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Selective inhibition of extracellular oxidants liberated from human neutrophils—A new mechanism potentially involved in the anti-inflammatory activity of hydroxychloroquine

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ABSTRACT

Hydroxychloroquine is used in the therapy of rheumatoid arthritis or lupus erythematosus. Although these diseases are often accompanied by activation of neutrophils, there are still few data relating to the impact of hydroxychloroquine on these cells.

We investigated the effect of orally administered hydroxychloroquine on neutrophil oxidative burst in rats with adjuvant arthritis. In human neutrophils, extra- and intracellular formation of oxidants, mobilisation of intracellular calcium and the phosphorylation of proteins regulating NADPH oxidase assembly were analysed.

Administration of hydroxychloroquine decreased the concentration of oxidants in blood of arthritic rats. The inhibition was comparable with the reference drug methotrexate, yet it was not accompanied by a reduction in neutrophil count. When both drugs were co-applied, the effect became more pronounced. In isolated human neutrophils, treatment with hydroxychloroquine resulted in reduced mobilisation of intracellular calcium, diminished concentration of external oxidants and in decreased phosphorylation of Ca^{2+}-dependent protein kinase C isoforms PKCα and PKCβII, which regulate activation of NADPH oxidase on plasma membrane. On the other hand, no reduction was observed in intracellular oxidants or in the phosphorylation of $p40^{phox}$ and PKCδ, two proteins directing the oxidase assembly to intracellular membranes.

Hydroxychloroquine reduced neutrophil-derived oxidants potentially involved in tissue damage and protected those capable to suppress inflammation. The observed effects may represent a new mechanism involved in the anti-inflammatory activity of this drug.

1. Introduction

Neutrophil NADPH oxidase (NOX2/$gp91^{phox}$) is the first identified and the best studied member of the NOX enzyme family. During activation, the cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac2 translocate to the plasma membrane or to membranes of specific granules, where they associate with the membrane-bound components $p22^{phox}$ and $gp91^{phox}$ to assemble the catalytically active oxidase [1,2]. Two different pools of NADPH oxidase products can be formed in neutrophils, extra- and intracellular, as the oxidase components $p22^{phox}$ and $gp91^{phox}$ were identified both in the plasma membrane (5%) and in granular membranes (95%) [3].These distinct oxidants are differently involved in neutrophil functions [4] and thus their pharmacological modulation should be considered separately.

Different mechanisms control the assembly of the oxidase, depending on the membrane in which the oxidase operates. The directing of cytosolic components to intracellular membranes was found to be mediated by several factors, namely by $p40^{phox}$, phosphoinositide PI(3)P and phosphoinositide 3-kinase (class III PI3K), by the isoform δ of protein kinase C (PKCδ) and cytoskeleton. The activation of NADPH oxidase on plasma membrane occurs without the participation of $p40^{phox}$ and is regulated by phosphoinositides $PI(3,4,5)P_3$, $PI(3,4)P_2$ formed through activation of class I PI3K and by the action of PKCβI, PKCβII and PKCζ [4,5].

Activated NADPH oxidase transfers an electron from NADPH to molecular oxygen, generating superoxide anion. This precursor of other reactive oxygen species (ROS) is immediately transformed into hydrogen peroxide (H_2O_2), spontaneously or through enzymatic dismutation by superoxide dismutase. Interaction between H_2O_2 and superoxide anion can give rise to the hydroxyl radical, one of the most powerful oxidants. Moreover, H_2O_2 is a substrate of myeloperoxidase, which catalyses its transformation into highly toxic molecules such as hypochlorous acid, chloramines and tyrosyl radicals [6–8]. These oxidants, capable to damage proteins, lipids and DNA, are directly involved in neutrophil host defence reactions and through formation

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of neutrophil extracellular traps, they can intensify and prolong bactericidal activity [9]. Moreover, neutrophil-derived oxidants can enhance inflammation by regulation of transcription factors and signal transduction pathways via cellular redox balance [10]. Finally, prolonged or excessive formation and liberation of NADPH oxidase products may increase the risk of tissue damage, block resolution and lead to permanent inflammation [11,12].

On the other hand, reactive oxygen species can stimulate neutrophil apoptosis and in this way act as anti-inflammatory agents [13,14]. The protective role of ROS and their capability to diminish inflammation was confirmed by hyper-inflammatory responses found in patients with chronic granulomatous disease. Phagocytes of these patients, deficient in $p47^{phox}$ or $gp91^{phox}$, displayed severely depressed production of oxidants, accompanied by an increased transcription of pro-inflammatory genes and by elevated cytokine release [4,15]. Recent findings suggest that oxidants keeping inflammation under control are formed inside neutrophils. A patient with $p40^{phox}$ deficiency was reported to exhibit substantially decreased intracellular ROS formation and to suffer from granulomatous colitis—a condition indicative of an inability to limit inflammatory reactions; extracellular oxidants were released normally and he had no history of recurrent infections [16]. Moreover, abnormalities of the gene encoding $p40^{phox}$ were shown to be associated with Crohn's disease and rheumatoid arthritis, which gives further support to the idea that intracellular ROS may act as anti-inflammatory agents [4,17].

All these data are confirming the dual role of neutrophil-derived oxidants—their direct contribution to tissue damage as well as involvement in intracellular signalling and capability to suppress inflammatory diseases. Since the optimum therapy is expected to minimise tissue damage without reduction of the physiological function of neutrophils, pharmacological agents eliminating preferentially extracellular ROS are of particular importance.

Hydroxychloroquine is a drug widely used in the treatment of rheumatoid arthritis or systemic lupus erythematosus, while the therapy is considered to be well-tolerated, safe and applicable to children or during pregnancy. The renewed interest in this old substance arose from its pronounced anti-inflammatory and immune-modulatory effects as well as from the recently revealed beneficial actions, such as reduced risk of thrombosis and diabetes, improvement of lipid abnormalities, anti-HIV and anti-tumour activities [18,19]. Despite the fact that hydroxychloroquine is applied in diseases connected with chronic inflammation, its effect on neutrophils has not yet been elucidated in detail. Nevertheless, neutrophils and neutrophil-derived oxidants participate substantially in the mechanisms that drive the onset of chronic inflammation—by inducing tissue damage and by modulating activities of other immune cells [11,20,21]. Under in vitro conditions, the effect of hydroxychloroquine on superoxide anion liberation was studied by Hurst et al. [22,23]. Since these authors assumed an interference with NADPH oxidase in specific granules of neutrophils, we analysed effects of hydroxychloroquine separately on extra- and intracellular formation of oxidants as well as on the phosphorylation of $p40^{phox}$, an oxidase component essential for intracellular ROS formation. Moreover, the phosphorylation of protein kinase C isoforms involved in oxidase activation and mobilisation of intracellular calcium were determined and the effect of hydroxychloroquine on neutrophils primed by experimental arthritis was evaluated.

2. Materials and methods

2.1. Chemicals and solutions

Hydroxychloroquine sulphate was purchased from Acros Organics (Geel, Belgium), methotrexate Ebewe 10 mg/ml from EBEWE Pharma Ges.m.b.H. (Unterach, Austria). *Mycobacterium butyricum* in incomplete Freund's adjuvant was obtained from Difco Laboratories (Detroit, MI, USA). Luminol, isoluminol, PMA (4β-phorbol-12β-myristate-13α-acetate), superoxide dismutase, dextran (average MW 464 000 kDa), hydrogen peroxide, Ca^{2+} ionophore A23187 and the protease inhibitor cocktail were from Sigma-Aldrich Chemie (Deisenhofen, Germany). HRP (horseradish peroxidase) and catalase were obtained from Merck (Darmstadt, Germany) and lymphoprep (density 1.077 g/ml) was purchased from Nycomed Pharma AS (Oslo, Norway). Phosphospecific rabbit anti-human antibodies versus PKC isoforms and versus $p40^{phox}$ were obtained from Cell Signalling Technology (Danvers, MA, USA). Secondary antibody conjugated to horseradish peroxidase (donkey anti-rabbit) and the Lumigen Detection Reagent were supplied by GE Healthcare Life Sciences (Little Chalfont, UK), Fluo-4 AM was from Life Technologies (Grand Island, NY, USA). All other products are available commercially or their origin is mentioned in the text.

Phosphate buffered saline (PBS) contained 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 1.8 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$, pH 7.4. Tyrode's solution consisted of 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l $NaH_2PO_4 \cdot 2H_2O$, 1 mmol/l $MgCl_2 \cdot 6\ H_2O$ and 5.6 mmol/l glucose, pH 7.4.

2.2. Chemiluminescence of whole blood in rats with experimental arthritis

Adjuvant arthritis was induced in male Lewis rats (160–180 g, Velaz, Prague, Czech Republic) by a single intradermal injection of heat-killed *M. butyricum* in incomplete Freund's adjuvant [24]. The study was performed in compliance with Principles of Laboratory Animal Care and was approved by the institutional Ethics Committee and by the State Veterinary and Food Administration of the Slovak Republic. It included healthy animals, arthritic animals without any medication and arthritic animals treated with hydroxychloroquine (40 mg/kg, daily, p.o.), with the reference drug methotrexate (0.4 mg/kg, twice a week, p.o.) or with both drugs. Each experimental group consisted of 10 animals and the substances tested were applied over a period 21 days from arthritis initiation. Then the formation of reactive oxygen species (spontaneous and stimulated with PMA) was determined on the basis of luminol-enhanced chemiluminescence [25,26]. The samples contained 50 μl aliquots of 1.25 mmol/l luminol, 40 U/ml horseradish peroxidase, rat blood diluted 200-times with Tyrode's solution, 0.05 μmol/l PMA (or PBS) and Tyrode's solution. Chemiluminescence was recorded for 1 h in a 96-well microplate luminometer (LM-01T Immunotech) at 37 °C and the area under curve was examined. The number of neutrophils was assessed using a Haemocytometer Coulter Counter. The production of oxidants by one cell was considered the parameter of neutrophil activity.

2.3. Blood collection and isolation of human neutrophils

Fresh blood was obtained at the blood bank by venepuncture from healthy male donors (20–50 years) who had not received any medication for at least 7 days. Erythrocytes were allowed to sediment in 1% dextran solution (35 min, 22 °C). Suspension of leukocytes and platelets was centrifuged (10 min, 170 ×*g*, 22 °C), the pellet was resuspended in PBS, layered on Lymphoprep and neutrophils were separated by centrifugation (30 min, 170 ×*g*, 22 °C). After hypotonic lysis of contaminating erythrocytes, neutrophils were washed and resuspended in PBS. The final suspension contained more than 96% of viable cells, as evaluated by trypan blue, and was used maximally for 2 h—as long as control chemiluminescence kept constant. Neutrophil count was determined by the Analyzer ABX Pentra 60 (Horiba Medical, Irvine, CA, USA), purity of isolated neutrophils was 91.9 ± 0.3%.

2.4. Extra- and intracellular formation of oxidants

Oxidative burst of isolated human neutrophils (5×10^5/sample), initiated by PMA (final concentration FC 0.05 μmol/l), was measured by a chemiluminescence method [27,28]. Oxidants released extracellularly were determined in the system containing isoluminol (FC 5 μmol/l) and HRP (FC 8 U/ml). Intracellular chemiluminescence was enhanced with