Suppression of oxidative burst in human neutrophils with the naturally occurring serotonin derivative isomer from *Leuzea carthamoides*

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Abstract

OBJECTIVE: Neutrophil leukocytes and macrophages represent professional phagocytic cells. When appropriately stimulated, they undergo dramatic physiological and biochemical changes resulting in phagocytosis, chemotaxis and degranulation with the activation of reactive oxygen species (ROS) production known as the respiratory burst.

DESIGN: In this study we analysed the effect of a crystalline complex fraction of four N-feruloyl-serotonin isomers isolated from the seeds of *Leuzea carthamoides* on the mechanism of oxidative burst of human neutrophils in vitro.

RESULTS: N-feruloyl-serotonin (N-f-5HT) inhibited dose-dependently oxidative burst of human whole blood and isolated neutrophils in vitro stimulated with phorbol-myristate-acetate (PMA) as measured by luminol/isoluminol enhanced chemiluminescence.In isolated neutrophils stimulated with PMA, N-f-5HT was effective against extracellular as well as intracellular reactive oxygen species. Western blot analysis documented that N-f-5HT in concentrations of 10 and 100 μM significantly decreased PMA-induced phosphorylation of protein kinase C alpha/beta II.

CONCLUSION: The results suggest that N-f-5HT represents an effective naturally occurring substance with potent effect on the oxidative burst of human neutrophils and could be further investigated for its pharmacological activity against oxidative stress in ischaemia-reperfusion, inflammation and other pathological conditions.

ORIGINAL ARTICLE

INTRODUCTION

"Professional" phagocytes, such as neutrophils and monocytes, when appropriately stimulated, undergo dramatic physiological and biochemical changes involving the abrupt consumption of oxygen termed respiratory burst (Robinson 2009). Due to the fact that activated phagocytes represent one of the major sources for free radicals and reactive oxygen metabolite generation, it may be beneficial to elucidate specific antioxidative defense mechanisms and suppress pathological and toxicological side effects (Splettstosser & Schuff-Werner 2002).

A number of therapeutically used medicaments and natural products were found to act not only as extracellular scavengers but they also suppressed intracellular formation of reactive oxygen species (ROS) in human neutrophils (Drábikova *et al.* 2007; Jančinová *et al.* 2001; Nosál *et al.* 2009). In accordance with the postulated reduction of autooxidative damage during respiratory burst of activated phagocytes, preincubation of polymorphonuclear leukocytes (PMN) as well as monocytes/macrophages with serotonin(5-HT) resulted in a concentration-dependent modulation of cell motility, chemotaxis and phagocytosis (Finocchiaro *et al.* 1988). In this paper we investigated the effect of a crystalline complex fraction of four N-f-5HT isomers isolated from the seeds of *Leuzea carthamoides* on the mechanism of oxidative burst of human neutrophils *in vitro.*

MATERIALS AND METHODS

Materials

Luminol, isoluminol, PMA (4β–phorbol-12β-myristate-α13-acetate), superoxide dismutase, dextran (average MW 464,000) were from Sigma-Aldrich Chemie (Deisenhofen, Germany), HRP (horseradish peroxidase) and catalase from Merck (Darmstadt, Germany), lymphoprep (density 1.077 g/mL) from Nycomed Pharma AS (Oslo, Norway).

Phosphate buffered saline solution (PBS) contained 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 0.5 mM $MgCl_2 \times 6\,H_20$, pH 7.4, Tyrode's solution consisted of 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaH_2CO_3, 0.4 mM $NaH_2PO_4 \times 2\,H_20$, 1 mM $MgCl_2 \times 6\,H_20$, and 5.6 mM glucose, pH 7.4.

Isolation and characterisation of N-feruloyl-serotonin isomers

The N-feruloyl-serotonin containing fraction was isolated from seeds of *Leuzea carthamoides* (Wild) DC by solvent extraction followed by column chromatography on silica gel, and by further HPLC separations under conditions reported previously (Harmatha *et al.* 2007).

Blood collection and neutrophil separation

Fresh human blood was obtained at the blood bank by venepuncture from healthy male volunteers (20–50 years) who had not received any medication for at least 7 days and it was anticoagulated with 3.8% trisodium citrate (blood:citrate ratio=9:1). Human neutrophils were isolated from whole blood as described earlier (Drábiková *et al.* 2006b; 2007).

Chemiluminescence (CL) assay of whole blood and isolated neutrophils

Oxidative burst in whole blood stimulated with PMA (0.05 μM), was measured by means of luminol enhanced chemiluminescence (CL - Jančinová *et al.* 2006b). The effect of N-f-5HT on extra- and intracellular ROS production was measured in unstimulated and PMA stimulated neutrophils (5×10^5 per sample) by isoluminol/luminol enhanced CL (Jančinová *et al.* 2006b) in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37 °C. Data were based on integral values of CL over 3 600 s (whole blood) or 1 800 s (isolated neutrophils) (RLU × s, RLU=relative light units).

Protein kinase C activation

Phosphorylation of protein kinase (PKC) isoenzymes α and βII was detected in isolated human neutrophils (5×10^6/sample) stimulated with PMA. Proteins were separated by electrophoresis and from two strips taken, one was detected for PKC and the second for detection of β-actin representing the internal control. Autoradiogram bands were quantified using the Image J programme and the optical density of each PKC band was corrected by the optical density of the corresponding β-actin band (for details see Jančinová *et al.* 2009).

Statistical analysis

Data represent the mean±SEM, unless stated otherwise. Statistical analysis was performed using ANOVA paired test to examine differences between treatments and control. Differences were considered to be statistically significant when $p<0.05$ (*) or $p<0.01$ (**).

RESULTS

Effect of N-f-5HT on whole blood and isolated neutrophil CL

Figure 1 demonstrates the dose-response effect of N-f-5HT on CL of whole human blood (panel A) and on isolated neutrophils (panel B), both stimulated with PMA (0.05 μM). A significant decrease in luminol-enhanced CL of whole blood is evident with N-f-5HT in concentration of 1 μM. In isolated neutrophils, N-f-5HT inhibited preferentially extracellular isoluminol-enhanced CL as compared with inhibition of intracellular luminol-enhanced CL.

Phosphorylation of PKC in the presence of N-f-5HT

The effect of N-f-5HT on PKC of isolated human neutrophils stimulated with PMA (0.15 μM) is demonstrated in Figure 2. Stimulation of isolated neutrophils increased PKC activity by 100%. In the concentration

of 10 μM, N-f-5HT decreased the activation of PKC to 50% of stimulated control. Increase of the concentration of N-f-5HT to 100 μM resulted in the recovery of PKC activity to spontaneous value.

DISCUSSION

N-feruloyl-serotonin, a condensation substance of 5-hydroxytryptamine and ferulic acid isolated from *Leuzea carthamoides* and characterised by HPLC, suppressed oxidative burst of human whole blood and isolated neutrophils. Stimulation of whole blood as well as of isolated neutrophils with PMA increased CL due to the oxidative burst of phagocytes. The effect was dose-dependent indicating that increase in N-f-5HT concentration resulted in decreased response of neutrophils to generate CL. A significant decrease in CL by N-f-5HT was similar yet much more effective compared with suppression of oxidative burst *in vitro* reported for cardiovascular or antihistaminic drugs (Drábiková *et al.* 2006a,b; Jančinová *et al.* 2006a; Nosál *et al.* 2009), stobadine (Drábiková *et al.* 2007), or natural substances like glucomannans (Drábiková *et al.* 2009), curcumin (Jančinová *et al.* 2009) and arbutin (Jančinová *et al.* 2007). Moreover, many pharmacologically active compounds tested decreased oxidative burst of blood phagocytes in an animal model of adjuvant arthritis (Drábiková *et al.* 2009; Nosál *et al.* 2009).

Since the mechanism of the N-f-5HT effect against oxidative stress and free radical generation has not yet been established, a method for differentiation between extra- and intracellular CL was applied (Jančinová *et al.* 2006a,b). It is evident from Figure 1 that N-f-5HT decreased in a dose-dependent way CL of isolated human neutrophils *in vitro,* both at extra- and intracellular level, similarly as described for other pharmacologically active compounds (Jančinová *et al.* 2009; Nosál *et al.* 2009). This finding indicates that N-f-5HT is active extracellularly (as scavenger), like serotonin, most probably due to its chemical struture consisting of 5-HT and ferulic acid (Betten *et al.* 2001; Jančinová *et al.* 2001). On the other side, inhibition of intracellular CL most probably depends on the interaction of N-f-5HT with regulatory pathways inside neutrophils.

The activation of PKC and consequently the respiratory burst in phagocytes by PMA resulted in oxidase activation and regulation of apoptosis (Morel *et al.* 1991). Since the role of PKC inactivation of phagocytes is considered predominant in oxidase activation any pharmacological interference/inhibition with PKC can substantially suppress ROS generation at intracellular level.

Activation of phagocytes with proinflammatory mediators play a role in ischaemia-reperfusion, arthritis, chronic gut inflammation, immune-complexes induced organ injury, etc. (Corner & Grisham 1996).

The detected CL resulted from activation of superoxide generating NADPH oxidase, which in turn

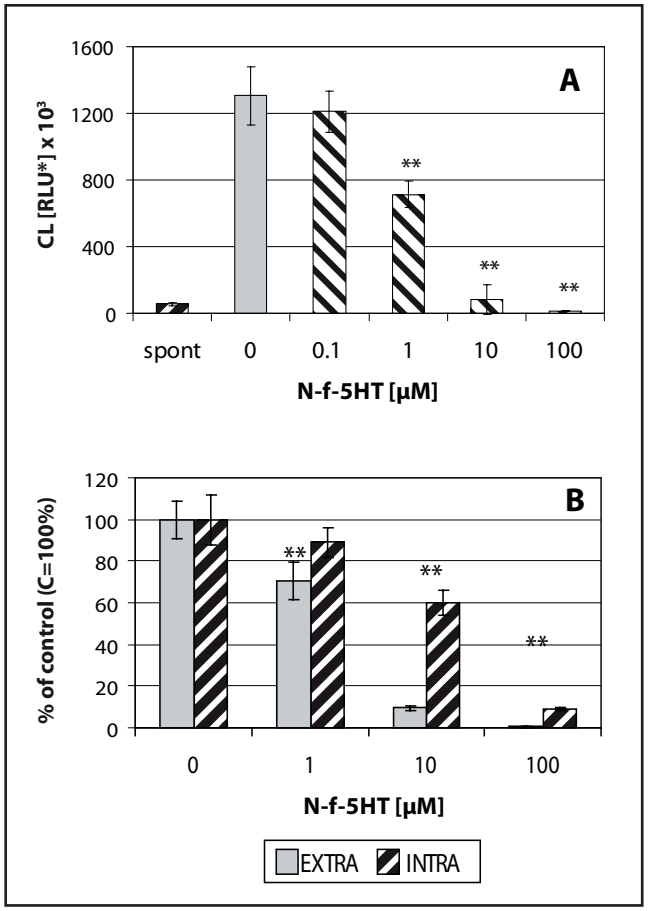

Fig. 1. Effect of N-f-5HT on whole human blood (panel A) luminol-enhanced chemiluminescence (CL) stimulated with phorbol myristate acetate (PMA 0.05 μM) and on isolated neutrophil CL (extracellular enhanced with isoluminol, intracellular enhanced with luminol - panel B) stimulated with PMA (0.05 μM); n=6;mean±SEM; $^{**}p \leq 0.01$

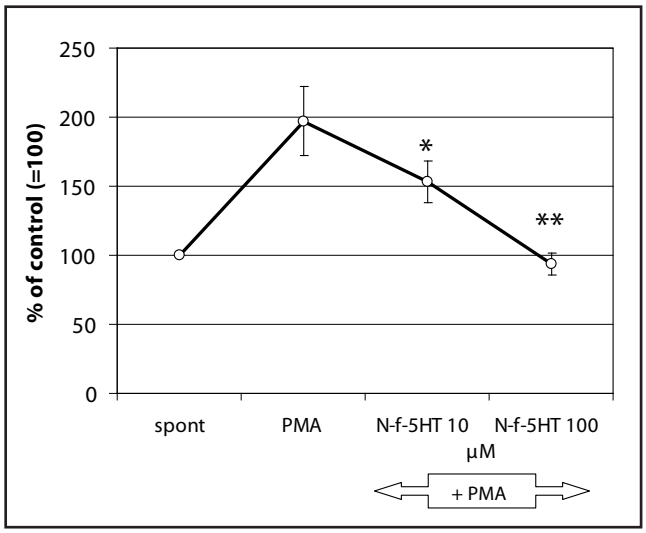

Fig. 2. Protein kinase C (PKC) phosphorylation in PMA (0.15 μM) stimulated neutrophils treated with 10 and 100 μM N-f-5HT. Increase of N-f-5HT concentration to 100 μM concentration resulted in the recovery of PKC phosphorylation to control (spontaneous) data. The values represent the percentage of resting control phosphorylated PKC isoenzymes (α and βII) were detected byWestern blotting and detected by phospho-PKC alpha/beta II (Thr638/641) antibody. n=8, mean± SEM.

depends on phosphorylation of the proteins due to PKC (Arnhold *et al.* 1999). N-f-5HT dose-dependently decreased PKC activation, presumably by interference with protein phosphorylation and NADPH oxidase activation.This is suggestive of a similar mechanism of action as described for diferuloylmethane on neutrophils (Mahmmoud 2007), confirmed by Jančinová *et al.* (2009). As PKC isoforms α and βII participate directly in the activation of neutrophil NADPH oxidase (Fontayne *et al.* 2002), their blockade may result in reduced oxidative burst and explain the decreased CL measured in the presence of N-f-5HT.

In addition to this suggested mechanism of action, N-f-5HT might interfere intracellularly with other mechanisms involved in neutrophil activation, such as phospholipase C and D (Liscovitch *et al.* 2000; Selvatici *et al.* 2006), lipooxygenase (Bonnans & Levy 2007) and particularly phospholipase A_2 (Levy 2006), as demonstrated for many cationic amphiphilic drugs (Nosáľ & Jančinová 2002) .

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