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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

luminol (FC 5 μmol/l) in the presence of the extracellular scavengers superoxide dismutase (FC 100 U/ml) and catalase (FC 2 000 U/ml). Concentration of oxidants was evaluated on the basis of integral values of chemiluminescence over 1800 s.

In rats, the complete cell isolation was not achievable due to the similar size of neutrophils and lymphocytes. Therefore, the measurements were performed in 1 000-times diluted whole blood, using the modified method of Rájecký et al [29]. Extracellular chemiluminescence was determined in samples containing blood (0.25 μl), hydroxychloroquine (FC 0.01–100 μmol/l), isoluminol (FC 100 μmol/l), HRP (FC 8 U/ml) and PMA (FC 0.05 μmol/l). When we measured the intracellular chemiluminescence, luminol (FC 100 μmol/l) was used as luminophore and HRP was replaced by superoxide dismutase (FC 100 U/ml) and catalase (FC 2000 U/ml). Chemiluminescence was recorded for 30 min and its integral value was evaluated.

2.5. Chemiluminescence of cell-free system

The scavenging activity of hydroxychloroquine was assessed in cell-free system, containing hydroxychloroquine (FC 0.01–100 μmol/l), HRP (FC 0.5 U/ml), luminol (FC 2.5 μmol/l), and hydrogen peroxide (FC 100 μmol/l). Chemiluminescence was determined for 10 min at 37 °C.

2.6. Phosphorylation of $p40^{phox}$, PKCα, PKCβII and PKCδ

Western blot analysis was performed as previously described [25,28]. Isolated human neutrophils (2.5×10^6/sample) were incubated at 37 °C for 60 s with hydroxychloroquine (FC 10 and 100 μmol/l) prior to addition of PMA (FC 0.15 μmol/l). Stimulation with PMA (5 min) was stopped by using a solubilisation buffer containing protease and phosphatase inhibitors. After sonication and centrifugation, the supernatant was boiled in a sample buffer. Proteins (20 μg per lane) were loaded on 10% polyacrylamide gel, separated by electrophoresis and transferred on a PVDF (polyvinylidene difluoride) membrane. The blot containing transferred proteins was blocked with 1% bovine serum albumin and incubated with primary rabbit anti-human antibodies (phospho-PKC-alpha/betaII Thr638/641 1:1000, phospho-PKC-delta Thr505 1:1000, phospho-p40phox Thr154 1:5 000 or beta-actin antibody 1:4000). After washing, the secondary antibody (donkey anti-rabbit, conjugated to horseradish peroxidase, 1:5000) was applied. The proteins investigated were detected with Lumigen Detection Reagent kit, scanned and measured densitometrically using the free ImageJ programme.

2.7. Flow cytometry measurement of intracellular calcium mobilisation

Isolated human neutrophils in Ca^{2+}–Mg^{2+}-free PBS (2×10^5/sample) were incubated for 9 min with hydroxychloroquine (FC 1, 10 and 100 μmol/l) and with Fluo-4 AM (FC 2 μmol/l). The baseline of Fluo-4 AM ratio was acquired prior to the addition of stimulus (calcium ionophore A23187, FC 1 μmol/l). Neutrophils were gated to record their transient increase of Fluo-4 AM ratio after stimulation. Measurements were performed on the flow cytometer Cytomics FC 500 (Beckman Coulter, Inc., Brea, CA, USA), the total acquisition time was 10 min. Data were analysed off-line using Winmdi 2.8 software. The rise of intracellular calcium concentration was evaluated on the basis of r_{max}/r_{min} value, where r_{max} and r_{min} represent the maximum and the minimum Fluo-4 AM mean ratio, respectively [30,31].

2.8. Data analysis

All values are given as the means ± SEM. The statistical significance of differences between means was established by Student's t-test, *P* values below 0.05 (*) and 0.01 (**) were considered to be statistically significant.

3. Results

Adjuvant arthritis was associated with an elevated concentration of reactive oxygen species in blood and with a remarkable increase in the number of neutrophils (Figs. 1–3). The activity of neutrophils was also increased. In comparison to healthy controls, the amount of oxidants produced by one cell was raised 7 times (spontaneous formation) or 14 times (PMA-stimulated formation). The administration of the reference drug methotrexate significantly reduced all of these changes and the inhibition became more pronounced when hydroxychloroquine was co-applied with methotrexate. The monotherapy with hydroxychloroquine diminished the concentration of oxidants in blood as effectively as did methotrexate, however this effect was not accompanied by a decreased neutrophil count.

Fig. 4 compares the kinetics of extra- and intracellular chemiluminescence produced by isolated human neutrophils in response to PMA stimulation. The external oxidant formation was much more intensive and reached maximum values sooner than did ROS production inside neutrophils. Hydroxychloroquine decreased the concentration of oxidants released from neutrophils, starting with the concentration of 0.01 μmol/l (Table 1). The radicals formed inside neutrophils were not reduced. At concentrations of 0.1–10 μmol/l, hydroxychloroquine slightly amplified the intracellular chemiluminescence. The scavenging effect of hydroxychloroquine was manifested only at the highest concentration used, as indicated by the chemiluminescence measured in cell-free system. The dose-dependent inhibition of extracellular chemiluminescence was observed in rat blood treated with hydroxychloroquine; chemiluminescence produced intracellularly was not affected (Table 2).

To analyse the differences between extra- and intracellular hydroxychloroquine effects, phosphorylation of selected regulatory proteins was examined (Fig. 5). The drug tested did not inhibit either the phosphorylation of $p40^{phox}$ (a component of NADPH oxidase essential for ROS formation inside neutrophils) or the phosphorylation of PKCδ (PKC isoform directing cytosolic oxidase components to intracellular membranes). On the other hand, the 27% decrease was found in the phosphorylation of PKCα and PKCβII, i.e. the PKC isoforms involved in oxidase activation on plasma membranes and regulating the production of extracellular oxidants.

Mobilisation of intracellular calcium represents another mechanism potentially involved in the effect of hydroxychloroquine. As shown by flow cytometry measurements of human neutrophils loaded with

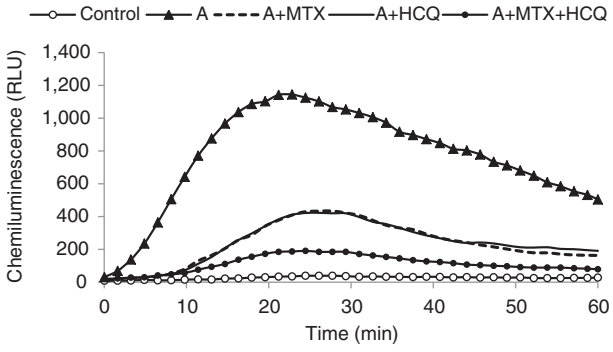

Fig. 1. Kinetics of PMA stimulated oxidant formation in rat blood—representative chemiluminescence curves. Adjuvant arthritis was induced in male Lewis rats by a single intradermal injection of heat-killed *Mycobacterium butyricum*. The study included healthy animals (Control), arthritic animals without any medication (A) and arthritic animals treated with hydroxychloroquine (A + HCQ, 40 mg/kg, daily, p.o.), with the reference drug methotrexate (A + MTX, 0.4 mg/kg, twice a week, p.o.) or with both drugs (A + MTX + HCQ). The substances tested were applied over a period 21 days from arthritis initiation. Then the formation of reactive oxygen species was determined on the basis of luminol-enhanced chemiluminescence in samples containing 1000-times diluted blood, luminol (FC 250 μmol/l), horseradish peroxidase (FC 8 U/ml) and PMA (FC 0.01 μmol/l), RLU—relative light units.

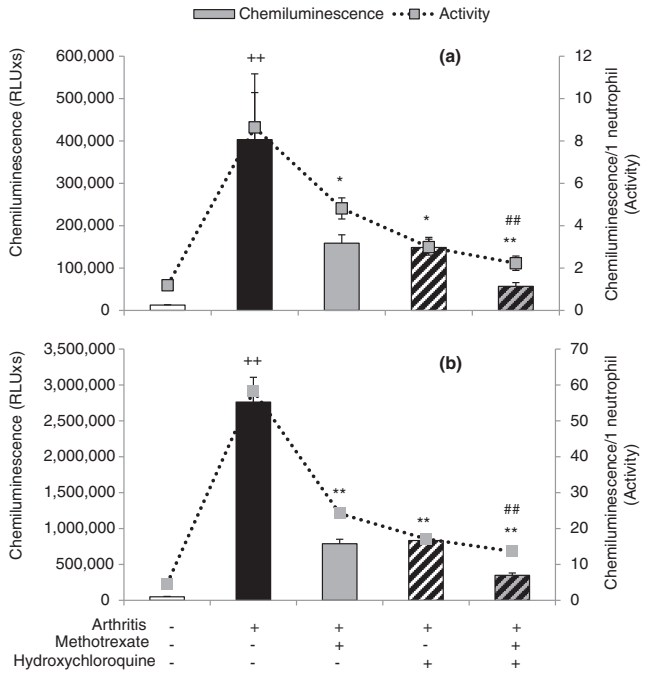

Fig. 2. Spontaneous and stimulated formation of oxidants in blood of arthritic rats. Spontaneous (a) and PMA-stimulated (b) whole blood chemiluminescence measured in healthy and arthritic animals, as well as in arthritic animals treated with hydroxychloroquine (40 mg/kg, daily, p.o.), with the reference drug methotrexate (0.4 mg/kg, twice a week, p.o.) or with both drugs. The substances tested were applied over a period 21 days from arthritis initiation. Then the formation of reactive oxygen species was determined on the basis of luminol-enhanced chemiluminescence in samples containing 1000-times diluted blood, luminol (FC 250 μmol/l), horseradish peroxidase (FC 8 U/ml) and PMA (FC 0.01 μmol/l). Columns represent mean integral values of chemiluminescence (area under curve), the dotted lines show neutrophil activity, i.e. ROS produced by one cell. Arthritic group was compared with control (+), treated animals were compared with arthritic (*), and the group treated with both drugs was compared with the methotrexate group (#). Mean ± SEM, n = 10, $^{*}P < 0.05$, $^{**}P < 0.01$, RLU—relative light units.

Fluo-4 AM, this drug in the concentration of 1, 10 and 100 μmol/l reduced concentration of intracellular Ca^{2+} ions by 49%, 63% and 64%, respectively (Fig. 6).

4. Discussion

Hydroxychloroquine is clinically used in the therapy of autoimmune diseases such as lupus erythematosus or rheumatoid arthritis [18,19],

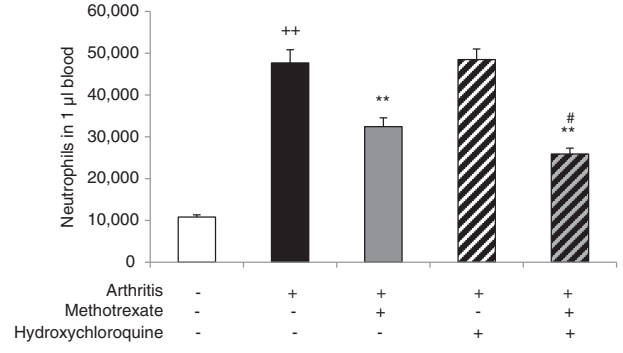

Fig. 3. Number of neutrophils in blood of animals tested. Neutrophil count assessed in blood of healthy and arthritic animals, as well as in arthritic animals treated with hydroxychloroquine (40 mg/kg, daily, p.o.), with the reference drug methotrexate (0.4 mg/kg, twice a week, p.o.) or with both drugs. The substances tested were applied over a period 21 days from arthritis initiation, number of neutrophils was measured by a Haemocytometer Coulter Counter. Arthritic group was compared with control (++), treated animals were compared with arthritic (**), and the group treated with both drugs was compared with the methotrexate group (#). Mean ± SEM, n = 10, $\#P < 0.05$, $^{**}P < 0.01$.

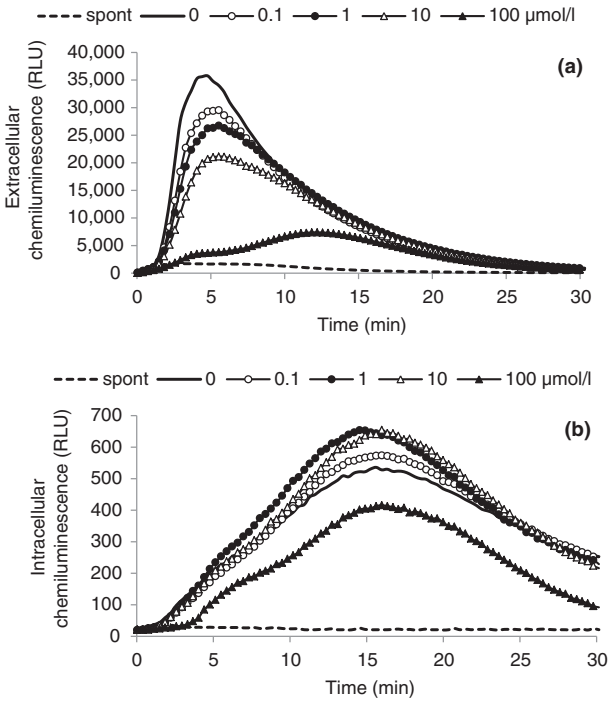

Fig. 4. Effect of hydroxychloroquine on extracellular (a) and intracellular (b) ROS formation in human neutrophils—representative chemiluminescence curves. Chemiluminescence of isolated human neutrophils stimulated with PMA (FC 0.05 μmol/l) in the presence of hydroxychloroquine (FC 0.1–100 μmol/l). Oxidants released extracellularly were determined in the system containing isoluminol (FC 5 μmol/l) and HRP (FC 8 U/ml), the chemiluminescence formed inside neutrophils was enhanced with luminol (FC 5 μmol/l) in the presence of the extracellular scavengers superoxide dismutase (FC 100 U/ml) and catalase (FC 2000 U/ml). RLU—relative light units, spont—spontaneous chemiluminescence.

which are often accompanied by activation of neutrophils [12,20,32]. Nevertheless, there are still few data relating to the influence of hydroxychloroquine on neutrophils modified by inflammation [33]. In the present study, this effect was analysed in rats with adjuvant arthritis, focussing on oxidants produced by neutrophils spontaneously or in the response to PMA stimulation. The measurements were performed in 1 000-times diluted blood, since the complete cell isolation was not achievable due to the similar size of rat neutrophils and lymphocytes. Moreover, using this method blood volumes and sample manipulation were substantially reduced [29]. In Lewis rats, neutrophils represent 50–58% of white blood cells and their number is 12 times higher than that of monocytes [34]. These facts, along with a considerable dilution of blood, minimised the interference of other blood cells with the chemiluminescence measurement. The dose of 40 mg/kg hydroxychloroquine was chosen on the basis of published data [35], as it provided rat plasma concentrations comparable to those found in patients [36], while the effectiveness of lower doses was uncertain in adjuvant arthritis [37,38].

Table 1
Effect of hydroxychloroquine (HCQ) on extra- and intracellular chemiluminescence of isolated human neutrophils stimulated with PMA (0.05 μmol/l) and on the chemiluminescence produced by cell-free system. Percentage of inhibition was calculated on the basis of integral values of chemiluminescence over 1800 s. Mean ± SEM, n = 4–8, $^{*}P < 0.05$, $^{**}P < 0.01$.

HCQ (μmol/l)	Inhibition of chemiluminescence (%)		
	Extracellular	Intracellular	Cell-free system
0.01	10.53 ± 3.69*	- 3.73 ± 1.49	- 0.71 ± 0.08
0.1	13.66 ± 1.96**	- 4.66 ± 1.27*	- 5.57 ± 1.50*
1	15.51 ± 3.55**	- 7.09 ± 1.53*	- 6.07 ± 2.53
10	21.36 ± 4.58**	−16.37 ± 2.23**	3.89 ± 1.27
100	53.68 ± 3.80**	9.74 ± 7.13	45.08 ± 1.76**

Table 2
Effect of hydroxychloroquine (HCQ) on extra- and intracellular chemiluminescence measured in rat blood stimulated with PMA (0.05 μmol/l). Percentage of inhibition was calculated on the basis of integral values of chemiluminescence over 1800 s. Mean ± SEM, n = 9–12, $^{*}P<0.05$, $^{**}P<0.01$.

HCQ (μmol/l)	Inhibition of chemiluminescence (%)	
	Extracellular	Intracellular
0.01	2.87 ± 1.51	−1.25 ± 0.73
0.1	4.22 ± 1.82*	−0.98 ± 0.69
1	3.30 ± 1.20*	−1.11 ± 0.56
10	6.10 ± 1.14**	−1.22 ± 0.78
100	38.75 ± 1.88**	−0.19 ± 0.96

Administration of hydroxychloroquine decreased the concentration of oxidants in blood of arthritic rats. The effect was comparable with that of methotrexate—a drug widely used in the therapy of rheumatoid arthritis—however, it was not accompanied by a reduction in neutrophil count. When both drugs were co-applied, the inhibition became more pronounced, indicating a synergy of several mechanisms of action. The anti-inflammatory activity of methotrexate arises from its ability to inhibit T cell proliferation and cytotoxicity, to decrease recruitment of monocytes and other cells to the inflamed joint, as well as to enhance the release of the endogenous anti-inflammatory mediator adenosine [39]. The latter mechanism is potentially involved in the inhibition of neutrophil oxidative burst [40]. Hydroxychloroquine is expected to decrease neutrophil activity through pro-inflammatory cytokines. The exposition of neutrophils to the effects of TNFα, GM-CSF or LPS results in partial $p47^{phox}$ phosphorylation and partial association of NADPH oxidase components. This configuration, not sufficient for oxidant generation, amplifies the response of neutrophils to subsequent stimulation [41,42]. Since hydroxychloroquine inhibits formation of pro-inflammatory cytokines [43], the observed reduction in spontaneous and stimulated ROS formation may be due to diminished neutrophil priming. Scavenging of oxidants by hydroxychloroquine seems to be less likely, owing to the weak activity of this drug, observed in cell-free system. Accumulation in neutrophils may represent another mechanism involved in the effect of hydroxychloroquine. As a weak base, it can pass through the lipid cell membrane and preferentially concentrate in lysosomes (acidic cytoplasmic vesicles). Once protonated, hydroxychloroquine can no longer freely diffuse and its concentrations within lysosomes may become 100-times higher than therapeutic plasma levels. In phagocytes, cellular levels comparable to those found during anti-rheumatic therapy were achieved by the 60-min treatment with 0.1 mmol/l hydroxychloroquine [19,44,45]. Alkalinisation of acidic intracellular vesicles inhibits a variety of enzymes and cell functions, e.g. activities of acidic proteases and phospholipase A_2, antigen processing and presentation, as well as calcium- and TLR-signalling [18].

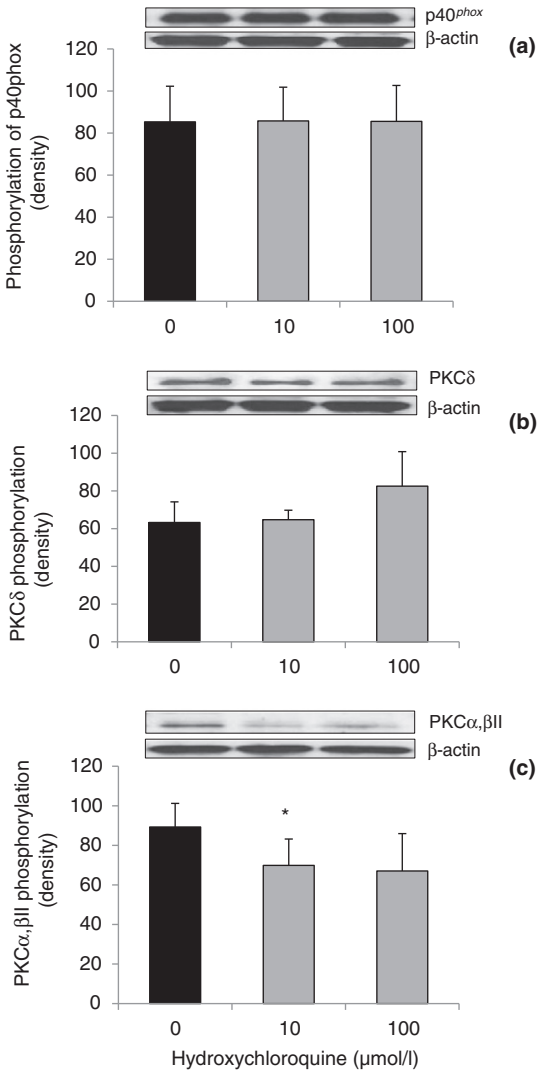

Fig. 5. Phosphorylation of $p40^{phox}$ (a) and protein kinase C isoforms PKCδ (b) and PKCα,βII (c). Isolated human neutrophils were treated with hydroxychloroquine (FC 10 and 100 μmol/l) and stimulated with PMA (FC 0.15 μmol/l). Phosphorylated proteins were separated by gel electrophoresis, transferred on PVDF (polyvinylidene difluoride) membrane and detected with phospho-$p40^{phox}$ (Thr154), phospho-PKC-δ (Thr505) or phospho-PKC-α,βII (Thr638/641) antibodies. Visualisation was made by the secondary antibody conjugated to horseradish peroxidase and by the Lumigen Detection Reagent kit. Phosphorylation is expressed as optical density corrected to β-actin content. Mean ± SEM, n = 5–7, $^{*}P<0.05$.

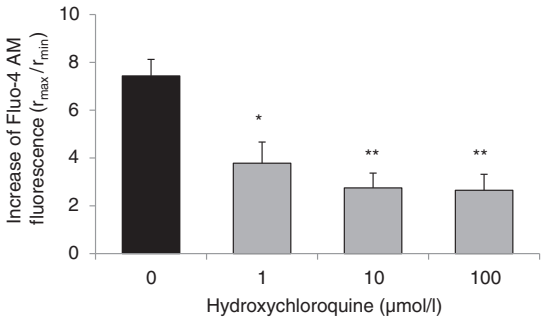

Fig. 6. Effect of hydroxychloroquine on intracellular calcium mobilisation in human neutrophils stimulated with Ca^{2+} ionophore A23187. Isolated human neutrophils were incubated with hydroxychloroquine (FC 1–100 μmol/l) and loaded with the fluorescent probe Fluo-4 AM. The baseline Fluo-4 AM ratio was acquired prior to the addition of A23187 (FC 2 μmol/l). Neutrophils were gated to record their transient increase of Fluo-4 AM ratio after stimulation. Measurements were performed on the flow cytometer Cytomics FC 500, the total acquisition time was 10 min. The rise of intracellular calcium concentration was evaluated on the basis of r_{max}/r_{min} value, where r_{max} and r_{min} represent the maximum and the minimum Fluo-4 AM mean ratio, respectively. Mean ± SEM, n = 6, $^{*}P<0.05$, $^{**}P<0.01$.

Our experiments indicated an interference of hydroxychloroquine with protein kinase C. Inhibition of PKC, potentially involved in the antimalarial activity of chloroquine, was studied on the malarial parasite *Plasmodium falciparum*. The experiments showed no direct interaction with the enzyme, indicating an upstream site of action [46]. One of the potential candidates may be calcium signalling. The interference of hydroxychloroquine with calcium availability was suggested by the partially decreased phosphorylation of Ca^{2+}-dependent protein kinases PKCα and PKCβII, while the activation of calcium independent PKCδ was not reduced. The inhibited intracellular calcium mobilisation was confirmed by flow cytometry measurement, based on the fluorescence of Fluo-4 AM loaded neutrophils. As hydroxychloroquine decreased the rise of Ca^{2+} ions initiated by A23187 (i.e. by a receptor-bypassing stimulus), its direct interaction with calcium mobilisation may be presumed. Similar effects were observed in T cells, where hydroxychloroquine

treatment was associated with inhibition of both calcium release and extracellular calcium influx as well as with a dose-dependent reduction of intracellular calcium stores [47].

Hydroxychloroquine slightly decreased phosphorylation of PKCα and PKCβII on their catalytic region. Since PKCβII participates directly in the activation of neutrophil NADPH oxidase on the plasma membrane [4], its inhibition may be involved in reduced extracellular chemiluminescence of neutrophils treated with hydroxychloroquine. In contrast to the assumption of Hurst et al. [22], hydroxychloroquine did not diminish the intracellular chemiluminescence and did not affect proteins which regulate the formation of oxidants inside neutrophils. Neither phosphorylation of $p40^{phox}$ (an enzyme component allowing the assembly of NADPH oxidase on intracellular membranes [48]), nor phosphorylation of PKCδ (the isoform of protein kinase C which controls the directing of cytosolic oxidase components to intracellular membranes [4]), was inhibited in the presence of this drug. These effects may partially explain the ability of hydroxychloroquine to decrease radicals liberated from human or rat neutrophils with minimal impact on oxidants inside the cell. Considering the protective role of intracellular oxidants and their anti-inflammatory activity [4,16,17], the observed selectivity of hydroxychloroquine effect should be advantageous, particularly as the drug is widely used in the therapy of chronic inflammation. The revealed properties assign hydroxychloroquine to optimal inhibitors which reduce the oxidants potentially involved in tissue damage and protect the oxidants fulfilling a regulatory role.

5. Concluding remarks

Reactive oxygen species produced by neutrophils can exert pro- or anti-inflammatory effects, with respect to their extra- or intracellular location. The optimal antioxidant should thus preferentially decrease external oxidants which may increase the risk of tissue damage, block resolution and lead to permanent inflammation. On the other hand, oxidants inside neutrophils would not be affected, as they are involved in intracellular signalling and can suppress inflammation. The anti-inflammatory drug hydroxychloroquine met these criteria and its antioxidant activity was confirmed in neutrophils modified by experimental inflammation. The interference with neutrophil-derived oxidants may represent a new mechanism potentially involved in the anti-inflammatory activity of this drug.

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