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Naturally appearing N-feruloylserotonin isomers suppress oxidative burst of human neutrophils at the protein kinase C level

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Abstract:
N-feruloylserotonin (N-f-5HT) isomers, isolated from seeds of *Leuzea carthamoides* (Wild) DC, inhibited dose-dependent oxidative burst in human whole blood and isolated neutrophils *in vitro*, which were measured by luminol- and/or isoluminol-enhanced chemiluminescence in the following rank order of stimuli: PMA > OpZ > calcium ionophore A23187. In isolated neutrophils that were stimulated with PMA, N-f-5HT isomers were effective against extracellular and intracellular reactive oxygen species. Liberation of ATP, analysis of apoptosis, and recombinant caspase-3 activity revealed that N-f-5HT isomers, used in concentrations up to 100 µM, did not alter the viability and integrity of isolated neutrophils. Western blot analysis documented that in concentrations of 10 and 100 µM, N-f-5HT isomers significantly decreased PMA-induced phosphorylation of PKC α/β II. The results suggest that N-f-5HT isomers are an effective, naturally occurring substance with a potent pharmacological effect on the oxidative burst of human neutrophils. It should be further investigated for its pharmacological activity against oxidative stress in ischemia-reperfusion, inflammation and other pathological conditions.

Keywords:
neutrophils, N-feruloylserotonin, extracellular and intracellular chemiluminescence, protein kinase C phosphorylation

Abbreviations: A23187 – calcium ionophore, CL – chemiluminescence, HRP – horseradish peroxidase, N-f-5HT – N-feruloylserotonin isomers, PMA – phorbol-myristate-acetate, RLU – relative light units, ROS – reactive oxygen species

Introduction

Neutrophils, leukocytes and macrophages represent professional phagocytic cells of the innate immune system that are important in host defense and inflammatory responses. When appropriately stimulated by a variety of agents, they undergo dramatic physiological and biochemical changes. This results in phagocytosis, chemotaxis and degranulation, with the activation of reactive oxygen species (ROS) production in a metabolic pathway, which is known as the respiratory burst [32], where they consume oxygen and produce superoxide anions [35]. The respiratory burst can be stimulated either by specific receptor-operated stimuli, such as the chemoattractant peptide n-formyl-methionyl-leucyl-phenylalanine (fMLP) and opsonized zymosan (OpZ), or it can be stimulated by receptor-bypassing stimuli like calcium ionophore A23187

or phorbol-myristate-acetate (PMA) [2, 15]. There are two concurrent events that occur in the stimulated neutrophils. The first step is the oxygen-dependent step known as the "oxidative burst" through the generation of ROS. The second step is the oxygen-independent step and consists of the release of enzymes from intracellular granules [15]. There is an increasing number of evidence for links between oxidative stress and a variety of pathological disorders like cardiovascular diseases, cancer, chronic inflammatory diseases and post-ischemic organ injury [5, 33]. The "two-faced" role of ROS is substantiated by an increasing amount of published information about ROS. Inside cells, ROS act as secondary messengers in intracellular signaling pathways, similar to protein and lipid kinases and phosphatases. They also act as phospholipases, thus regulating phagocytosis, apoptosis, gene expression, vascular tone, etc. [13, 40]. The ROS released during the oxidative burst of professional phagocytes can be conveniently detected by chemiluminescent techniques [6]. The development of pharmacological procedures to ameliorate undesirable ROS production may be one of the central issues in research on aging and oxidative stress-related diseases in the near future. A number of therapeutically used medicines and natural products act not only as extracellular scavengers, but they also suppress the intracellular formation of ROS in human neutrophils [9, 11, 17, 26, 27].

N-feruloylserotonin (N-f-5HT; N-feruloyl-5-hydroxytryptamine) isomers, isolated from the seeds of *Leuzea carthamoides* (Wild) DC [16], were found to possess antioxidative, antiatherosclerotic and antimelatogenic effects. It was also found to reduce anxiety in high-pain thresholds *in vivo* and *in vitro* [22, 36, 41].

In this study, we analyzed the effect of a crystalline complex fraction of four N-f-5HT isomers, which were isolated from the seeds of *Leuzea carthamoides*, on the mechanism of oxidative burst of human neutrophils *in vitro*.

Materials and Methods

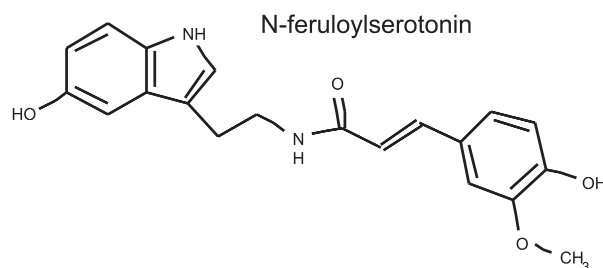
Isolation and characterization of N-f-5HT isomers

The fraction containing N-f-5HT was isolated from the seeds of *Leuzea carthamoides* (Wild) DC by solvent extraction. This was then followed by column chromatography on silica gel and HPLC separations under conditions previously reported [16, 34]. The authors described the structural identification of separated single isomers as well as the analytical characteristics of the obtained crystalline fractions. In this study, a crystalline fraction with a naturally occurring composition was selected (as indicated in Tab. 1). Recrystallizations did not change the isomeric composition, which was probably due to the facile and common *Z/E* (*cis/trans*) isomerization of the feruloyl- (or isoferuloyl-) moiety in crystallization solutions. The isolation of single isomers in a high purity requires extensive and laborious preparation [16], and therefore it is more easily to be substituted by synthesis.

N-f-5HT (1.76 mg) was dissolved in a mixture of 20 µl of 1 M NaOH and 980 µl of redistilled water. The stock solution (5 mM) was further diluted with Tyrode's solution to yield N-f-5HT sample concentrations of 0.01–100 µM. The corresponding final concentrations of NaOH were in the range of 0.04–400 µM. At these concentrations, the solvent agent alone did not reduce the activity and the viability of neutrophils.

Tab. 1. Composition of the crystalline N-feruloylserotonin complex fraction, where the content of N-feruloyl- and N-isoferuloyl-(*E* = *trans*- and *Z* = *cis*-) serotonin isomers was determined by HPLC analysis [34]. For isolation and structural identification, see [16]

Compound	Content [%]
N-(*E*)-feruloylserotonin	18.3
N-(*E*)-isoferuloylserotonin	67.4
N-(*Z*)-feruloylserotonin	6.1
N-(*Z*)-isoferuloylserotonin	8.2



Drugs and chemicals

The following other chemicals were used: luminol, isoluminol, PMA (4β-phorbol-12β-myristate-α13-acetate), Ca^{2+}-ionophore A23187, superoxide dismutase, dextran (average MW 464,000), zymosan A (from *Saccharomyces cerevisiae*), luciferase from the firefly *Photinus pyralis* and D-luciferin sodium salt from Sigma-Aldrich Chemie (Deisenhofen, Germany). HRP (horseradish peroxidase) and catalase from Merck (Darmstadt, Germany) and lymphoprep (density 1.077 g/ml) from Nycomed Pharma AS (Oslo, Norway) were also used.

The phosphate buffered saline solution (PBS) used in this study 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 $MgCl_2 \times 6H_20$ and had a pH of 7.4. Tyrode's solution used in this study consisted of 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaH_2CO_3, 0.4 mM $NaH_2PO_4 \times 2H_20$, 1 mM $MgCl_2 \times 6H_20$ and 5.6 mM glucose and had a pH of 7.4.

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. It was anticoagulated with 3.8% trisodium citrate (blood : citrate ratio = 9:1). The Ethical Committee license for blood sampling at National Transfusion Service NTS-KRA/2008/SVI, was registered.

Human neutrophils were isolated from whole blood, as described previously [9, 10]. The blood was gently mixed, and erythrocytes were allowed to sediment in 3% dextran solution by centrifugation (10 × g, 25 min, 22°C). The neutrophil suspension (3 ml) was layered on Lymphoprep (3 ml) and centrifuged (500 × g, 30 min, 22°C). After hypotonic lysis (3 ml of ice-cold deionized H_2O, followed after 45 s by 3 ml of 1.8% NaCl and 6 ml of Ca^{2+}- and Mg^{2+}-free PBS) and centrifugation (500 × g, 10 min, 22°C), neutrophils in Ca^{2+}- and Mg^{2+}-free PBS were counted. They were then adjusted to a final concentration of 10^4 cells/μl (Coulter Counter, Coulter Electronics, England) and kept on ice. The final suspension of neutrophils contained more than 96% of viable cells, as evaluated by trypan blue exclusion, and was used within 2 h, as long as the control chemiluminescence remained constant.

Chemiluminescence (CL) assay of whole blood and isolated neutrophils

The oxidative burst in whole blood was stimulated with phorbol myristate acetate (PMA 0.05 μM), opsonized zymosan (OpZ; 0.5 mg/ml; for preparation see [8]) or Ca ionophore A23187 (1 μM). It was measured in 250 μl samples consisting of 50 μl aliquots that contained blood (50× diluted), luminol (200 μM), N-f-5HT (0.01–100 μM) and phosphate buffer [20]. Horseradish peroxidase (HRP 8 U/ml) was added to the system and maintained a sufficient extracellular peroxidase concentration [12]. The effect of N-f-5HT on extra- and intracellular ROS production was measured in unstimulated and PMA-(0.05 μM) stimulated neutrophils (5×10^5 per sample) by isoluminol/luminol-enhanced CL [18]. Extracellular CL was determined in the system containing isoluminol (5 μM) and HRP (8 U/ml). Intracellular CL was measured with luminol in the presence of extracellular scavengers – superoxide dismutase (100 U/ml) and catalase (2,000 U/ml) [12]. The CL of both whole blood and isolated neutrophils was evaluated in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37°C. The 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).

Analysis of apoptosis

Citrated whole human blood was collected as described above. Dextrane (3%) was added (blood : dextrane = 2:1) and centrifuged at 10 × g at room temperature. Before use, 1 ml of buffy coat that contained leukocytes was collected and stored on ice. The cells were counted on the hemocytometer (Coulter Counter), which focused on granulocytes. The cell suspension was dissolved to get 2×10^5 neutrophils per sample. Three different concentrations of N-f-5HT (1, 10 and 100 μM) were applied and incubated with a control sample at 37°C for 10 min. The cells were stained with Annexin V, conjugated with FITC (BenderMedSystems) in the dark at 4°C for 10 min, followed by staining with propidium iodide (1 μg/ml), and then analyzed immediately by the Beckman Coulter Cytomics FC500 cytometer. All samples were analyzed under the same conditions (gains, volts). From the granulocytic area, 5,000 cells were gated and analyzed.

Caspase-3 activity

To determine the caspase-3-activity, a method by Notebaert et al. [31] was modified. Briefly, the cleavage of the Z-DEVD-amino-luciferin substrate by caspase releases amino-luciferin. The following reaction with luciferase was detected by CL. The light production was measured on the Luminometer Immunotech LM-01T. According to the manufacturer's instructions, 10 µl of 0.1 IU caspase was added to 20 µl aliquots of different N-f-5HT concentrations and buffered solution. Finally, 50 µl of Caspase-Glo 3/7 Reagent was added and the mixture was measured for 60 min to determine the caspase-3 activity. The vehicle solutions for N-f-5HT, which contained NaOH, were also evaluated.

Neutrophil integrity

The cytotoxic effect of N-f-5HT was evaluated by means of ATP liberation by the luciferin-luciferase CL [20]. Using this method, ATP was enzymatically assessed with firefly luciferase, which catalyzed the hydrolysis of ATP and the oxidation of luciferin. The neutrophil suspension (30 µl; 30,000 cells/sample) and 20 µl of Tyrode's solution were incubated with 50 µl of N-f-5HT (1–100 µM) for 15 min at 37°C. Ten microliters of a mixture of luciferin (1.6 µg/sample) and luciferase (45,000 U/sample) was added, and the CL was recorded for 60 s. In each experiment, the CL of ATP standards (1–500 nM) was measured, and the concentrations of ATP in samples were calculated from the calibration curve. The total ATP content was assessed immediately after the sonication of neutrophils for 10 s.

Protein kinase C activation

Phosphorylation of protein kinase (PKC) isoenzymes α and βII was detected using the method described by Jančinová et al. [20]. Isolated human neutrophils (5×10^6) were incubated at 37°C with N-f-5HT for 1 min, stimulated with PMA (0.15 µM, 1 min) and lysed by the addition of solubilization buffer (20 mM Tris-HCl, 5 mM EDTA, 1% Triton, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 200 µM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml aprotinin, pH 7.4). After sonication on ice, samples were centrifuged (18,625 × g, 5 min, 4°C) to remove unbroken cells, the supernatant was boiled for 5 min with sample buffer (50 mM Tris-HCl, 2% SDS, 7.5% glycerol, 2.5% mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and samples were loaded on 9.8% SDS polyacrylamide gels. Proteins (20 µg per lane) were separated by electrophoresis and transferred to Immobilon-P Transfer Membrane (Millipore Corp., USA). From the two strips taken, one was detected for PKC (area between 60 and 100 kD) and the second for detection of β-actin (30–60 kD), which represented the internal control. Membrane strips were blocked for 60 min with 1% bovine serum albumin in Tris buffered saline (TBS, 20 mM Tris-HCl, 154 mM NaCl, 0.05% Tween-20, pH 7.5). This was then followed by 60 min incubation in the presence of the Phospho-PKC α/β II (Thr638/641) antibody (rabbit anti-human, 1:8,000, Cell Signaling Technology) or β-actin Antibody (rabbit anti-human, 1:4,000, Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed six times with TBS and incubated 60 min with the secondary antibody conjugated to horse-radish peroxidase (anti-rabbit from donkey, 1:10,000, Amersham, UK), and the activity of horseradish peroxidase was visualized using Enhanced Chemiluminiscence Western Blotting Detection Reagents (Amersham, UK), followed by autoradiography. Autoradiogram bands were quantified using the Image J program. The optical density of each PKC band was corrected by the optical density of the corresponding β-actin band.

Statistical analysis

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

Results

Figure 1 demonstrates dose-response representative luminol-enhanced CL curves of whole human blood (from one donor) which was treated with N-f-5HT and subsequently stimulated with PMA (0.05 µM). PMA was taken as a representative stimulus. Other stimuli showed similar CL curves.

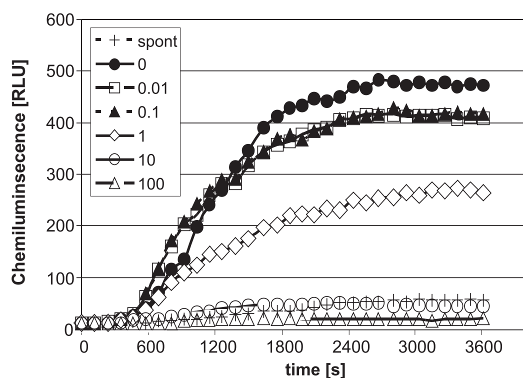


Fig. 1. Whole blood chemiluminescence (CL) (samples from one donor) exposed to N-f-5HT and stimulated with PMA (0.05 μM). Representative dose-response luminol-enhanced chemiluminescence curves. Spont = spontaneous (control value)

The effect of N-f-5HT on whole human blood CL, which is stimulated by three stimuli, is summarized in Figure 2. The most potent stimulus was PMA, which was then followed by OpZ and calcium ionophore A23187. The percentage ratio between spontaneous and stimulated whole blood CL with PMA, OpZ and A23187 was 4.16, 14.6 and 36.03, respectively. The rank order potency of stimuli for N-f-5HT to inhibit stimulated CL in whole blood was PMA > OpZ > A23187.

It is evident from Figure 3, representing the IC_{50} values, that large differences exist between N-f-5HT concentrations that inhibit the chemiluminescence of whole human blood in the rank order of stimuli PMA < OpZ < A23187.

Figure 4 shows the representative dose-response chemiluminescence curves for isolated neutrophils stimulated with PMA at the extra- and intracellular level. The values of extracellular CL were 2 to 14 times higher compared to intracellular CL, which depended on the N-f-5HT concentration used.

The dose-dependent effect of N-f-5HT on the extra- and intracellular CL, evaluated as percentage of the control values, is summarized in Figure 5. The ratio between the percentage inhibition of extra- to intracellular CL for N-f-5HT concentrations of 1, 10 and 100 μM were 30:11, 90:40 and 99:91 percent, respectively.

The exposition of isolated human neutrophils to increasing concentrations of N-f-5HT, was not associated with their damage because no increase of spontaneous ATP liberation was recorded (Fig. 6). During the 15 min incubation, neutrophils liberated 19.48 ±

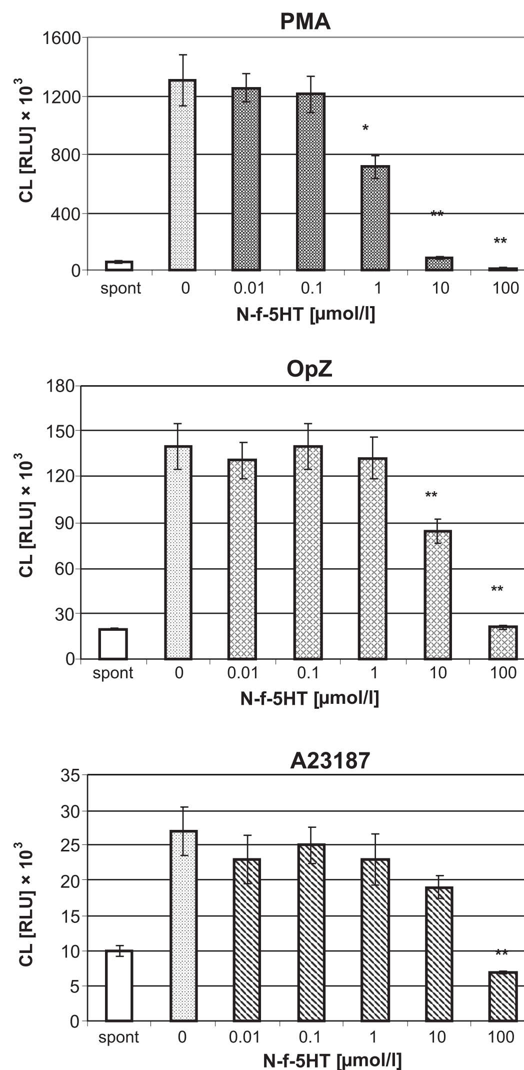


Fig. 2. Effect of N-f-5HT on stimulated whole human blood chemiluminescence (CL) with phorbol myristate acetate (PMA 0.05 μM), opsonized zymosan (OpZ 0.5 mg/ml) and calcium ionophore A23187 (1 μM). Spont = CL of whole blood without PMA and N-f-5HT; 0 = CL of whole blood stimulated with PMA; n = 6; the mean ± SEM; * p ≤ 0.05, ** p ≤ 0.01

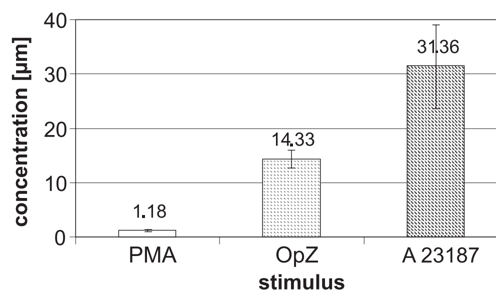


Fig. 3. Mean effective concentrations of N-f-5HT producing 50% inhibition of whole blood chemiluminescence (CL), which was stimulated with phorbol myristate acetate (PMA 0.05 μM), opsonized zymosan (OpZ 0.5 mg/ml), and Ca ionophore A23187 (1 μM); n = 4–6; the mean ± SEM

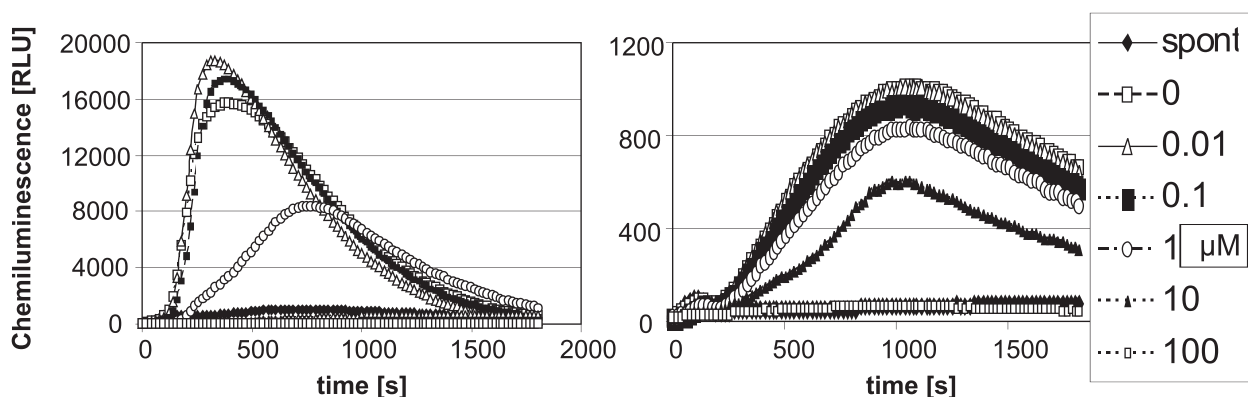


Fig. 4. Representative dose-response curves for extracellular (left panel) and intracellular (right panel) chemiluminescence in isolated human neutrophils treated with N-f-5HT and stimulated with PMA (0.05 μM). Spont = spontaneous (control value)

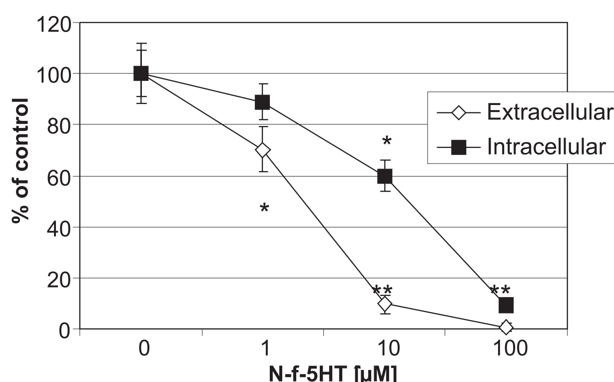


Fig. 5. Effect of N-f-5HT on extracellular and intracellular chemiluminescence in isolated human neutrophils stimulated with PMA (0.05 μM) and exposed to N-f-5HT; n = 6; the mean ± SEM; * $p \leq 0.05$; ** $p \leq 0.01$

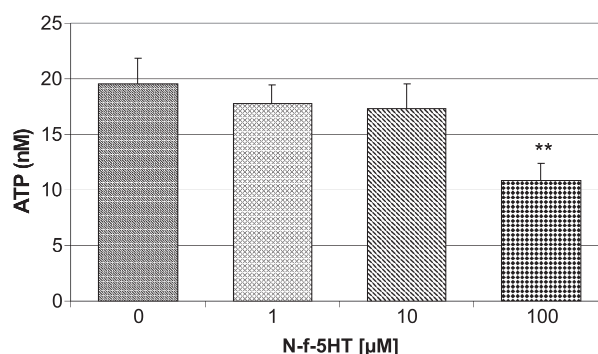


Fig. 6. ATP liberated from isolated neutrophils exposed to N-f-5HT. Total ATP content assessed after neutrophil sonication was 547.72 ± 112 nM; n = 6; the mean ± SEM; ** $p \leq 0.01$

2.3 nM ATP, which represented 3.56% of the total ATP content (547.72 ± 112 nM) that was determined after complete neutrophil disintegration. For the concentration of 100 μM, N-f-5HT decreased the spontaneous ATP liberation by 44.36%.

The percentage of viable and apoptotic isolated neutrophils exposed to N-f-5HT, as measured by flow cytometry, is demonstrated in Figure 7. Measurements were evaluated after staining the cells with FITC-conjugated Annexin-V plus propidium iodide (PI). Only Annexin-positive cells are considered apoptotic (mentioned also as pre-apoptotic), and double-positive cells (Annexin+/PI+) are considered as late-apoptotic or dead cells. N-f-5HT did not induce significant apoptosis of isolated human neutrophils in any concentration tested (1, 10 and 100 μM). The percentage of viable cells did not change significantly.

Figure 8 shows the effect of N-f-5HT on the caspase-3 activity. In the concentrations tested, it did not significantly affect the activity of the caspase-3 enzyme. Appropriate vehicle solution concentrations (4–400 μM NaOH) did not significantly change the activity of caspase-3 (data not shown).

The stimulation of neutrophils with PMA increased PKC phosphorylation by 100%. In concentrations of 10 and 100 μM, N-f-5HT decreased the phosphorylation by 50 and 100%, respectively (Fig. 9). Moreover, 100 μM concentration suppressed the phosphorylation to the control (basal) value. This is clear evidence that N-f-5HT provides a significant concentration-dependent effect on PMA-stimulated neutrophil phosphorylation. This effect correlated with the effect of N-f-5HT on the dose-dependent inhibition of CL and oxidative burst in human neutrophils.

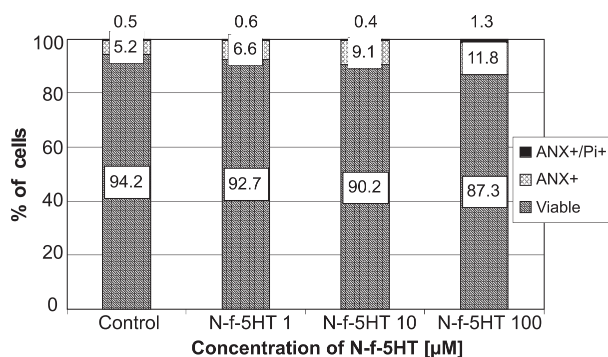


Fig. 7. The dose-response effect of N-feruloylserotonin (N-f-5HT) on the viability of human neutrophils. ANX = annexin; Pi = propidium iodide; n = 5; the mean ± SEM

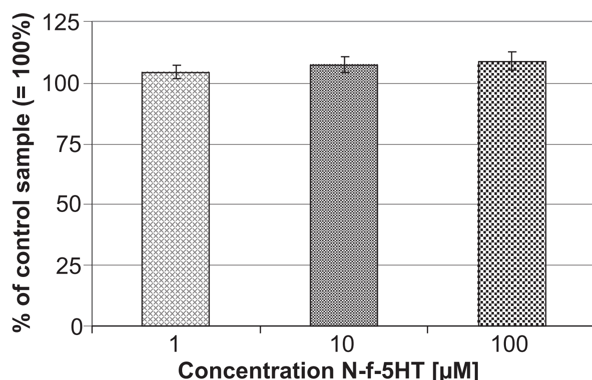


Fig. 8. Effect of different concentrations of N-feruloylserotonin (N-f-5HT) (1–100 µM) on human recombinant caspase-3 activity shown as a percentage of the control sample (the mean ± SEM, n = 5)

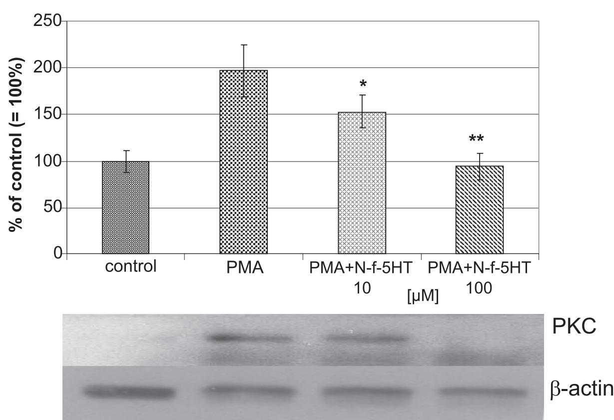


Fig. 9. Protein kinase C (PKC) phosphorylation in phorbolmyristate (PMA = 0.15 µM) stimulated neutrophils treated with 10 and 100 µM N-f-5HT. The values represent the percentage of resting control phosphorylated PKC isoenzymes (α and βII) detected by Western blotting and by phospho-PKC α/β II (Thr638/64) antibody. n = 8, the mean ± SEM. * p < 0.05; ** p < 0.01

Discussion

The N-f-5HT isomeric complex mixture, isolated from the seeds of *Leuzea carthamoides*, dose-dependently decreased the oxidative burst of human whole blood and isolated neutrophils.

"Professional" phagocytes, such as neutrophils and monocytes, when appropriately stimulated, undergo dramatic physiological and biochemical changes that involve the abrupt consumption of oxygen, termed respiratory burst [35]. 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 [39].

Oxidant production was first detected by means of luminol- and/or isoluminol-enhanced CL after stimulation with a receptor operated stimulus (OpZ). It was also detected with receptor-bypassing stimuli (PMA, A23187). A significant decrease in CL by N-f-5HT was similar, yet much more effective, when compared with the suppression of oxidative burst *in vitro*. This was reported for cardiovascular or antihistamine drugs [9, 11, 17, 18, 28], stobadine [10], or with natural substances like glucomannans [12], curcumin [20] and arbutin [21]. Moreover, many pharmacologically active compounds that were tested decreased the oxidative burst of blood phagocytes in animal models of adjuvant arthritis [12, 21, 30].

Because the mechanism of 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 [17, 18]. It is evident from Figures 4 and 5 that N-f-5HT decreased, in a dose-dependent way, the CL of isolated human neutrophils *in vitro* at both the extra- and intracellular level. This was similarly described for other pharmacologically active compounds [17, 28]. This finding indicates that N-f-5HT is active extracellularly (as a scavenger) like serotonin, probably due to its chemical structure, which consists of 5-HT and feruloyl acid [3, 19]. Inhibition of intracellular CL most probably depends on the interaction of N-f-5HT with regulatory pathways inside neutrophils. In the concentrations of 1 to 100 µM, N-f-5HT did not disintegrate neutrophils as demonstrated by any increase in ATP liberation from isolated neutrophils (Fig. 6). Moreover, N-f-5HT did not alter neutrophil viability and the number of proapoptotic and apoptotic cells exposed to 100 µM N-f-5HT only slightly increased by 6.6 and

0.8%, respectively, as compared with control cells. Caspase-3 activity also demonstrated that N-f-5HT did not alter the vitality of neutrophils.

N-f-5HT was most effective against the stimulation of whole blood and isolated neutrophils with PMA. The stimulation of respiratory burst in neutrophils by PMA and other stimuli resulted from the intracellular activation of the superoxide-generating NADPH oxidase system, which was dependent on the phosphorylation of 47 and 67 kD proteins [7, 38]. N-f-5HT significantly decreased the activity of PKC in isolated neutrophils in 10 and 100 μM concentrations by 25 and 50% of the PMA value, respectively.

The suppression of oxidative burst with N-f-5HT on all the stimuli applied indicated an interference with a process involved in different mechanisms of neutrophil activation. The CL detected resulted from the activation of superoxide-generating NADPH oxidase, which is dependent on the phosphorylation of the proteins due to PKC [1]. N-f-5HT dose-dependently decreased PKC activation, suggesting interference with protein phosphorylation and NADPH oxidase activation. This suggests a similar mechanism of action described for diferuloylmethane on neutrophils [25], confirmed by Jančinová et al. [20]. As PKC isoforms α and βII directly participate in the activation of neutrophil NADPH oxidase [14], their blockade could 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 [24, 37], lipooxygenase [4] and, particularly, phospholipase A_2 [23]. This was demonstrated for many cationic amphiphilic drugs [29].

In light of the presented results, N-f-5HT isomers are interesting naturally occurring compounds with the pharmacological effect of inhibiting respiratory burst of human neutrophils at extracellular and intracellular compartments. This substance is easy to synthesize, and our results can inspire other researchers to characterize this substance from a pharmacotoxicological point of view and test it in several models, like those for ischemia-reperfusion, inflammation, tumor growth, diabetic complications, etc.

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