Coll. Antropol. **36** (2012) 2: 409–412
Original scientific paper

Tetrahydrophthalazine Derivative »Sodium Nucleinate« Exert its Anti-Inflammatory Effects through Inhibition of Oxidative Burst in Human Monocytes

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A B S T R A C T

We described the use of a new chemical substance Sodium nucleinate (SN) as an immunomodulatory substance exhibiting antiinflammatory properties. Sodium nucleinate (SN) registrated in Russian Federation as Tamerit®, is 2-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione sodium salt dihydrate, derivative of well known chemical substance luminol. To comprehend the mechanisms of SN immunomodulatory activity, we examined the SN modulation of the oxidative burst responses of whole blood human monocytes and polimorphonuclear cells (PMC) stimulated with phorbol 12-myristate 13-acetate (PMA) or E. coli suspension in vitro. SN did not inhibit the proportion of neutrophils and monocytes phagocytosing E. coli. Oxidative burst responses of monocytes stimulated with PMA were strongly inhibited at SN concentration ranging from 10–500 mg/ml, less efficient inhibitor was SN in E. coli stimulated monocytes (inhibition range was from 50–500 mg/ml SN). SN inhibited PMC oxidative burst only in range 100–500 mg/ml SN. In conclusion, we found SN as an efficient inhibitor of oxidative burst in monocytes. Since ROS generation in monocytes/macrophages has been found to be important for LPS-driven production of several proinflammatory cytokines, SN may exsert its antiinflammatory effects through monocyte/macrophage oxidative burst inhibition.

Key words: *oxidative burst, granulocytes, monocytes, Sodium nucleinate*

Introduction

Neutrophils and monocytes/macrophages play an important role in non-specific immune response and organism resistance, specifically in anti-bacterial resistance and as effectors, inducing and regulating immune cells[1]. Free oxygen radicals kill bacteria in phagosomes and partially are released into the environment, intensifying killing microorganisms and simultaneously injuring the surrounding tissues. It is specifically intensified in acute inflammation, less in chronic course of the disease[2,3].

Active oxygeninduced and free-radical-mediated oxidation of biological molecules, membranes, and tissues is closely related to a variety of inflammatory diseases. Once free radicals are produced, they multiply geometrically in chain reactions unless they are quenched by antioxidants or other free-radical scavengers. Antioxidants are compounds that react with free radicals and protect neighboring structures from oxidant damage. Common protective antioxidant nutrients include vitamins C, E, and P-carotene, however their action is limited due to their low capacity to enter tissue and cell compartments[3,4].

This study was performed to determine whether Sodium Nucleinate (SN; 2-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione sodium salt dihydrate, derivative of well known chemical substance luminol)) registrated in Russian Federation as antiinflammatory drug Tamerit®, but in EU or USA not avaliable until recently, may play a role as inhibitor of respiratory burst in polymorphonuclear cells (PMCs) and/or monocytes. In previous study we demonstrated that SN is an efficient inhibitor of sepsis development in mice model of LPS-induced sepsis[3]. The

Received for publication January 2, 2012

changes induced by SN included decreased mice plasma inflammatory cytokine production. Similarly we demonstrated a decreased TNF-α, IFN-γ and IL-6 response in human LPS-stimulated PBMNCs.

Several authors have recently confirmed that ROS are important for normal lipopolysaccharide (LPS)-driven production of several proinflammatory cytokines and for the enhanced responsiveness to LPS seen in cells from patients with chronic inflammatory diseases[4,5]. Multiple cytokine secretion may be triggered by a vast number of pathological conditions, many of them begin with activation of PMNs and/or monocytes[4,5].

Since ROS was found to be important for lipopolysaccharide (LPS)-driven production of several proinflammatory cytokines, we have focused on the in vitro influence of the SN when applied in blood samples stimulating with agonists leading to an activation of respiratory burst in PMCs and/or monocytes.

Materials and Methods

Blood samples

Peripheral blood samples were drawn from 9 healthy individuals after obtaining their signed informed consents.

Phago-Burst Test

Phagocytosis and oxidative burst capacity was measured using Phago-Burst test (Orphegen Pharma, 341060+ 341058, Heidelberg, Germany). Whole blood test samples were divided into six plastic test tubes (BD, Falcon, 353052) in volume of 100 μl. All the tubes were held in ice bath during the preparation of the test. The test reagents were added as follows: in the first tube FITC labelled *E. coli* (phagocytosis control – FK); in the second FITC labelled *E. coli* (phagocytosis test – FT); in the third wash solution and 123 dihydrorhodamin (wash solution control); in the fourth unlabeled stimulating opsonized *E. coli* and 123 dihydrorhodamin (*E. coli* stimulated test – EC); in the fifth fMLP and 123 dihydrorhodamin (fMLP stimulated control – FMLP); and in the sixth PMA (phorbol myristate acetate) and 123 dihydrorhodamin (PMA stimulated test – PMA). Tube 1 was incubated at 0°C (FK) and all others on 37°C for 10 minutes. Whole blood test samples were then lysed with lysing solution and washed three times with wash solution. Cells were than stained with propidium iodide. Measuring was performed using flow cytometer (BD, FACSort, USA) equipped with argon-ion laser of 488 nm excitation wavelength.

FITC labeled E. coli suspension

FITC labeled *E. coli* (Phago-Burst test (Orphegen Pharma, Heidelberg, Germany) were used for the stimulation of monocytes/PMC. *E. coli* suspension (5×10^7/ml) was quantitated by using Cell Viability Kit with Liquid Counting Beads (BD Biosciences). The kit contains a liquid suspension of fluorescent beads, which are added to the flow sample to calculate absolute counts of bacterial suspension.

Statistical analysis

Descriptive statistics were calculated for all the measured parameters. Differences between control and SN pre-treated samples were tested both using paired *t*-test and exact Wilcoxon signed-rank matched-pairs test (EWMPT). Differences were taken as statistically significant if $p<0.05$. Statistical analyses were performed using SPSS for Windows 15.0.1.1 software (SPSS Inc., Chicago, IL, 2007).

Results

Phagocytic activity of monocytes/neutrophils

The ability of neutrophils to phagocytize opsonized *E. coli* was assessed. No inhibitory effects of SN (range 0.1–500 mg/ml) were found in monocytes/PMC stimulating either with PMA or *E. coli* in vitro (data not shown).

Inhibitory effects of SN on monocyte/neutrophil oxidative burst

To determine the effect of SN on oxidative burst of monocytes/MNCs blood was preincubated with 0 (control), 0.1, 10, 50, 100 and 500 mg/ml of SN for 10 min.

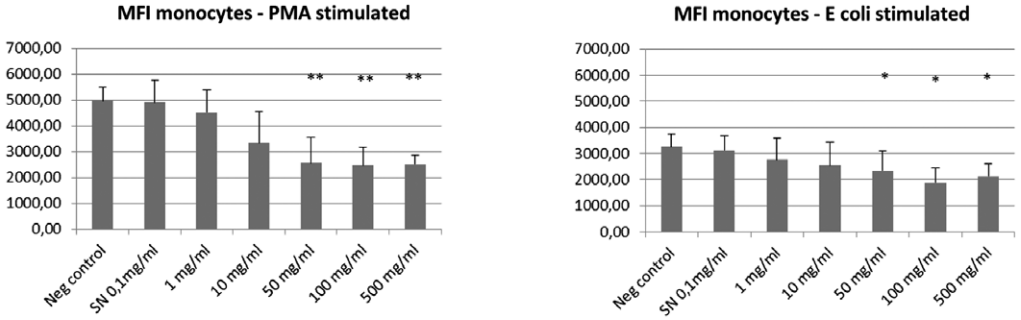

*Fig. 1. Dose effect for the inhibition induced by SN on the monocyte oxidative burst in PMA and E. coli stimulated blood. Data are presented as X±SD (N=9). SN was added to whole blood 10 min before phago-burst test. Monocyte oxidative burst activity of normal blood (Neg control,) and blood containing SN in concentrations 0.1; 1; 10; 50 and 500 mg/ml). * $p<0.05$; ** $p<0.01$.*

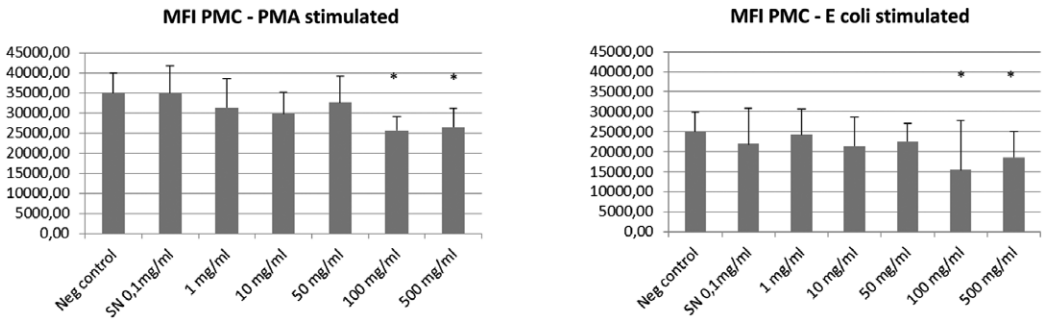

*Fig. 2. Dose effect for the inhibition induced by SN on the PMC oxidative burst in PMA and E. coli stimulated blood. Data are presented as X±SD (N=9). SN was added to whole blood 10 min before phago-burst test. PMC oxidative burst activity of normal blood (Neg control,) and blood containing SN in concentrations 0.1; 1; 10; 50 and 500 mg/ml). * p<0.05; ** p<0.01.*

Blood was then stimulated with PMA or *E. coli* suspension (5×10^7/ml). We assessed the effects of SN on monocyte/PMC oxidative burst activity as measured by the conversion of DHR 123 to rhodamine 123. When compared to PMA-activated monocytes which were cultured alone, monocytes preincubated with SN showed a significant decrease in oxidative burst activity (Figure 1). Oxidative burst responses of monocytes stimulated with PMA were strongly inhibited at SN concentration ranging from 10–500 mg/ml, however SN was less efficient inhibitor in *E. coli* stimulated monocytes (inhibition range was from 50–500 mg/ml SN). SN inhibited PMC oxidative burst only in very high concentration range (100 and 500 mg/ml SN).

Discussion

The oxidative burst assay employed in this study utilizes dichlorofluorescein diacetate as a fluorescent probe. This assay detects mainly superoxide anion and hydrogen peroxide, and to a lesser extent, other reactive oxygen species such as hydroxyl radicals[4]. The inhibition of oxidative burst by SN would suggest its ability to scavenge superoxide anion and hydrogen peroxide.

In the previous study, we first demonstrated that SN is an efficient inhibitor of sepsis development in mice model of LPS-induced sepsis. The changes induced by SN include decreased mice plasma inflammatory cytokine production. Similarly we demonstrated a decreased TNF-α, IFN-γ and IL-6 response in human LPS-stimulated PBMNCs. SN was therefore shown to be a promising inhibitor of multiple inflammatory cytokine secretion.

Inflammatory mediators, cytokines (e.g. TNF-α) increase the ability of monocytes/PMC to localize at the site of inflammation. Phagocytic ability is elevated by intensification of hydroxylic radical production and lysosomal enzyme release (6). TNFα is an important factor strengthening granulocyte phagocytic and cytotoxic activity. Activated monocytes and granulocytes secrete cytokines. IL-1, which stimulates monocytes, endothelial cells, and fibroblasts to secrete IL-8, in turn increases the expression of CD11b/CD18 adhesive molecules and granulocyte oxygen metabolism[7,8].

This study was performed to determine whether SN may exert the anti-inflammatory effects by acting as as inhibitor of respiratory burst in PMCs and/or monocytes. Stimulation of monocytes/PMCs by phorbol 12-myristate 13-acetate (PMA) results in induction of superoxide anion production through assembly of the NADPH-oxidase complex. This so-called respiratory burst is fundamentally important in monocytes/PMC since it is implicated in the killing of microbial intruders and in the tissue damage secondary to the induced inflammatory response[9]. The activation status of monocytes/PMCs can range from quiescent over primed to fully activated[10].

To determine the effect of SN on oxidative burst of monocytes/MNC blood was preincubated with 0 (Control), 0.1, 10, 50, 100 and 500 mg/ml of SN for 10 min. We assessed the effects of SN on monocyte/PMC oxidative burst activity as measured by the conversion of DHR 123 to rhodamine 123. Blood was incubated without or with SN and after 10 min whole blood was stimulated with PMA or *E. coli* suspension (5×10^7/ml). When compared to monocytes which were cultured alone, PMA-activated monocytes showed a significant decrease in oxidative burst activity. Oxidative burst responses of monocytes stimulated with PMA were strongly inhibited at SN concentration ranging from 10–500 mg/ml. SN was less efficient inhibitor in *E. coli* stimulated monocytes (inhibition range was from 50–500 mg/ml SN). On contrary SN inhibited PMC oxidative burst only in range 100–500 mg/ml SN, that concentration of SN is probably a lot over the achievable range during therapy and inhibitory effects may be a consequence of some relatively unspecific process.

We found SN as an efficient inhibitor of oxidative burst in monocytes. The data from this study are encouraging as researchers across the world are searching for ways to prevent the development of free-radical-mediated disorders such as atherosclerosis, arthritis, cancer, and aging[11,12]. Furthermore our data may also explain intriguing clinical data described in Russian pharmacopeia where SN is declared as a potent anti-inflammatory drug with excellent body-distribution and minimal side effects[3]. As we found SN effective mainly as a potent ROS scavenger in strongly activated monocytes (by PMA as strong ROS activator), SN may exsert its antiinflammatory effects through oxidative burst inhibition in stron-

gly activated monocytes/macrophages[13]. Since ROS generation in monocytes/macrophages has been found by many authors to be important for LPS-driven production of several proinflammatory cytokines, the antiinflamatory action of SN may be a consequence of ROS inhibition in activated monocytes/macrophages[2,13,14]. Although SN used as a drug is widely distributed within tissues and cells through the body, its ROS scavenger function is phisiologically important mainly in cells where ROS prodaction reaches substantial levels, e.g. in monocytes/macrophages and neutrophils.

Side effects of SN may be therefore expected in inhibition od neutrophil killing of bacteria, an essential process in defence against infectious agents. However, we assesed the ability of neutrophils to phagocytize opsonized *E. coli* was assessed. No inhibitory effects of SN (range 0,1–500 mg/ml) were found in monocytes/PMC stimulating either with PMA or *E. coli* in vitro. On the other hand, SN inhibited PMC oxidative burst only in range 100–500 mg/ml SN, that concentration of SN is probably a lot over the achievable range during therapy. It is therefore probable that SN does not affect neutrophil killing of bacteria while it is capable to act as ROS scavenger and anti-inflammatory agent in monocytes/macrophages[3,4].

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TETRAHIDROFTALAZINSKI DERIVAT »NATRIJ NUKLEINAT« ISPOLJAVA PROTUUPALNO DJELOVANJE KROZ INHIBICIJU OKSIDATIVNE AKTIVNOSTI HUMANIH MONOCITA

SAŽETAK

Opisali smo korištenje nove kemijske tvari Natrij nukleinata (SN) kao imunomodulatorne tvari koja pokazuje protuupalna svojstva. Natrij nucleinat (SN) registriran u Ruskoj Federaciji kao Tamerit® je 2-amino-1,2,3,4-tetrahidroftalazin-1,4-dion dihidrat natrijeve soli, derivat poznate kemijske tvari luminol. Da bi shvatiti mehanizme SN imunomodulatorne aktivnosti, ispitali smo SN modulaciju naglog oksidativnog odgovora monocita i polimorfonuklearnih stanica (PMC) cijele ljudske krvi stimuliranih s forbol-12-miristat-13-acetatom (PMA) ili E. coli suspenzijom in vitro. SN nije spriječio udio neutrofila i monocita u fagocitiranju E. coli. Nagli oksidativni odgovori monocita stimuliranih s PMA snažno su inhibirani sa SN u rasponu koncentracija od 10–500 mg/ml, manje učinkoviti inhibitor je SN u monocita stimuliranih s E. Coli (inhibicijski raspon od 50–500 mg/ml SN). SN je inhibirao PMC oksidativni odgovor samo u rasponu od 100–500 mg/ml. Kao zaključak, otkrili smo da je SN učinkoviti inhibitor naglog oksidativnog odgovora u monocita. Od kako se pokazalo da je produkcija ROS u monocitima/makrofaga važna za LPS-potaknutu proizvodnju nekoliko proupalnih citokina, SN može vršiti svoje protuupalno djelovanje kroz inhibiciju monocitnog/makrofagnog naglog oksidativnog odgovora.

Abbrevations

LPS – lipopolysaccharide
ROS – reactive oxigen species
PBMNC – peripherial blood mononuclear cell
PMC – polymorphomononuclear cell
TNF-α – tumor necrosis factor alpha
IFN-γ – gamma interferon
IL-6 – interleukin 6