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Antioxidant activity and cytotoxicity as mediators of the neutrophil chemiluminescence inhibition by butylated hydroxytoluene

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The tissue damage found in some inflammatory and autoimmune diseases has been shown to be mediated by an increased activation of neutrophil effector functions. In this study, we investigated the inhibitory effect of the phenolic compound butylated hydroxytoluene (BHT) on reactive oxygen species (ROS) generation by opsonized zymosan-stimulated neutrophils, assessed by luminol- and lucigenin-enhanced chemiluminescence (CL-lum and CL-luc, respectively), and some aspects of its mechanism of action. BHT showed concentration-dependent: (a) inhibitory effect on CL-lum and CL-luc; (b) cytotoxic effect, expressed by increased lactate dehydrogenase leakage by the cells; (c) interaction with neutrophil membranes; (d) ROS scavenger activity. These biological effects were observed in the same range of concentrations ($0-5 \times 10^{-5}$ mol/l). Taken together, the results suggest that inhibition of neutrophil chemiluminescence by BHT was a result of multiple mechanisms, especially a cytotoxic effect probably mediated by BHT interaction with neutrophils membranes, and the ROS scavenging effect.

1. Introduction

Neutrophils are fundamental components of the non-specific immune response, being recruited rapidly to sites of inflammation. These cells respond to injurious agents by phagocytosis, release of preformed granular enzymes and proteins, and production of a range of potentially damaging reactive oxygen species (ROS) (Malech and Gallin 1987; Johnson et al. 1992). A large neutrophil infiltration has been found in a variety of clinical scenarios, such as the acute respiratory distress syndrome, systemic lupus erythematosus, rheumatoid arthritis, ischemia-reperfusion injury and atherosclerosis, where the increased activation of these cells and release of ROS have been implicated in the inflammatory tissue injury (Weiss 1989; Ross 1999; Babior 2000).

One of the strategies for the pharmacological control of neutrophil-mediated tissue damage has been the modulation of ROS generation by the use of antioxidant compounds (Hoult and Payá 1996; Middleton et al. 2000; Ng et al. 2000; Ostrakovitch and Afanas'ev 2001). Some synthetic antioxidants confer substantial benefits on man, not only by the increased preservation and palatability of food products, but also by affording protection against the ROS pathological effects (Parke and Lewis 1992). One of them is the phenolic compound butylated hydroxytoluene (BHT), widely used as preservative in processed foods, pharmaceutics and cosmetics (Richardson and Gangolli 1992). BHT has been shown to protect lipid membranes from mycotoxin-induced oxidative damage (Atroshi et al. 2002) and to prevent low-density lipoprotein oxidation (Cathcart et al. 1985; Panasenko et al. 1991).

The *in vitro* measurement of neutrophil ROS generation by luminol- and lucigenin-amplified chemiluminescence (CL-lum and CL-luc, respectively) has been a widely used technique for investigation of cellular and humoral abnormalities in patients with increased susceptibility to infections, such as chronic granulomatous diseases and myeloperoxidase deficiency (Van Dyke and Castranova 1987), and for evaluation of the role of neutrophil membrane receptors in autoimmune diseases patients (Marzocchi-Machado et al. 2002; Alves et al. 2003; Russo-Carbolante et al. 2005). More recently, amplified-chemiluminescence methods have emerged as new tools for investigation of antioxidant activity of low-molecular weight compounds on neutrophil-mediated ROS generation triggered by different stimuli, e.g. phorbol myristate acetate, *n*-formyl-methionyl-leucyl-phenylalanine, opsonized zymosan and immune complexes (Limasset et al. 1999; Liu et al. 2000; Lucisano-Valim et al. 2002; Kanashiro et al. 2004).

In the present study, we used chemiluminescence-based assays to evaluate the modulatory effect of BHT on the neutrophil ROS generation process. In addition, we investigated some of the mechanisms that could contribute to this biological effect, such as cytotoxicity, free radical scavenger activity and alteration of the neutrophil plasma membrane fluidity.

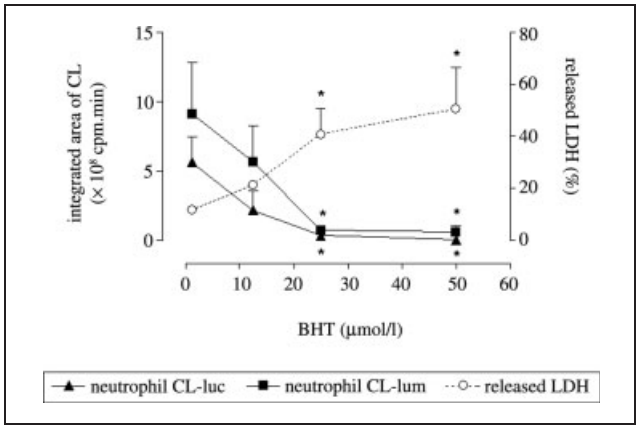

Fig. 1: Double-plot graph showing the concentration-dependent BHT effect on the opsonized zymosan-stimulated neutrophil chemiluminescence (left axis, full lines) and lactate dehydrogenase (LDH) release by these cells (right axis, dotted line, open circle). CL-luc: lucigenin-enhanced chemiluminescence (closed triangle). CL-lum: luminol-enhanced chemiluminescence (closed square). Percentage of released LDH was calculated considering a positive control where complete lysis of 10^6 neutrophils was achieved with 0.2% Triton X-100. Data shown are means ± standard deviation (n = 9). * $p < 0.001$ vs. DMSO-treated control (ANOVA and Dunnett's *post-hoc* test)

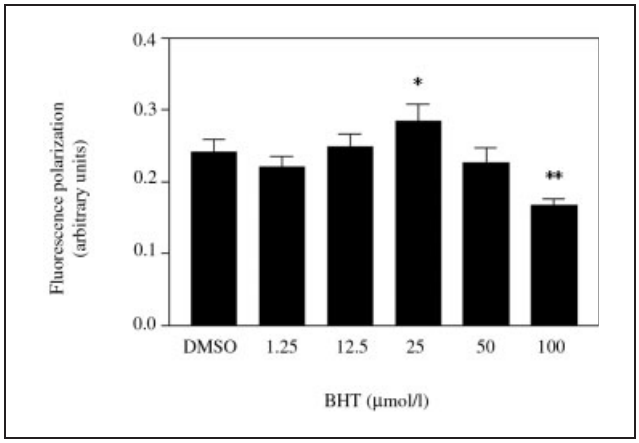

Fig. 2: BHT interaction with neutrophil membranes, evaluated by the degree of DPH fluorescence polarization. The inverse of fluorescence polarization correspond to the value of membrane fluidity. Excitation and emission wavelengths were respectively 377 nm and 431 nm. Values shown are means ± standard deviation (n = 6). *$p < 0.01$ and **$p < 0.001$ vs. DMSO-treated control (ANOVA and Dunnett's *post-hoc* test)

2. Investigations and results

2.1. Effects on neutrophil chemiluminescence

BHT suppressed both CL-luc and CL-lum produced by the opsonized zymosan-stimulated neutrophils (Fig. 1). BHT inhibitory effects were dependent on concentration but significantly different from control in concentrations higher than 25×10^{-6} mol/l.

2.2. Cytotoxicity evaluation

BHT promoted a concentration-dependent increase in the LDH release by neutrophils (Fig. 1) and decreased the cellular viability, as assessed by the Trypan Blue test (data not shown). Both effects were significantly different from the untreated control at concentrations higher than 25×10^{-6} mol/l, showing that BHT was cytotoxic to neutrophils, under the assessed conditions. Moreover, the high correlation between CL-lum ($r^2 = 0.94$) and CL-luc ($r^2 = 0.87$) inhibition by BHT and LDH release by neutrophils indicated a significant contribution of BHT cytotoxic effects to the inhibition of the neutrophil chemiluminescence and ROS generation by these cells.

2.3. Interaction with neutrophils membranes

The DPH fluorescence polarization was used to evaluate BHT interaction with neutrophils membranes. A significant change on fluorescence polarization was also observed at BHT concentrations higher than 25×10^{-6} mol/l, suggesting that interaction between BHT and neutrophils membranes could also be part of its mechanisms of action (Fig. 2). There were no significant changes in DPH fluorescence polarization over the time of incubation, under the assessed conditions (data not shown).

2.4. ROS scavenger activity

BHT showed a concentration-dependent ROS scavenger activity, expressed by inhibition of the chemiluminescence produced by the luminol-H_2O_2-horseradish peroxidase reaction (CL^{HRP}) (Fig. 3). BHT activity was assessed at a similar range of concentrations used for neutrophil chemiluminescence assay, and showed a significant inhibitory effect on CL^{HRP}) at concentrations higher than 12.5×10^{-6} mol/l.

In addition, it was observed a high correlation between CL^{HRP} inhibition by BHT and its inhibitory effect on neutrophil CL-lum ($r^2 = 0.86$) and CL-luc ($r^2 = 0.87$), suggesting a significant contribution of BHT free radical scavenger properties to the neutrophil chemiluminescence inhibition.

3. Discussion

In the present study, the modulatory effect of BHT on ROS generation by opsonized zymosan-stimulated neutrophils was investigated, by using CL-luc and CL-lum assays. The former specifically measures generation of

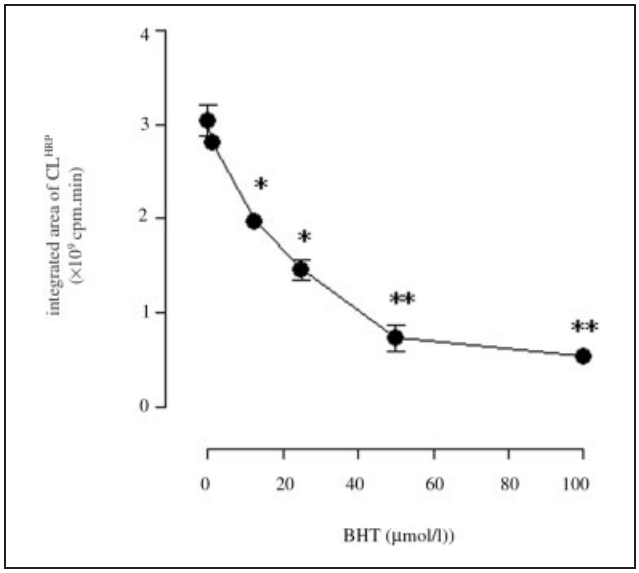

Fig. 3: Concentration-dependent reactive oxygen species scavenger activity of BHT, expressed as inhibition of the chemiluminescence produced by the luminol-H_2O_2-horseradish peroxidase (HRP) reaction (CL^{HRP}). Values shown are means ± standard deviation (n = 6). *$p < 0.01$ and **$p < 0.001$ vs. DMSO-treated controls (ANOVA and Dunnett's *post-hoc* test)

superoxide anion, the first ROS produced by NADPH oxidase-complex of activated neutrophils, whereas CL-lum measures the overall ROS production, especially those involved in the myeloperoxidase-H_2O_2-halide system. The neutrophil respiratory burst is a complex multi-step process, and many mechanisms can be involved in its modulation, such as: (a) cytotoxicity, with a consequent cell death; (b) scavenging of the generated ROS, avoiding oxidation of the chemiluminescence probes; (c) alteration in the plasma membrane fluidity; (d) inhibition of the ROS-generating enzymes, such as NADPH oxidase and myeloperoxidase; (e) interference in the intracellular machinery responsible for the respiratory burst activation, constituted by a series of kinases (Van Dyke and Castranova 1987; Lucisano-Valim et al. 2002). Some of these mechanisms were investigated in the present work.

We observed that BHT inhibited both CL-luc and CL-lum. However, at the same range of concentrations where a significant chemiluminescence inhibition was observed (over 25×10^{-6} mol/l), BHT also had a cytotoxic effect on neutrophils. This suggests that BHT inhibitory effect was in part mediated by cell death. Moreover, at BHT concentrations higher than 25×10^{-6} mol/l, a significant change on neutrophil membrane fluidity was observed. This suggests that BHT interaction with neutrophil membranes affected their integrity, leading to the release of cellular constituents. This cytotoxic effect contributed to the decrease in chemiluminescence, as discussed above. It is also possible that BHT interaction with neutrophils membranes, at lower concentrations, led to small changes on membrane fluidity and a consequent partial impairment of neutrophil functions, such as phagocytosis and chemotaxis, as previously reported by other authors for steroids (Lamche et al. 1990), non-steroidal anti-inflammatory drugs (Abramson et al. 1990) and pentoxifilline (Buescher et al. 1990).

BHT interaction with membranes has been shown to promote protective effects in some situations and deleterious effects in others. For example, BHT was reported to protect cells from killing at low temperatures and erythrocytes from osmolysis (Shertzer et al. 1991), but also to promote cell death by permeabilization of plasma and mitochondrial membranes of rat hepatocytes (Thompson and Moldéus 1988). Interestingly, rabbit neutrophils seemed to be more susceptible to BHT toxic effects than other cell lines, since lower concentrations and incubation time were need to achieve cell death in the present study (Klein and Brusen 1992; Rabini et al. 1999; Reed at al. 2001).

BHT has been reported to exert multifaceted effects on cellular functions, both *in vitro* and *in vivo*. This compound has been shown to enhance carcinogenesis and cause pulmonary and liver damage in animals fed with high doses of BHT and/or long-term diets. On the other hand, BHT is well known for its ability to inhibit the autocatalytic chain reactions which initiate and propagate lipid peroxidation and has been studied for its ability to induce xenobiotic metabolizing enzymes (Thompson and Moldéus, 1988; Richardson and Gangolli, 1992). Regarding the *in vitro* studies, BHT has been reported to protect cell membranes against cytotoxic effects of mycotoxins (Atroshi et al. 2002), and also a variety of cell lines such as Caco-2 cells (Courtois et al. 2003), cultured murine hepatocytes (Han et al. 2004) and *Saccharomyces cerevisiae* (Soares et al. 2003) against free radical-induced oxidative stress. BHT has been shown to prevent low-density lipoprotein oxidation by scavenging superoxide anion (Lamche et al. 1990; Abramson et al. 1990).

We also observed a significant ROS scavenger effect of BHT, which probably contributed to the neutrophil chemiluminescence inhibition since a high correlation was found between these biological activities. However, despite of its ROS-scavenger activity, which could be helpful to prevent oxidative damage, BHT caused a loss of neutrophils integrity, leading to the release of cellular constituents. Therefore, this toxic effect could limit the use of BHT in cellular models involving neutrophils for investigation of *in vitro* antioxidant activity. Such toxic effect could further contribute to increase *in vivo* tissue damage, by the activation and recruitment of more cells to the site of inflammation.

In the present work, the high correlation of the neutrophil chemiluminescence inhibition by BHT with both the cytotoxic and ROS scavenger activity of this compound did not allow us to identify which one of these mechanisms was more relevant to the modulation of the neutrophil function studied. Surprisingly, these biological effects of BHT occurred at the same range of concentrations, and the compound was not effective at non-toxic concentrations.

Taken together, the results of the present work suggest that the inhibitory effect of BHT on chemiluminescence produced by stimulated neutrophils was the result of multiple actions of this compound on the cells: a toxic effect, probably mediated by BHT interaction with neutrophils membranes and the loss of membrane integrity, and scavenging of the ROS generated by these cells.

4. Experimental

4.1. Chemicals

Butylated hydroxytoluene (BHT; 2,6,di-*tert*-butyl-4-methylphenol), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), lucigenin (bis-*N*-methylacridinium nitrate), zymosan A and horseradish peroxidase type VI-A (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LDH Liquiform kit was from Labtest Diagnostica (Lagoa Santa, MG, Brazil). The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Fluka Chemie (Sigma-Aldrich, Steinheim, Switzerland), and hydrogen peroxide from Labsynth (Diadema, SP, Brazil). All other chemicals were of the highest available quality.

4.2. Preparation of neutrophils

Blood from New Zealand White rabbits (females, young adults, weight about 3 kg) was collected from the central ear artery into Alsever solution (v/v), pH 6.1, as an anticoagulant. Neutrophils were isolated by the method described in Lucisano and Mantovani (1984). Cell pellets were suspended in Hanks balanced salt solution (HBSS) pH 7.2 supplemented with gelatin 0.1% (w/v) (HBSS-gel). The preparations contained 80–90% neutrophils and viability higher than 95% was established by Trypan Blue exclusion.

4.3. Preparation of opsonized zymosan

Zymosan A was prepared and opsonized with normal rabbit serum as described by Kabeya et al. (2002). Opsonized zymosan was suspended in HBSS-gel for use.

4.4. Measurement of ROS production by stimulated neutrophils

Concentrations of each component in the final reaction volume of 1 ml are indicated in parentheses. Aliquots of neutrophils (10^6 cells/ml) were mixed with the chemiluminescence probe (2.8×10^{-4} mol/l of luminol or 1.5×10^{-4} mol/l of lucigenin) and BHT or DMSO (control). Luminol, lucigenin and BHT were prepared in DMSO. Reaction tubes were incubated for 3 min at 37 °C. The reaction was started by adding serum-opsonized zymosan (1 mg/ml). Luminol- or lucigenin-enhanced chemiluminescence (CL-lum and CL-luc, respectively) was measured in a luminometer (Auto-Lumat LB953, EG & G Berthold, Bad Wildbad, Germany) for 10 min at 37 °C. The integrated areas of chemiluminescence (area under the curve) were calculated.

4.5. Cytotoxicity evaluation

The cytotoxic effects of BHT on neutrophils were evaluated by measuring the activity of LDH released by the cells into the reaction medium (HBSS-gel) as described by Lucisano-Valim et al. (2002).

4.6. Membrane interaction

A slightly modified method from Beccerica et al. (1988) was used. The final concentration of each component in a reaction volume of 2 ml is indicated in parentheses. Aliquots of 2 ml of neutrophils in HBSS-gel (10^6 cells/ml) were incubated for 15 min at 37 °C with DMSO (control) or BHT. Cells were labeled by addition of DPH (1×10^{-6} mol/l) dissolved in tetrahydrofuran. After a second period of incubation of 30 min at 37 °C, in the dark, DPH fluorescence polarization (P) was measured in a fluorescence spectrophotometer (Hitachi, F-4500, Tokyo, Japan). The excitation and emission wavelengths were 377 nm and 431 nm, respectively. Fluorescence intensity measurements were performed at 22 °C, 5–10 s after exposing the samples to the excitation light. The degree of DPH fluorescence polarization was obtained by the following equation: $P = (I_{\parallel} - I_{\perp} \cdot G)/(I_{\parallel} + I_{\perp} \cdot G)$, where G is an instrument correction factor and $I_{\parallel}$ and $I_{\perp}$ are respectively the emission intensities polarized vertically and horizontally to the direction of the polarized light. Background fluorescence values without DPH in each plane of polarization were very low and they were not subtracted from the samples. BHT and the other constituents of the reaction medium did not influence background fluorescence, at the concentrations tested.

4.7. Evaluation of ROS scavenger activity

The ROS scavenger activity of BHT was evaluated by the modified method of Krol et al. (1994). Briefly, aliquots of H_2O_2 (5×10^{-5} mol/l), luminol (2.8×10^{-4} mol/l) and BHT ($0–1 \times 10^{-4}$ mol/l) or DMSO were incubated at 30 °C for 3 min. The reaction was initiated by adding horseradish peroxidase (0.2 IU/ml). The values in parentheses are final concentrations of each component in a reaction volume of 1 ml. Luminol and BHT were dissolved in DMSO, whereas H_2O_2 and horseradish peroxidase solutions were prepared in 0.1 mol/l phosphate buffer pH 7.4. Chemiluminescence (CL^{HRP}) was measured in a luminometer for 15 min at 30 °C. The integrated areas of chemiluminescence were calculated.

4.8. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA). When indicated by ANOVA, differences between groups were evaluated by Dunnett's test. P-values of less than 0.05 were considered significant. Statistical analysis was performed using the GraphPad Prism, version 3.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

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