

ORIGINAL ARTICLE

Pharmacological intervention with oxidative burst in human neutrophils

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ABSTRACT

In this study we investigated the effect of five therapeutically used drugs and four natural polyphenolic compounds on the mechanism of oxidative burst of human neutrophils concerning their participation in the generation of reactive oxygen species (ROS). The compounds investigated decreased the oxidative burst of whole blood in the rank order of potency: N-feruloylserotonin > quercetin > curcumin > arbutin > dithiaden > carvedilol. The generation of intracellular reactive oxygen species in isolated neutrophils decreased in the same rank order, while carvedilol was ineffective. Scavenging of extracellular oxygen radicals followed the rank order of potency: N-feruloylserotonin > curcumin > quercetin > dithiaden. Arbutin and carvedilol had no effect. All compounds tested increased the activity of caspase-3 in cell-free system indicating a positive effect on apoptosis of neutrophils. Activation of protein kinase C was significantly decreased by dithiaden, curcumin, quercetin and N-feruloylserotonin. Carvedilol, dithiaden, quercetin and arbutin reduced activated neutrophil myeloperoxidase release more significantly compared with their less pronounced effect on superoxide generation. The presented results are indicative of pharmacological intervention with neutrophils in pathological processes. Of particular interest was the effect of natural compounds. Intracellular inhibition of oxidative burst in isolated neutrophils by the drugs tested and natural antioxidants has to be further analysed since ROS play an important role in immunological responses of neutrophils.

KEY WORDS: human neutrophils; oxidative burst; therapeutical drugs; natural antioxidants

Introduction

Professional phagocytic cells play a central role in defending the host against microorganisms by producing reactive oxygen species (ROS) *via* the NADPH oxidase enzyme complex (Raad *et al.*, 2009). A number of therapeutically used drugs and natural compounds with antioxidative properties have been demonstrated to interfere with oxidative burst of human whole blood and isolated neutrophils (Nosál *et al.*, 2015, 2014, 2011, Jančinová *et al.*, 2015, 2009). When appropriately stimulated by a variety of stimuli, they undergo dramatic physiological and biochemical changes. These are resulting in phagocytosis, chemotaxis and degranulation with the activation of ROS production in a metabolic pathway known as respiratory burst (O'Dowd *et al.*, 2004). These radicals are involved in

the elimination of pathogens, however an excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep apnea, and other diseases (Droge 2002) and result in damage of surrounding tissues (Halliwell and Whiteman 2004, Lonkar and Dedon 2011). In addition, the intracellular oxidants fulfill a regulatory role and participate in the initiation of phagocytosis and immunological processes (Holmdal *et al.* 2016).

Materials and methods

Chemicals

Arbutin (hydroquinone β -D-glucopyranoside), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, 3,3',4',5,6-pentahydroxyflavone), PMA (phorbol-4 β -12 β -myristate-13 α acetate), luminol, isoluminol, superoxide dismutase, dextran (average MW 464,000), luciferase (from firefly *Photinus pyralis*),

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gallic acid, cytochalasin B, curcumin, o-dianosidine and D-luciferinsodium salt were from Sigma Aldrich Chemie GmbH (Steinheim, Germany), horseradish peroxidase (HRP), catalase, cytochrome C and Folin Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany), Lymphoprep (density 1.077 g/ml) from NycomedPharma AS (Oslo, Norway).

H₁ antihistamines: Dithiaden: 4-[3-(dimethyl-amino) propylidene]-4,9-dihydrothieno [2,3-b]benzo[e] thiepin, Zentiva, Czech Republic; Brompheniramine (BPA): 3-(4-bromophenyl)-N,N-dimethyl-3-pyridine-2-yl-propan-1-amine, Weyth, Austria; Chlorpheniramine (CPA): 3-(4-chlorophenyl)-N,N-dimethyl-3-pyridin-2-yl-propan-1-amine, Glaxo, Smithkline, Great Britain; Pheniramine (PA): N,N-dimethyl-3-phenyl-3-pyridin-2-yl-propan-1-amine, Hoechst, Germany; carvedilol (±)-[3-(9H-carbazol-4-yloxy)-2-hydroxypropyl][2-(2-methoxyphen-oxo) ethyl]am from La Roche (Mannheim, Germany), N-feruloylserotonin (N-f-5HT) was isolated from the seeds of *Leuzea carthamoides* (Wild DC) by solvent extraction (Harmatha *et al.*, 2007). All other chemicals used were of analytical grade and obtained from commercial sources.

Blood collection and neutrophil separation

Fresh human blood was obtained at the bloodbank by venepuncture from healthy male volunteers (aged 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 was registered at the National Transfusion Service NTS-KRA/2012/SVI. Human neutrophils were isolated from whole blood, as described previously (Jančinová *et al.*, 2009, Nosál *et al.*, 2011). 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 was layered on Lymphoprep (3 ml) and centrifuged (500×g, 30 min, 22°C). After hypotonic lysis and centrifugation (500×g, 10 min, 22°C), the neutrophils were resuspended in Ca²⁺-Mg²⁺-free PBS. After counting, they were adjusted to a final concentration of 10⁵ 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 phorbolmyristate acetate (PMA 0.05 μM). CL was measured in 250 μl samples consisting of 50 μl aliquots that contained blood (50× diluted), luminol (250 μM), compounds tested (10 μM) and phosphate buffer (Jančinová *et al.*, 2009). Horseradish peroxidase (HRP 8 U/ml) was added to the system and maintained a sufficient extracellular peroxidase concentration. The effect of the compounds tested on extra- and intracellular reactive oxygen

species (ROS) production was measured in unstimulated and PMA (0.05 μM)-stimulated neutrophils (5×10⁵ per sample) by isoluminol/luminol-enhanced CL. 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 (2000 U/ml) (Drábiková *et al.*, 2009). 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 3600 s (whole blood) or 1800 s (isolated neutrophils) (RLU×s; RLU, relative light units).

Protein kinase C activation

Phosphorylation of protein kinase C (PKC) isoenzymes α and βII was detected (for details see Jančinová *et al.*, 2009). Isolated human neutrophils (5×10⁶) were incubated at 37°C with the compounds tested for 1 min, stimulated with PMA (0.15 μM, 1 min) and lysed by the addition of solubilization buffer. After sonication on ice, the samples were centrifuged to remove unbroken cells, the supernatant was boiled for 5 min with sample buffer and the samples were loaded on 9.8% SDS polyacrylamide gels. Proteins were separated by electrophoresis, transferred to Immobilon-P Transfer Membrane (Millipore Corp., USA). From the two strips taken, one was detected for PKC and the second for β-actin, which represented the internal control. Membrane strips were blocked for 60 min with 1% bovine serum albumin in Tris buffered saline. The membranes were subsequently washed six times with TBS and incubated for 60 min with the secondary antibody conjugated to horse-radish peroxidase. The activity of horseradish peroxidase was visualized using Enhanced Chemiluminescence Western Blotting Detection Reagents (Amersham, UK), followed by autoradiography. The optical density of each PKC band was corrected by the optical density of the corresponding β-actin band.

Superoxide determination

Superoxide formation (Pečivová *et al.*, 2007) was measured in isolated human neutrophils as superoxide dismutase inhibitable reduction of cytochrome C. The suspension (1×10⁶ neutrophils in PBS with 0.9 mmol/l CaCl₂, 0.5 mmol/l MgCl₂) was preincubated for 5 min at 37°C with the compounds tested (10 μM) and subsequently stimulated by PMA (1 μM) for 15 min at 37°C. Controls were included for the effect of the stimulus of the drugs tested on cytochrome C reduction. After centrifugation (4200×g for 4 min at 4°C), absorbance was measured at 550 nm in a microplate spectrophotometer (Labsystem Multiscan RC, MTX LabSystems, Inc., Vienna, Wyoming, USA).

Myeloperoxidase (MPO) release

For determination of MPO release (Pečivová *et al.*, 2007), neutrophils were preincubated with cytochalasin B (5 μg/ml) for 5 min at room temperature. Then the neutrophils (2×10⁶ /sample) were preincubated with the

compounds tested (10 μ M) in a shaker bath at 37 °C for 5 min, followed by 15 min exposure to PMA (1 μ M). The activity of MPO was assayed in the supernatant after centrifugation (983 \times g for 10 min at 4 °C) by determining the oxidation of *o*-dianisidine in the presence of hydrogen peroxide in a microplate spectrophotometer (Labsystem Multiscan RC, MTX LabSystems, Inc., Vienna, Wyoming, USA) at 450 nm.

Recombinant caspase-3 activity

To determine the caspase-3 activity, a modified method was applied (Perečko *et al.*, 2010). Briefly, the cleavage of the Z-DEVD-amino-luciferin substrate by caspase releases amino-luciferin. The subsequent reaction with luciferase was detected by CL. The light production was measured in the Luminometer Immunotech LM-01T. According to the manufacturer's instructions, 10 μ l of 0.1 IU caspase was added to 20 μ l aliquots of the compounds tested (10 μ M) and buffered solution. Finally, 50 μ l of Caspase-Glo 3/7 reagent was added and the mixture was measured for 60 min to determine caspase-3 activity.

Statistical analysis

Data represent the mean \pm 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 $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Results

The effect of medicinal drugs and natural antioxidants on oxidative burst of whole human blood is demonstrated in Figure 1. In isomolar concentration (10 μ M), the greatest inhibitory effect on stimulated ROM (reactive oxygen

metabolites) was demonstrated by natural antioxidants arbutin, curcumin, quercetin and N-f-5HT. Carvedilol and dithiaden showed an effect of more than 50%. Phenyramine compounds from the H1 antihistamines significantly decreased CL only for halogenized derivatives chlor- and bromphenyramine.

Figure 2 demonstrates the effect of the compounds tested on extra- and intracellular ROS generation of isolated neutrophils stimulated with PMA. Carvedilol in 10 μ M concentration proved less effective on both ROS formation. All compounds tested affected more the activity of extracellular ROS. Most active were natural compounds, dithiaden was the most effective from the group of therapeutic drugs. The effect of phenyramines was significant at 10% level. The generation of intracellular ROS was decreased in the rank order of potency: curcumin > N-f-5HT > quercetin. Arbutin was without effect. From the therapeutically used drugs dithiaden was effective at 20%, the other drugs tested were without effect.

From the group of the compounds tested at 10 μ M concentration, dithiaden, curcumin and quercetin significantly increased the activity of caspase. Arbutin and N-f-5HT did not affect caspase activity (Figure 3).

The activity of stimulated neutrophil protein kinase C was significantly affected with dithiaden in 10 μ M concentration, decreasing it by less than 20% (Figure 4).

Figure 5 demonstrates the activity of superoxide anion (SO⁻) and the release of myeloperoxidase in isolated neutrophils treated with dithiaden (DIT), carvedilol (CARV), arbutin (ARB) and quercetin (QUER) in 10 μ M concentration stimulated with PMA (1 μ M). The generation of SO⁻ was decreased at 10% level by carvedilol and arbutin. The liberation of myeloperoxidase was decreased by dithiaden by 60%, quercetin by 25% and by carvedilol and arbutin close to 10%.

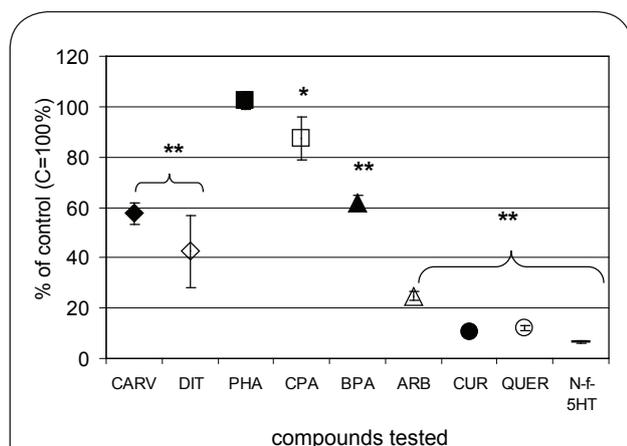


Figure 1. Effect of carvedilol (CARV), dithiaden (DIT), arbutin (ARB), curcumin (CUR), quercetin (QUER), phenylalanine (PHA), chlorphenylalanine (CPA), bromphenylalanine (BPA) and N-feruloyl serotonin (N-f-5HT) in 10 μ M concentration on chemiluminescence of whole human blood stimulated with phorbolmyristate acetate (PMA – 0.5 μ M). n=6, $x \pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$

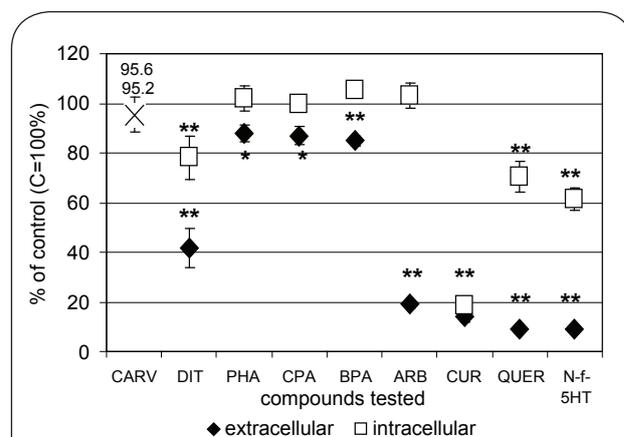


Figure 2. Effect of carvedilol (CARV), dithiaden (DIT), arbutin (ARB), curcumin (CUR), quercetin (QUER), phenylalanine (PHA), chlorphenylalanine (CPA), bromphenylalanine (BPA) and N-feruloyl serotonin (N-f-5HT) in 10 μ M concentration on chemiluminescence of isolated human neutrophils stimulated with phorbolmyristate acetate (PMA – 0.5 μ M) measured extracellularly and intracellularly. n=6, $x \pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$

Discussion

We reported previously that in *in vitro* studies a number of therapeutically used drugs as well as natural antioxidants significantly decreased oxidative burst in human blood and in isolated human neutrophils (Drábiková *et al.*, 2009; Jančinová *et al.*, 2016; Nosál *et al.*, 2015; Pečivová *et al.*, 2007; Perečko *et al.*, 2010). Polymorphonuclear leukocytes in a process of phagocytosis represent the primary defense line against invading microorganisms (Bjorkman *et al.*, 2008). Moreover, these cells play a crucial role in the initiation and development of pathological events, like ischemia-reperfusion, hyperuricemia, lupus, acute respiratory distress syndrome, rheumatoid arthritis and others, when generated increased the amount of reactive oxygen and nitrogen species and proteolytic enzymes (Fialkow *et al.*, 2007; Cascao *et al.*, 2009; Wight *et al.*, 2010). A number of therapeutically used drugs act as free radical scavengers by decreasing the activity of ROS produced by neutrophils *in vivo* as well as *in vitro*. These are represented by the H1-antihistamines (Drábiková *et al.*, 2006a,b; Jančinová *et al.*, 2006 a,b; Nosál *et al.*, 2005) and carvedilol (Nosál *et al.*, 2009). The pyridol derivative stobadine (Drábiková *et al.*, 2007) and a number of natural compounds like glucomannans (Drábiková *et al.*, 2009), arbutin (Jančinová *et al.*, 2007), curcumin (Jančinová *et al.*, 2009) and N-feruoyl serotonin (Nosál *et al.*, 2015) demonstrated antioxidant activity on stimulated neutrophils *in vitro*. Many pharmacologically active drugs, like glucomannans, significantly decreased oxidative burst of blood phagocytes in the model of rat adjuvant polyarthritis (Drábiková *et al.*, 2009; Jančinová *et al.*, 2007).

All drugs tested in the concentration of 10 μ M increased the activity of caspase-3 in cell-free system. Since the activation of caspase-3 indicates apoptotic activity in cells and tissues (Pan *et al.*, 2007), it is suggested that the effect of dithiaden, arbutin, curcumin and quercetin decreased the life span of neutrophils like pterostilbene (Perečko *et al.*, 2010). This effect might be useful in rheumatoid arthritis with prolonged apoptosis of activated neutrophils (Hallett *et al.*, 2008).

Dithiaden and quercetin in the concentration tested significantly decreased stimulated activity of neutrophil PKC by 38 and 26%, respectively. The effect of curcumin and N-f-5HT was less significant with 26 and 17 percent decrease, respectively. Phosphorylation of PKC represents an activation marker for NADPH-oxidase phosphorylation responsible for generation of ROS in cells and tissues (Pan *et al.*, 2009; Klink *et al.*, 2009).

A further analysis is required to verify the ratio between the inhibition of extra- to intracellular ROS by the drugs tested. The role of ROS is currently changing from being seen as toxic agents that will promote inflammation toward a more complex view with ROS as crucial regulators of immune and inflammatory pathways. Now that ROS have been shown to regulate also autoimmune responses, it is of interest to study which pathways are oxidation dependent and how this affects autoimmunity (Holmdahl *et al.*, 2016). Spontaneous ROS production

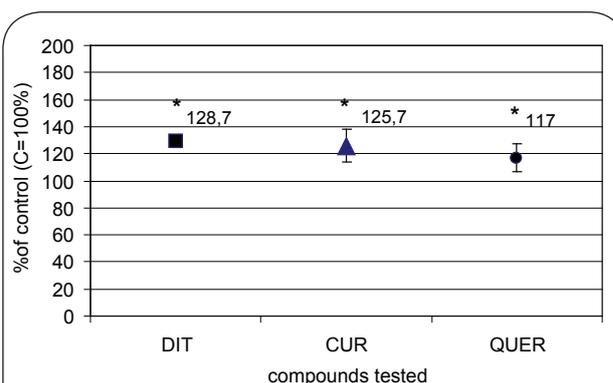


Figure 3. Caspase 3 activity in cell-free system in the presence of dithiaden (DIT), curcumin (CUR) and quercetin (QUER) in 10 μ M concentration. n=6, $\bar{x} \pm \text{SEM}$, * $p \geq 0.05$

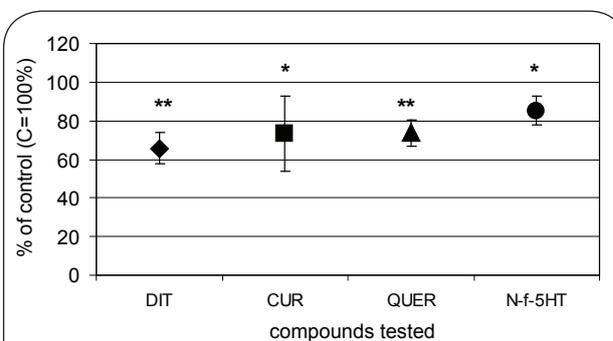


Figure 4. Proteinkinase C (PKC) activity in isolated human neutrophils after incubation with dithiaden (DIT), curcumin (CUR), quercetin (QUER) and N-feruloyl serotonin (N-f-5HT) in 10 μ M concentration stimulated with phorbolmyristate acetate (PMA). n=4, $\bar{x} \pm \text{SEM}$, * $p \leq 0.05$, ** $p \leq 0.01$

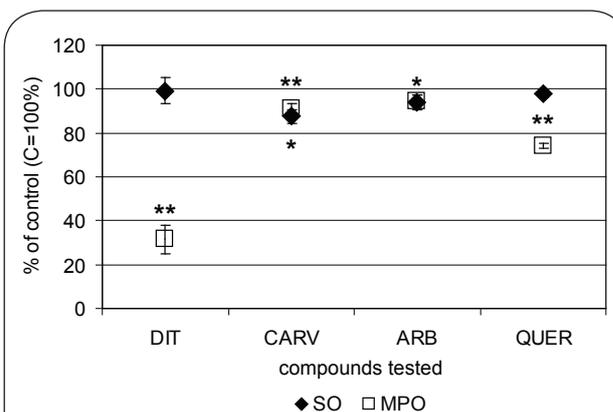


Figure 5. Effect of dithiaden (DIT), carvedilol (CARV), arbutin (ARB) and quercetin (QUER) in 10 μ M concentration and subsequent stimulation with PMA, on superoxide (SO⁻) generation and myeloperoxidase liberation in isolated human neutrophils. n=6, $\bar{x} \pm \text{SEM}$, * $p \leq 0.05$, ** $p \leq 0.01$

by normal phagocytes may suppress proinflammatory gene transcription. These nonphagosomal, intracellular radicals are key suppressors of inflammation. Exactly how nphROS could dampen inflammatory reactions is still unknown. Cellular oxidants are known to influence a variety of cell signaling pathways, some of which are highly involved in inflammation, by alteration of cellular

redox balance (Bylund *et al.*, 2010.). Intracellular inhibition of oxidative burst in isolated neutrophils by the drugs tested and natural antioxidants has to be further analyzed since ROS play an important role in immunological responses of neutrophils.

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