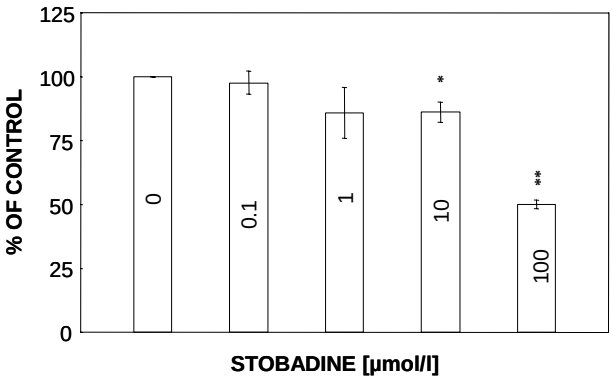

Fig. 4. Effect of stobadine on opsonized zymosan-stimulated myeloperoxidase release from isolated human PMNL versus control without the drug. Results are mean ± S.E.M., n=6, **p<0.01, *p<0.05. Absolute average control value was 4.11 ± 3.07 ΔA/Δt (calculated as area under curve).

Results

To study the effect of stobadine in whole blood, we used chemiluminescence enhanced with luminol, a convenient and sensitive method allowing measurement of both the extra and intracellular part of the chemiluminescence signal.

In unstimulated cells, stobadine (0.1-100 µmol/l) had no effect on chemiluminescence measured free radical generation or on $O_2^{\cdot-}$ generation and myeloperoxidase release. The following results concern OZ stimulated cells only.

Stobadine in the concentrations of 10 and 100 µmol/l significantly decreased chemiluminescence of whole blood to 66.2±11.8 % and 16.7±2.5 % of control values, respectively (Fig. 1).

The effect of platelets on opsonized zymosan stimulated PMNL in the ratio close to physiological conditions (1:50) was investigated. OZ-stimulated superoxide generation was enhanced in the presence of platelets by 22.4±3.5 % compared to PMNL control. Stobadine in the concentration of 100 µmol/l significantly decreased superoxide generation in the mixture of PMNL : platelets (93.4±1.9 %) (Fig. 2) compared to the control (PMNL: platelets) without the drug.

Figure 3 shows the effect of stobadine on opsonized zymosan activated $O_2^{\cdot-}$ generation in isolated human PMNL. In the concentration of 100 µmol/l stobadine significantly decreased $O_2^{\cdot-}$ generation to

91.9±2.6 %. OZ stimulation of isolated PMNL caused the release of 12.1±1.6 % of total myeloperoxidase. Stobadine in the concentrations of 10 and 100 μmol/l significantly decreased myeloperoxidase release from opsonized zymosan-stimulated isolated PMNL to 86.1±3.9 % and to 49.9±1.7 %, respectively (Fig. 4).

Discussion

Apart from their beneficial role in the defense against bacterial infections, reactive oxygen species (ROS) produced by stimulated neutrophils may exert severe side effects (Weiss 1989).

Cellular processes initiated in response to the occupancy of unique surface receptors (Ben-Baruch *et al.* 1995) are translated into specific cellular activities *via* such signals as activation of calcium-mediated protein kinases and phosphorylation of critical proteins.

After phagocytosis, the engulfed particle is subjected to killing mechanisms, involving reactive oxygen species, acid pH, and antimicrobial proteins (Hampton *et al.* 1998). Insight into these molecular events may allow modulation of neutrophil activity, although the benefit of *in vitro* assays will be restricted to revealing potential interrelations, which will then require careful evaluation in *in vivo* models.

In comparison with natural antioxidants, such as vitamin E or vitamin C (Gotoh and Niki 1992), stobadine is not an efficient scavenger of superoxide radicals (Kagan *et al.* 1993), as established by chemiluminescence. The potency of antioxidants to interact with stable free radical increased in the order: stobadine, ascorbic acid, and trolox (Horáková *et al.* 1995).

Effective concentrations of stobadine (10-30 μmol/l), which are able to protect one of the essential functions of neurons (Štolc *et al.*1997), were in a similar range as those in which this compound inhibited lipid peroxidation under various *in vitro* and *in vivo* conditions (Ondriaš and Hromadová 1992, Štefek *et al.* 1992, Kagan *et al.* 1993, Horáková *et al.* 1994). The neuroprotective activity of antioxidants was in the following order: stobadine > melatonin >> trolox (Vlkolinský and Štolc 1999).

To predict the action of stobadine in the whole organism more precisely, we studied its effect on opsonized zymosan-stimulated free radical generation in whole blood, superoxide generation in a mixture of PMNL : platelets (1:50), as well as superoxide generation in and myeloperoxidase release from isolated PMNL. OZ stimulation causes superoxide generation and myeloperoxidase release.

In our experiments the effect of stobadine was tested in the concentration range of 0.1-100 μmol/l. Stobadine alone did not induce chemiluminescence, superoxide generation or myeloperoxidase release from unstimulated cells. Higher concentrations were not used since stobadine in concentrations exceeding 300 μmol/l (Štolc *et al.* 1997) seems to be membrane stabilizing agent, similarly as described for drugs with antiarrhythmic effect (Štolc 1994).

In comparison with its effect on isolated blood cells, stobadine in whole blood exerted a higher inhibitory effect to OZ stimulation (33.8±10.8 % in 10 μmol/l and 83.3±2.5 % in 100 μmol/l concentration). We ascribed the marked decrease of OZ-stimulated chemiluminescence induced by stobadine to its effective scavenging of free radicals derived from superoxide, since the ability of stobadine to scavenge hydroxyl, peroxyl, alkoxyl radicals and singlet oxygen, was reported for other experimental models (Horáková and Štolc 1998). *In vivo*, blood platelets, accumulated and activated simultaneously with PMNL, liberate substances that can eliminate or decrease generation of oxygen metabolites: serotonin (Schuff-Werner *et al.* 1995), β-thromboglobulin, adenosine and/or AMP (Moon *et al.* 1990), platelet catalase and glutathione peroxidase (Clark and Klebanoff 1980). On the other hand, serotonin at concentrations normally present at sites of tissue injury and consecutive thrombus formation increases the bactericidal function of PMNL (Schuff-Werner and Splettstoesser 1999). Mutual platelet-neutrophil interactions (inhibitory and/or stimulatory) can weaken the effect of drugs (Siminiak *et al.*1995). Differences in the results concerning PMNL and platelet interactions depend on the stimulus used, thus involving distinct metabolic pathways triggered in the cells tested, on the ratio of cells tested and on the experimental conditions applied. Consequently, even in spite of the apparent contrast between the reports, caution is needed in considering the data as actually conflicting.

In our experimental conditions, the coexistence of platelets with PMNL in OZ-stimulated mixture increased superoxide generation approximately by 25 % in comparison to OZ-stimulated PMNL and partly decreased the effect of stobadine.

Kagan *et al.* (1993) reported a poor ability of stobadine to quench superoxide. In our experiments, however, stobadine in the concentrations of 10 and 100 μmol/l decreased opsonized zymosan-stimulated

superoxide generation by 2.3 % and 8.1 %, respectively, and release of myeloperoxidase (a hemoprotein located within azurophilic granules) by 13.9 % and 50.0 %, respectively.

Our present results, along with our previous findings obtained using soluble stimuli such as A 23187 or PMA (Pečivová *et al.* 1999), suggest that stobadine is effective on the level of membrane signal transfer and does neither interfere with protein kinase C nor does it quench superoxide.

In this way stobadine effectively participates in the decreased generation of superoxide and of subsequently derived free radicals. Moreover, it can be beneficial in diminishing the damage of surrounding tissues by inhibiting MPO release, production of HOCl and of further toxic chloramines, such as NH_2Cl. In addition, the known pharmacological properties of the pyridoindole stobadine may be used in the search for other molecules with an even more specific and appropriate profile of action than that exhibited by currently available compounds.

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Reprint requests

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