

ORIGINAL ARTICLE

The effects of berberine on reactive oxygen species production in human neutrophils and in cell-free assays

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ABSTRACT

The health benefits of berberine have been recognized for years. Even so, its effects on human neutrophils, the first line of immune defense, have not been reported. The purpose of this study was to investigate the effects of berberine on the human neutrophil oxidative burst. Reactive oxygen species production was analyzed by luminol-enhanced chemiluminescence. The analysis was performed in spontaneous and stimulated (phorbol myristate acetate (PMA) or opsonized zymosan particles (OZP)) whole blood and isolated neutrophils in the presence or absence of berberine. The effects of berberine on oxidant production in cell-free assays were evaluated using luminescence (H₂O₂-peroxidase-luminol) and fluorescence (Oxygen Radical Absorbance Capacity – ORAC) techniques. Berberine decreased the production of reactive oxygen species in human whole blood and isolated neutrophils stimulated with either PMA or OZP with a different efficiency (EC₅₀ was 69 μM and 197 μM for PMA and OZP, respectively). The effect was more pronounced in isolated neutrophils. Cell-free assays showed the antioxidant activity of berberine against peroxy radicals and hydrogen peroxide. Based on our results, we suggest that the effects of berberine on reactive oxygen species production in human neutrophils are due to its antioxidant activity.

KEY WORDS: berberine; chemiluminescence; neutrophil; reactive oxygen species

Introduction

Berberine, a constituent of some traditional Chinese medicinal plants, belongs to the isoquinoline alkaloids, found in high concentrations mainly in the roots, rhizomes, and stem bark of various plants of the *Berberis* species (Birdsall & Kelly, 1997). The metabolic and pharmacological activities of berberine have been widely investigated over the past decade. It has been recorded that berberine promotes antidiabetic activity through stimulating the secretion of insulin, supporting pancreatic β-cell proliferation, and activating insulin/insulin-like growth factor signaling cascades, leading to reduction of blood sugar (Ko *et al.*, 2005). Berberine promotes anti-hyperlipidemia activity through reducing the levels of serum cholesterol, triglycerides and low density

lipoproteins (Kong *et al.*, 2004). It may also rescue neuronal cells after global brain ischemia through the phosphorylation of the protein kinase B /glycogen synthase kinase-3 beta pathway and the inhibition of extracellular signal regulated kinases ERK1/2, c-Jun NH2-terminal kinases and caspase-3 (Simoes Pires *et al.*, 2014). The anti-inflammatory properties of berberine may be due to the inhibition of IκB-α phosphorylation and degradation, which will lead to the suppression of pro-inflammatory cytokines production (Lee *et al.*, 2007). Berberine has also been shown to inhibit nitric oxide (NO) production in lipopolysaccharide treated murine macrophages, by significantly reducing the expression of inducible NO synthase in these cells. Such inhibition of NO production was found to be mediated via an AMP-activated protein kinase sensitive pathway (Jeong *et al.*, 2009). However, there is no published data about the effect of berberine on neutrophils. Professional phagocytes such as neutrophils and macrophages play a key role in innate immunity. They are able to recognize, ingest, and destroy pathogens by oxidative and non-oxidative mechanisms, and represent the first line of immune defense in the human body. In

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response to a variety of stimuli, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present in neutrophils is activated in a phenomenon described as the “respiratory burst”, characterized by the production of the superoxide anion, which gives rise to other forms of reactive oxygen species (ROS) which are among the major microbicidal agents of inflammation during the fight against pathogenic microorganisms (Fontayne *et al.*, 2002).

Therefore, in the current work we investigated the effects of berberine on the production of ROS in whole blood and isolated neutrophils. Opsonized zymosan particles (OZP) and phorbol myristate acetate (PMA) were used as activators having different means of activation. OZP triggers signaling during phagocytosis via complement and/or Fc receptors, whereas PMA directly activates protein kinase C (PKC) (Vasicek *et al.*, 2014). This study also aimed to evaluate the possible free radical scavenging activity of berberine in cell-free systems: Oxygen Radical Absorption Capacity (ORAC) and the hydrogen peroxide – horseradish peroxidase – luminol system (H₂O₂-peroxidase-luminol).

Materials and methods

Chemicals

Berberine hydrochloride (B3251, Sigma) stock solution was dissolved in DMSO (D8418, Sigma), working concentrations were prepared in Hank’s balanced salt solution (HBSS, 14025, Invitrogen), with DMSO below 1%. 2,2-azobis-(2-methylpropionamide) dihydrochloride (AAPH, 440914), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 238813), cobalt(II) fluoride tetrahydrate (399876), dextran (average molecular weight 464,000) (31392), fluorescein sodium salt (F6377), gallic acid (G7384), luminol (123072), phorbol12-myristate13-acetate (PMA) (P8139), and zymosan particles (Z4250) were purchased from Sigma-Aldrich (Germany). Horseradish peroxidase (HRP, 77332) was purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) contained 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na₂HPO₄, 1.5 mmol/l KH₂PO₄, 1.8 mmol/l CaCl₂ and 0.5 mmol/l MgCl₂, pH7.4.

Blood collection and neutrophil isolation

Heparinized (50 IU/ml) blood samples were obtained by venipuncture from healthy male volunteers, with their informed consent and in accordance with the Helsinki Declaration and with approval of the Local Ethic Committee. Human neutrophils were isolated from whole blood. Briefly, after sedimentation with 3% dextran (blood/dextran ratio was 2/1) for 30 minutes at room temperature, the leukocyte rich buffy coat was laid over Histopaque 1077 (10771, Sigma-Aldrich) and spun down 390 g for 30 minutes without brake. Remaining erythrocytes were removed with hypotonic hemolysis. The cell palette was washed with PBS. The final suspension of neutrophils contained more than 96% viable cells (CASY, Roche, Switzerland), and the cells were used within 2h.

Chemiluminescence (CL) assay of whole blood and isolated neutrophils

The CL of human whole blood and isolated neutrophils was evaluated in a 96-well plate luminometer Orion II (Berthold Detection Systems GmbH, Germany). The principle of the method has been previously described (Denev *et al.*, 2014). Briefly, each reaction mixture consisted of 100× diluted whole blood in HBSS, 1 mM luminol, and berberine in various concentrations between 10 and 500 μM. HBSS was used to adjust the total reaction volume to 250 μl. The reaction was started using 25 μl of one of the activators (OZP in final concentration of 0.5 mg/ml or PMA in final concentration of 0.05 μM), and the samples were immediately measured for 60 min at 37 °C.

In the case of isolated neutrophils, the effect of berberine (10–100 mM) on ROS production was measured in the similar conditions, with 250 000 neutrophils per sample, stimulated with the activators OZP (final concentration 0.5 mg/ml) or PMA (final concentration 0.05 μM). The concentration of luminol was 5 μM. The samples were measured at 37 °C for 30 min. The data were acquired as integral values of a CL signal over time.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC was measured according to the method previously described (Denev *et al.*, 2014). Solutions of AAPH, fluorescein, Trolox, and the samples were prepared in a phosphate buffer (75 mM, pH7.4). The reaction mixture contained 170 μl of fluorescein (53.6 nM), 20 μl of AAPH (51.51 mM), and 10 μl of the tested compound (20, 35 and 50 μM). The fluorescein solution and sample were incubated at 37 °C for 20 min, directly in a microplate reader, and AAPH (dissolved in buffer at 37 °C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μl of phosphate buffer was used instead of the tested compounds. Trolox solutions were used for defining the standard curve. The antioxidant activity was expressed in Trolox equivalence.

ROS scavenging in the H₂O₂-peroxidase-luminol cell-free system

H₂O₂ and horseradish peroxidase (HRP) were used as a source of CL signal. According to Banasova *et al.*, aliquots of 50 μl of the tested compounds (1, 10, 50, 100, 250 and 500 μM), HRP (2 U/ml), and luminol (10 μM) were mixed in a 96-well luminescence plate (Banasova *et al.*, 2012). The reaction was started by adding hydrogen peroxide to a final concentration of 100 μM. The final volume of the sample was adjusted to 200 μl by HBSS. CL was measured for 10 min at 37 °C by a Luminometer Immunotech LM-01T (Beckman Coulter). The results were expressed as integral values of CL over 10 min.

Statistical analysis

Experiments with whole blood and isolated neutrophils were measured in triplicates, from 5 independent donors. Cell free assays were measured in triplicates, in three independent measurements. The data represent the

mean \pm SEM, unless stated otherwise. The data were statistically analyzed using a one-way analysis of variance (ANOVA), which was followed by Dunnett's multiple comparison test. Differences were considered to be statistically significant when $p < 0.05$ (*) or $p < 0.01$ (**). The correlation between whole blood and cell-free assays was calculated using the Pearson Correlation Coefficient.

Results

Effect of berberine on neutrophil-derived CL

PMA or OZP were used for the stimulation of ROS production in whole blood and isolated neutrophils. The production of ROS was evaluated using luminol-enhanced CL. Figure 1 shows the effects of berberine

(in the 10–500 μM concentration scale) on whole blood neutrophils stimulated with PMA (0.05 μM) or OZP (0.5 mg/ml). It is obvious that the tested concentrations inhibited CL in a dose-dependent manner at $p < 0.05$, when compared to the control values. The EC_{50} for berberine in whole blood stimulated with PMA was 69 μM , and that stimulated with OZP was 197 μM . Moreover, the immunomodulatory activity of berberine was also evaluated in the isolated neutrophils stimulated with PMA or OZP. The tested concentrations (10–100 μM) of berberine strongly inhibited ROS production in comparison with whole blood CL. The 50 and 100 μM concentrations of berberine were able to inhibit the CL signal to the level of the spontaneous production of ROS (Figure 2). Calculated EC_{50} values are 10 μM for PMA and 5 μM for OZP, respectively.

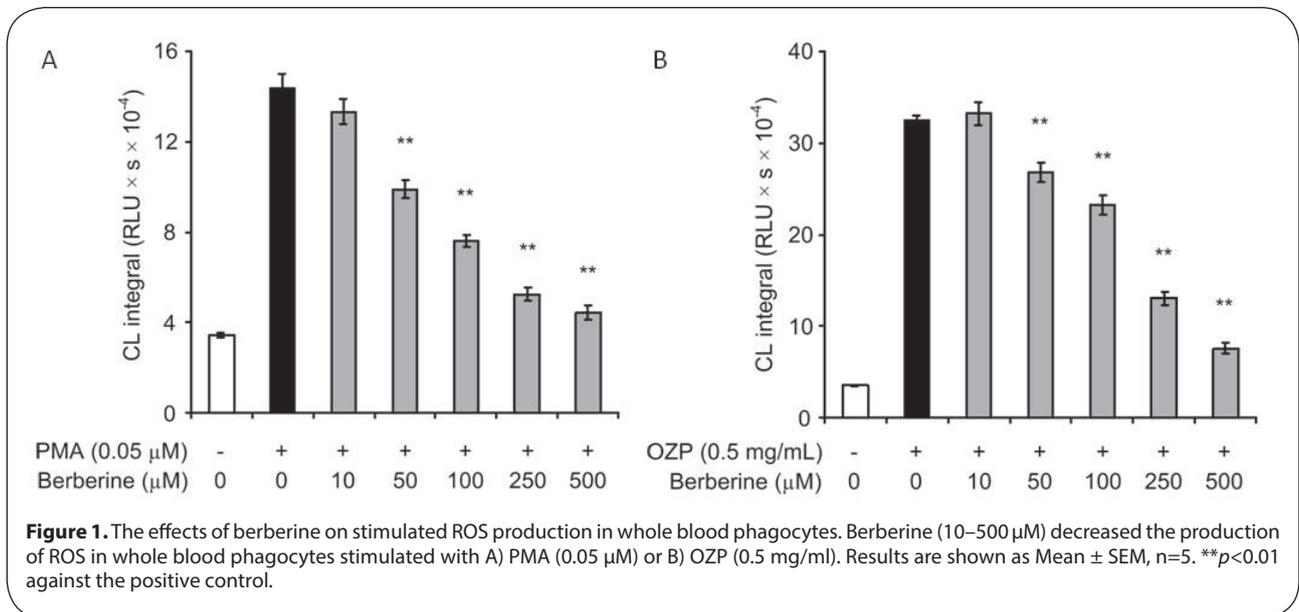


Figure 1. The effects of berberine on stimulated ROS production in whole blood phagocytes. Berberine (10–500 μM) decreased the production of ROS in whole blood phagocytes stimulated with A) PMA (0.05 μM) or B) OZP (0.5 mg/ml). Results are shown as Mean \pm SEM, $n=5$. ** $p < 0.01$ against the positive control.

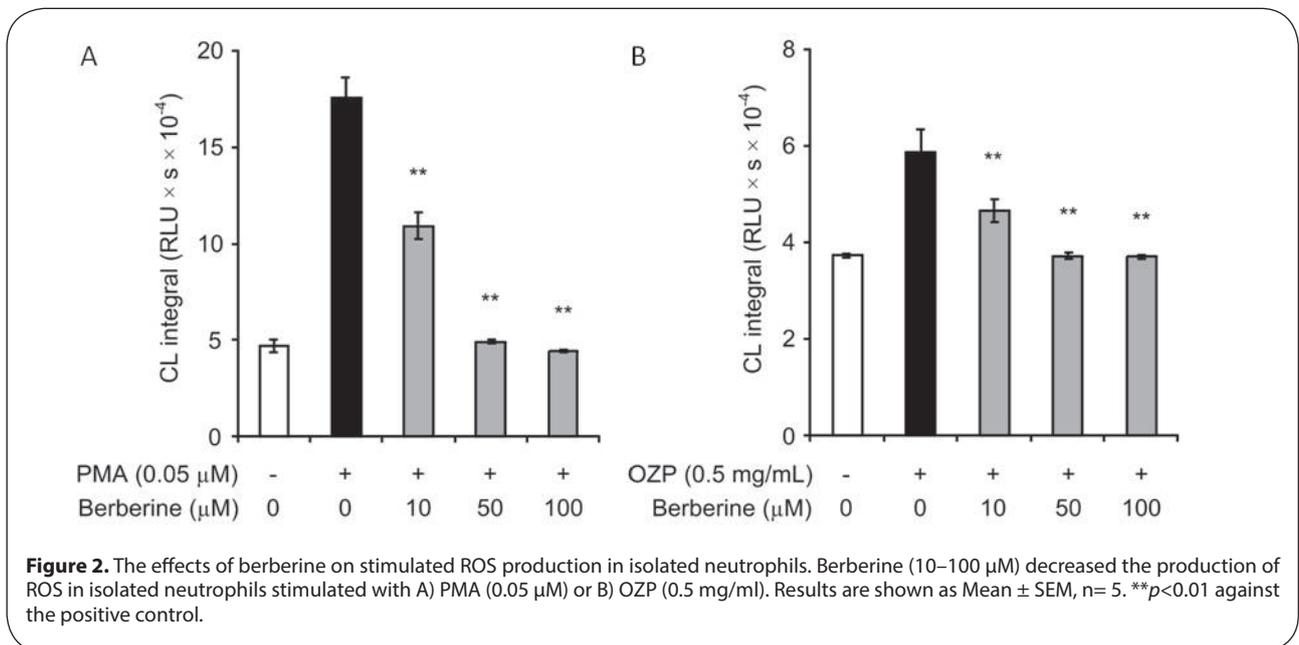


Figure 2. The effects of berberine on stimulated ROS production in isolated neutrophils. Berberine (10–100 μM) decreased the production of ROS in isolated neutrophils stimulated with A) PMA (0.05 μM) or B) OZP (0.5 mg/ml). Results are shown as Mean \pm SEM, $n=5$. ** $p < 0.01$ against the positive control.

Antioxidant activity in a cell-free system

In this part, we focused on the ROS scavenging activity of berberine as one of the possible CL inhibition mechanisms. The antioxidant activity of berberine in the cell-free system was evaluated using ORAC and H₂O₂-peroxidase-luminol assays. ORAC assay provides data to measure the ability to scavenge peroxy radicals, expressed as a trolox equivalent. Here it was found that berberine, in the tested concentrations of 20, 35 and 50 μM, is equivalent to 6.5, 9.2 and 12.1 μM of Trolox.

The CL probe luminol is oxidized by H₂O₂ in the presence of HRP to yield light emission. In this assay, berberine in a concentration dependent manner inhibited the production of ROS, with EC₅₀ value of 96 μM (Figure 3). A significant correlation has been recorded between ROS inhibition by berberine in H₂O₂-peroxidase-luminol assay and whole blood stimulated with PMA or OZP. At *p*<0.01, the R values of Pearson Correlation Coefficient were 0.9939 and 0.9791 for PMA and OZP, respectively.

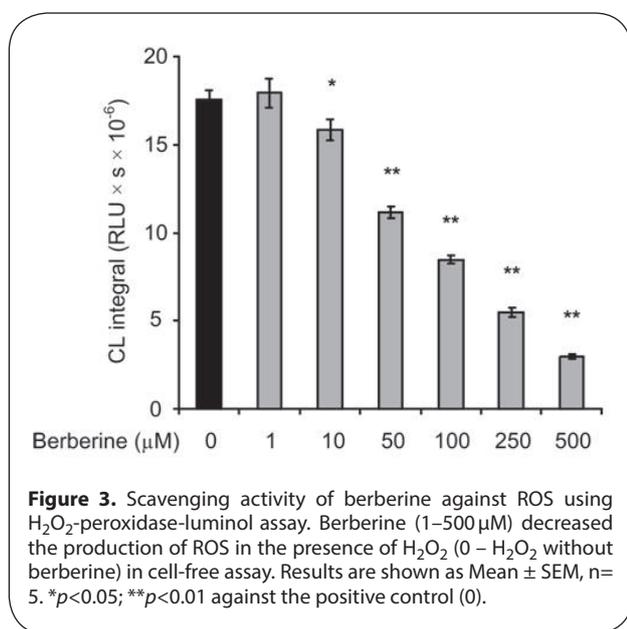
Discussion

Neutrophils are present in high numbers in areas of inflammation, where they constitute the first line of defense against pathogens. Neutrophils act by production of antimicrobial ROS (oxidative burst) and release of proteolytic peptides. However, neutrophils are also implicated in tissue-damaging inflammatory reactions that underlie the pathogenesis and exacerbation of many inflammatory diseases (Fontayne *et al.*, 2002). Our findings showed that berberine inhibited ROS production in whole blood phagocytes and isolated neutrophils stimulated with PMA or OZP. According to EC₅₀, berberine was more effective in decreasing ROS production stimulated with PMA, as compared to OZP in whole blood. This may be due to less ROS production after stimulation with

PMA. Nevertheless, there was no significant difference between the stimuli applied and the chemiluminescence decrease of whole blood, suggesting that berberine may not act only as an extracellular scavenger but also as a suppressor of oxidative burst intracellularly. Comparing the activity of berberine on whole blood and isolated neutrophils, the inhibitory effect of berberine on ROS production was more profound in isolated neutrophils, as compared to whole blood. This may be due to a lack of regulatory molecules and other cell types found in whole blood, which may affect the response of neutrophils to berberine.

Possible mechanisms of CL inhibition by berberine are the effects on the PKC or NADPH oxidase activation pathway or the scavenging activity of berberine. It has been reported that berberine has antioxidant capacity and can scavenge ROS in *in vitro* and *in vivo* systems (Yokozawa *et al.*, 2004; Luo & Fan, 2011). To elucidate the mechanisms how berberine influenced the production of ROS in neutrophils we performed cellular and cell-free experiments. PMA stimulates ROS generation by phosphorylation of PKC isoenzymes α and β II, which directly participate in the activation of neutrophil NADPH oxidase (Fontayne *et al.*, 2002). OZP binds to surface opsonin (either complement or Fc) receptors on phagocytes and triggers a signaling cascade that leads finally to the activation of PKC, with subsequent activation of NADPH oxidase. It has been reported that berberine could reduce oxidative stress by attenuating the expression level of NADPH oxidase, particularly gp91phox (subunit of NADPH oxidase), which was a major source of ROS production in macrophages and endothelial cells upon stimulation with inflammatory stimuli (Tan *et al.*, 2007; Sarna *et al.*, 2010; Cheng *et al.*, 2013). The anti-inflammatory activity of berberine was also associated with its inhibitory effect on mitogen-activated protein kinase signaling pathways that were activated by inflammatory stimuli (Jia *et al.*, 2012), suggesting that p38 was associated with berberine, which was considered important for berberine's efficacy against oxidative stress and inflammation (Lee *et al.*, 2013). Based on our results and published data we were tempted to think about the PKC-NADPH oxidase-ROS pathway. However, our cell-free-assays revealed the scavenging effect of berberine.

Several methods are used for the determination of the antioxidant activity of different extracts and pure compounds. Most of these methods depend mainly on the generation of various radicals (Huang *et al.*, 2005); and, according to Ciz *et al.*, (Ciz *et al.*, 2010), it is recommended that the antioxidant activities of the investigated compounds should be evaluated using different methodologies. In the present study the antioxidant properties of berberine were analyzed via ORAC and H₂O₂-peroxidase-luminol assays. ORAC assay is a good indicator of the free-radical scavenging ability of antioxidants against peroxy radical, using the hydrogen atom transfer mechanism. The tested concentrations of berberine (20, 35 and 50 μM) in this assay were found to have lower antioxidant properties in comparison to trolox. H₂O₂-peroxidase-luminol assay



is a sensitive method for screening antioxidant activity (Yildiz & Demiryurek, 1998), where luminol is oxidized by the peroxidase to produce luminol radicals which then undergo a further reaction to form the endoperoxide which decomposes to form 3-aminophthalate dianion, emitting light on return to its ground state (Thorpe & Kricka, 1986). The present data showed that berberine has the ability to inhibit the ROS generated by this reaction. Therefore, the effect of berberine may be due to the inhibition of luminol radical formation by HRP enzyme inhibition, H₂O₂ scavenging, or by reducing the oxidized HRP enzyme (Georgetti *et al.*, 2003). The inhibition of ROS in H₂O₂-peroxidase-luminol assay could be one of the mechanisms responsible for the inhibition of ROS produced by OZP-stimulated neutrophils (Grael *et al.*, 2010). Moreover, our results showed significant correlation between ROS inhibition by berberine in cell-free assay and whole blood stimulated with PMA or OZP. In conclusion, we suggest that berberine has an anti-inflammatory effect via scavenging neutrophil-derived ROS which are included in the propagation of inflammatory disorders.

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Author contribution

Rami Kassab performed the experiments, Ondrej Vasicek and Tomas Perecko contributed to the experimental planning and design, analyzed and interpreted data, and drafted the manuscript; Milan Ciz and Antonin Lojek conceived the study and coordinated and supervised the experiments.

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