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Zinc activates neutrophils' oxidative burst

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Abstract Zinc has been shown to disturb the innate host defense response by interfering in the activation of neutrophils and subsequent oxidative burst, although the exact role of this metal, either as an activator or inhibitor, remains a matter of controversy among research groups. These apparent discrepancies may be due to experimental settings, through modification of zinc availability to neutrophils, or to inaccurate detections of reactive species. Thus, the main objective of the present study was to provide clarification on the role of zinc on the activation of human neutrophils and the subsequent oxidative burst. For that purpose, different detection methods and incubation media were used. The obtained results showed that phosphate buffers (PBS and HBSS) complex with zinc and interfere with the results obtained with this metal. By using Tris-G, it was clearly demonstrated that zinc, at low concentrations (5–12.5 μM), activates NADPH oxidase, mainly via protein kinase C, leading to the formation of superoxide radical ($O_2^{\bullet -}$). Higher concentrations of zinc results on a rapid dismutation of $O_2^{\bullet -}$ to oxygen and hydrogen peroxide, which in turn is used by myeloperoxidase to generate hypochlorous acid (HOCl).

Keywords Human neutrophils · Oxidative burst · Zinc · Protein kinase C · NADPH oxidase

Abbrevations

PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
PKC	Protein kinase C
ABAH	4-Aminobenzoyl hydrazide
PMA	Phorbol myristate acetate
PBS	Dulbecco's phosphate buffer saline
HBSS	Hank's balanced salt solution
Tris-G	Tris buffer saline
APF	6-(4′-Amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid
HRP	Horseradish peroxidase
Amplex Red	10-Acetyl-3,7-dihydroxyphenoxazine
DPI	Diphenyleneiodonium chloride
Gö6983	3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione

Introduction

Zinc is one of the most abundant transition metals in the earth's crust. It is also a relevant trace element in

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the human body, through its involvement in many biological pathways as a constituent of functional proteins, namely as a catalytic component of approximately 300 enzymes (Parkin 2004). The ensuing relevance for cell division and differentiation, as well as for programmed cell death, for gene transcription, for biomembrane functioning, and obviously for many enzymatic activities, has led zinc to be considered as a leading element in assuring correct functioning of various tissues, organs and organ systems in man and animals (Maret and Sandstead 2006; Parkin 2004; Rink and Gabriel 2001; Stefanidou et al. 2006). Most reports in the literature referring to zinc indicate this metal as being relatively non-toxic and that animals, including humans, exhibit considerable tolerance to high zinc intake (Fosmire 1990). Nevertheless, in spite of its safety, some reports suggest that zinc may disturb the innate host defense response, by interfering in the activation of neutrophils and subsequent oxidative burst (Benoni et al. 1998; Lindahl et al. 1998; Takeyama et al. 1995). Furthermore, is has also been argued that zinc inhibits superoxide radical ($O_2^{\bullet -}$) production (DeCoursey et al. 2003; Henderson et al. 1988). We hypothesised that these apparent discrepancies may be related to possible zinc concentration-dependent effects on neutrophils' NADPH activation pathways, or in the quantitative and/or qualitative ensuing formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). A frequently overlooked bias in metal research is that even when similar concentrations of a metal are tested in vitro assays, its effective concentrations may be highly dependent on the metal-complexation potential of the incubation media (Frasco et al. 2005). Zinc research corresponds to a paradigmatic example, as it was previously shown that phosphate avidly complexes zinc (Zhang 1996).

The aim of the present study was to provide clarification on the role of zinc chloride in the activation human neutrophils and the consequent oxidative burst. For that purpose, we used different methods for the identification of the ROS and RNS produced during the oxidative burst, and applied incubation media with different phosphate concentrations, in order to understand the apparent contradictory results reported in literature. Having confirmed the zinc stimulatory effect, the pathways leading to zinc-induced activation of NADPH oxidase were evaluated, underlining its involvement with protein kinase C (PKC) activation.

Materials and methods

Reagents

The following reagents were obtained from Sigma Chemical Co. (St. Louis, USA): Dulbecco's phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS), luminol, cytochrome c from horse heart, potassium chloride, zinc chloride, *N*-nitro-L-arginine methyl ester (L-NAME), diphenyleneiodonium chloride (DPI), 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF), horseradish peroxidase (HRP), 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), 3-[1-[3-(dimethylamino)-propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (Gö6983), and phorbol myristate acetate (PMA). *Tris*(hydroxymethyl)-aminomethane (Tris) and mannitol was obtained from Riedel de Haën (Germany). 4-Aminobenzoyl hydrazide (ABAH) was purchased from Calbiochem (San Diego, CA, USA). The chemical composition of buffering media is presented in Table 1.

Equipment

All determinations were performed in a microplate reader (Synergy HT, BIO-TEK), using colorimetric, fluorimetric or chemiluminometric detection.

Table 1 Composition of incubation media used to test neutrophils (pH = 7.4)

Composition (mM)	PBS	HBSS	Tris-G
$CaCl_2 \cdot 2H_2O$	0.90	1.26	1.26
$MgCl_2 \cdot 6H_2O$	0.49	–	–
KCl	2.68	5.37	5.37
$MgSO_4$	–	0.81	0.81
NaCl	140	140	140
KH_2PO_4	1.21	0.36	–
Na_2HPO_4	8.10	0.34	–
$NaHCO_3$	–	4.17	–
D-Glucose	–	5.55	5.55
Tris	–	–	25

Isolation of human neutrophils by the gradient density centrifugation method

Venous blood was collected from healthy human volunteers by antecubital venipuncture, into vacuum tubes with EDTA. The isolation of human neutrophils from whole blood was performed by the gradient density centrifugation method, using Histopaque solutions 1077 and 1119 in polypropylene 12 mL centrifuge tubes, as reported by Freitas et al. (2008). Briefly, 3 mL of Histopaque 1077 was carefully layered on top of 3 mL of Histopaque 1119 in a 12 mL polypropylene tube. Subsequently, 6 mL of the collected blood was decanted on this discontinuous density gradient. The tube was centrifuged at 890*g* for 30 min at 20°C. Once the centrifugation was complete the neutrophils were carefully removed using a Pasteur pipette. The neutrophils pellet was removed and doubled in volume using PBS without Ca^{2+} and Mg^{2+} (this reduces the viscosity of the Histopaque-neutrophils suspension so that the cells can be centrifuged without the need for high *g* forces). The neutrophils suspension was then centrifuged at 870*g* for 5 min at 4°C. The supernatant was decanted and a mixture of 1.25 mL of PBS without Ca^{2+} and Mg^{2+} + 5.25 mL of sterile distilled water was added to the neutrophils pellet to lyse any remaining red blood cells. The tube was gently inverted for 1.30 min, after which isotonicity was re-established by adding 2.2 mL of 3% NaCl. This suspension was then submitted to a new centrifugation at 870*g* for 5 min at 4°C after which the supernatant was decanted and the neutrophils pellet resuspended in PBS with Ca^{2+} and Mg^{2+}. The time taken from venipuncture to the start of the different assays was about the same in all experiments (2 h). Isolated neutrophils were kept in ice until use. The neutrophils were used from one volunteer per experiment. Cell viability and cell yield were evaluated by the Trypan blue exclusion method, using a neubauer chamber and an optic microscope (40×).

Oxidative burst in human neutrophils

Upon activation, neutrophils initiate an oxidative burst by consuming molecular oxygen, resulting in the formation of $O_2^{\bullet-}$ through the activity of the plasma membrane bound enzyme NADPH oxidase. $O_2^{\bullet-}$ rapidly undergoes either spontaneous- or enzyme-catalyzed dismutation (by microorganism-derived superoxide dismutase, SOD) to hydrogen peroxide and oxygen ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$; Babior 1999). Concomitantly, myeloperoxidase (MPO), a heme protein present in azurophil granules of neutrophils is released, upon cell activation, into the phagolysosome or into the extracellular space. This enzyme considerably contributes to the bactericidal capabilities of these cells via formation of HOCl from H_2O_2 and chloride ions (Hampton et al. 1998; Witko-Sarsat et al. 2000). H_2O_2 also contributes to the formation of hydroxyl radicals ($HO^{\cdot}$), especially in the presence of metal ions (usually iron), through the Fenton or Haber–Weiss reactions (Mladenka et al. 2006). In addition, H_2O_2 can react with HOCl, to yield another highly reactive ROS, singlet oxygen ($^1O_2$; Costa et al. 2008; Miyamoto et al. 2007).

Likewise ROS, the production of RNS is also highly increased in the event of inflammatory processes, and contributes to the antimicrobial activity. Inducible nitric oxide synthase (iNOS) transforms L-arginine into nitric oxide ($^{\cdot}NO$) and L-citrulline. The rapid reaction of $^{\cdot}NO$ with $O_2^{\bullet-}$ leads to the formation of peroxynitrite anion ($ONOO^-$; Denicola and Radi 2005). $^{\cdot}NO$ or $ONOO^-$ could also react with H_2O_2 to generate $^1O_2$ (Costa et al. 2008; Miyamoto et al. 2007).

Some of the above mentioned reactive species were measured by using their ability to react with chemiluminometric, fluorimetric and colorimetric probes as described below.

Luminol amplified chemiluminescence assay

The chemiluminometric probe luminol has been thoroughly studied and used for monitoring reactive species production by neutrophils, namely $O_2^{\bullet-}$, H_2O_2, $HO^{\cdot}$, HOCl, $^{\cdot}NO$, and $ONOO^-$ (Freitas et al. 2009a). The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring the oxidation of luminol by neutrophil-generated reactive species, according to a previously described procedure (Freitas et al. 2008). Reaction mixtures contained neutrophils and the following reagents at the indicated final concentrations (in a final volume of 200 μL): luminol (50 μM when HBSS was used and 500 μM with the other buffers, as proposed by Freitas et al. (2009b), buffer used to test neutrophils (PBS, HBSS, Tris-G), zinc (0–1,000 μM) and

neutrophils (final suspension = 1×10^6 cells/mL). In a set of experiments, the effect of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; 10 or 20 μM, 5 min before zinc) was tested against the highest zinc concentration studied (1,000 μM). The reaction mixture was subjected to continuous soft shaking and incubated at 37°C during the course of the assays. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve, which varied depending on the buffer saline used. Effects are expressed as chemiluminescence arbitrary units/min.

Cytochrome c reduction assay

The cytochrome c reduction assay consists in following, colorimetrically, the reduction of cytochrome c by $O_2^{\bullet -}$ (Freitas et al. 2009a). The measurement of neutrophils' oxidative burst was undertaken by colorimetry, through monitoring the reduction of cytochrome c by $O_2^{\bullet -}$, at 550 nm, according to a previously described procedure, with some modifications (Freitas et al. 2009b). Reaction mixtures contained neutrophils and the following reagents at the indicated final concentrations (in a final volume of 200 μL): cytochrome c (50 μM), buffer used to test neutrophils (PBS, HBSS, Tris-G), zinc (0–1,000 μM) and neutrophils (final suspension = 1×10^6 cells/mL). In a set of experiments, the effect DPI (10 or 20 μM, 5 min before zinc) was tested against the zinc concentration that stimulates neutrophils (5 μM). The reaction mixture was subjected to continuous soft shaking and incubated at 37°C during the course of the assays. Kinetic readings were initiated after a lag time of 5 min. Effects are expressed as absorbance arbitrary units/min.

Amplex Red assay

Amplex Red is a highly sensitive and chemically stable fluorimetric probe for the extracellular detection of H_2O_2 (Freitas et al. 2009a). Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 200 μL): neutrophils (final suspension = 1×10^6 cells/mL) resuspended in Tris-G, zinc (0–1,000 μM), HRP (0.25 U/mL), Amplex Red (25 μM). In a set of experiments, the effect of catalase (which catalyzes the reaction $2H_2O_2 \rightarrow 2\ H_2O + O_2$), (500 U/mL), 5 min before zinc, was tested against the zinc concentration that provided the best response. The excitation and emission wavelengths used were 530 and 590 nm, respectively. The reaction mixture was subjected to continuous soft shaking and incubated at 37°C during the course of the assays. Kinetic readings were initiated after a lag time of 5 min. Effects are expressed as fluorescence arbitrary units/min.

APF (2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid) assay

2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) is a non-fluorescent derivative of fluorescein, which is oxidized by highly reactive ROS and RNS like $HO^{\cdot}$, HOCl and $ONOO^-$ (Freitas et al. 2009a). To distinguish which specie(s) is(are) involved in the measured oxidative burst we used a MPO inhibitor (ABAH), a NOS inhibitor (L-NAME) and a specific scavenger of $HO^{\cdot}$ (mannitol). Reaction mixtures contained neutrophils and the following reagents at the indicated final concentrations (in a final volume of 275 μL): neutrophils (final suspension = 3×10^6 cells/mL) resuspended in Tris-G incubated at 37°C with one of the inhibitors of the enzymes responsible for the generation of reactive species [ABAH (500 μM) or L-NAME (5 mM) or the scavenger of reactive species mannitol (30 mM)]. After 5 min, APF (2 μM) and zinc (1,000 μM) were added. The excitation and emission wavelengths used were 485 and 528 nm, respectively. The reaction mixture was subjected to continuous soft shaking and incubated at 37°C during the course of the assays. Kinetic readings were initiated after a lag time of 5 min. Effects are expressed as fluorescence arbitrary units/min.

Influence of zinc on the activation of neutrophils by phorbol myristate acetate or formyl-methionyl-leucyl-phenylanine

The influence of zinc on the activation of neutrophils by phorbol myristate acetate (PMA) or formyl-methionyl-leucyl-phenylanine (fMLP) was evaluated at the same conditions as above, except that neutrophils (final suspension = 1×10^6 cells/mL or 2×10^6 cells/mL, in case of PMA or fMLP, respectively) were activated by PMA (16 nM) or fMLP (5 μM) at

37°C, just before the addition of zinc (0–1,000 μM). Luminol (500 μM) was used as probe.

Evaluation of PKC contribution to the activation of human neutrophils by zinc

The purpose of this study was to evaluate the contribution of PKC to the zinc stimulatory effect, by using the PKC specific inhibitor 3-[1-[3-(dimethylamino) propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (Gö6983). Neutrophils (final suspension = 1×10^6 cells/mL) were pre-incubated with two concentrations of Gö6983 (250 and 500 nM), for 5 min, at 37°C, before stimulation by zinc. After incubation, the following reagents were added at the indicated final concentrations (in a final volume of 200 μL): luminol (500 μM) and zinc (1,000 μM). The reaction mixture was subjected to soft shaking and the temperature of incubation was maintained at 37°C during the course of the assays. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. Effects are expressed as chemiluminescence arbitrary units/min.

Statistical analysis

Statistics were calculated using GraphPad Prism™ (version 5.0; GraphPad Software). Results are expressed as mean ± standard error of the mean (SEM; from at least five individual experiments, performed in triplicate in each experiment). Statistical comparisons between groups were estimated using the one-way analysis of variance (ANOVA), followed by the Bonferroni's *post-hoc* test. In all cases, P values lower than 0.05 were considered as statistically significant.

Results

Activation of human neutrophils' oxidative burst by zinc

A crucial observation of the present study was that the activation of human neutrophils' oxidative burst by zinc varies according to the incubation media used to resuspend neutrophils and the methodology used to detect reactive species. When HBSS and PBS were used, no stimulation of neutrophils by zinc (0–1,000 μM) was detected by any of the methodologies tested (graphical data not shown). However, when Tris-G was used as incubation medium, a significant activation was observed with the different probes used. It was demonstrated that the concentrations of the metal that originate the oxidative burst signal are highly dependent on the technique used. The chemiluminescence technique revealed that neutrophils were activated at concentrations of zinc from 250 to 1,000 μM (Fig. 1). Under these experimental conditions, the NADPH oxidase inhibitor DPI (10 or 20 μM, 5 min before zinc) completely prevented the oxidative burst induced by zinc at the 1,000 μM concentration. The use of cytochrome c to measure neutrophils' oxidative burst showed that the stimulation of neutrophils by zinc occurs at lower concentrations, 5 to 12.5 μM. Also in this study, DPI (10 μM) decreased the signal close to the blank assay. In turn, higher concentrations of zinc (>100 μM) reduced the oxidative burst mediated signal (Fig. 2). Figure 3 shows that 5–12.5 μM of zinc tendentially increases the amount of H_2O_2 produced by neutrophils. The signal provided by 5 μM of zinc was inhibited by catalase (500 U/mL) showing that Amplex Red is detecting H_2O_2. In contrast, higher zinc concentrations (from 500 to 1,000 μM) decrease the H_2O_2-mediated fluorescence signal. Figure 4

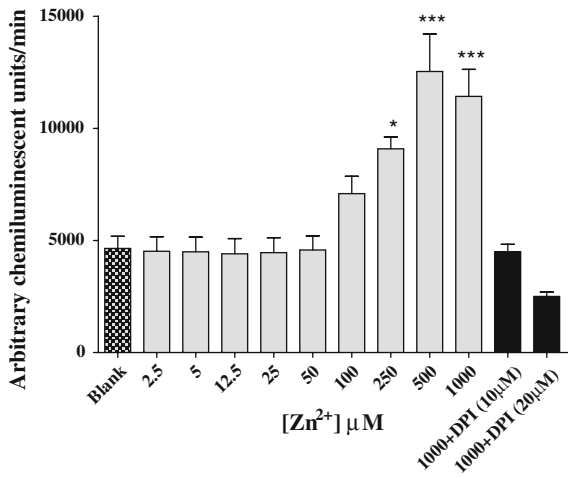

Fig. 1 The effect of zinc on human neutrophils' oxidative burst, measured by luminol and the effect of the NADPH oxidase inhibitor DPI (10 and 20 μM). * $P < 0.05$, *** $P < 0.001$ compared to blank assay. Values are given as mean ± SEM ($n \geq 6$)

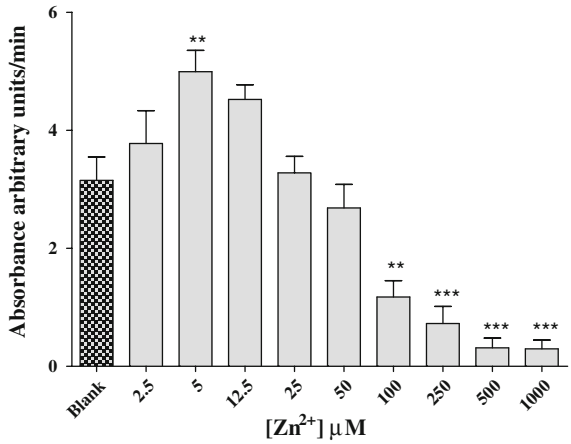

Fig. 2 The effect of zinc on human neutrophils' oxidative burst, measured by cytochrome c and the effect of the NADPH oxidase inhibitor DPI (10 μM); *** $P < 0.001$ and ** $P < 0.01$ compared to blank assay. Values are given as mean ±SEM ($n \geq 6$)

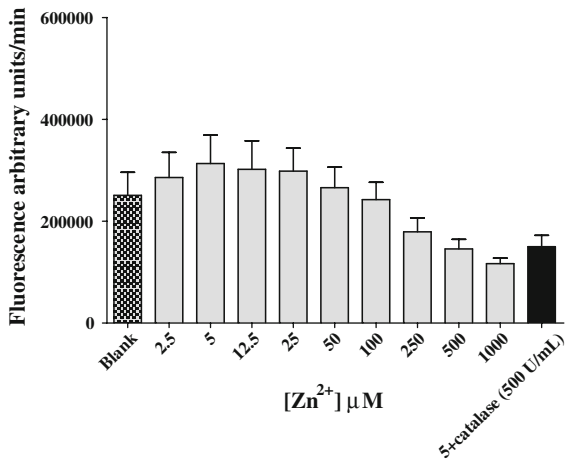

Fig. 3 The effect of zinc on the production of H_2O_2 by human neutrophils, measured by Amplex Red and the inhibitory effect of catalase (500 U/mL). Values are given as mean ± SEM ($n \geq 6$)

reveals that zinc, from 500 to 1,000 μM, significantly increased the APF-mediated fluorescence signal. A MPO inhibitor- ABAH, a NOS inhibitor-L-NAME and a specific scavenger of $HO^{\cdot}$-mannitol were used to distinguish which species is involved on burst induced by zinc. ABAH (5 mM) and mannitol (30 mM) had no effect on neutrophils activated up to 1,000 μM of zinc (graphical data not shown). Only the inhibitor of MPO decreased the signal to values close to the blank assay (without zinc; Fig. 4).

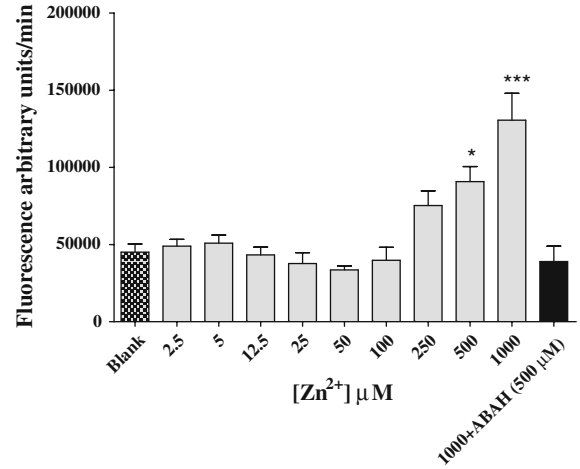

Fig. 4 The effect of zinc on human neutrophils'oxidative burst, measured by APF and the inhibitory effect of ABAH (500 μM) in neutrophils activated by 1,000 μM of zinc. * $P < 0.05$, *** $P < 0.001$ compared to blank assay. Values are given as mean ± SEM ($n \geq 6$)

Influence of zinc on the activation of human neutrophils by PMA or fMLP

Figure 5 shows the results obtained from the study of the effect of zinc on the activation of human neutrophils by PMA (16 nM) or fMLP (5 μM). This study was performed using Tris-G as incubation media and the technique used to measure the oxidative burst was luminol amplified chemiluminescence. Figure 5a shows that zinc had no effect on neutrophils stimulated by PMA. In contrast, Fig. 5b reveals that zinc tendentially augments the signal provided by the activator fMLP. As a representative example, neutrophils stimulated by fMLP originated 10,551 ± 2,076 arbitrary chemiluminescence units while fMLP + 500 μM of zinc increased the signal to 22,108 ± 2,469 (mean ± SEM).

Contribution of PKC to the activation of human neutrophils by zinc

The results obtained from the study of the contribution of PKC to the activation of human neutrophils by zinc shows that the PKC specific inhibitor Gö6983 inhibited the response of neutrophils to zinc in a concentration-dependent manner. At 500 nM, Gö6983 provided a complete inhibition of zinc-induced luminol oxidation (Fig. 6).

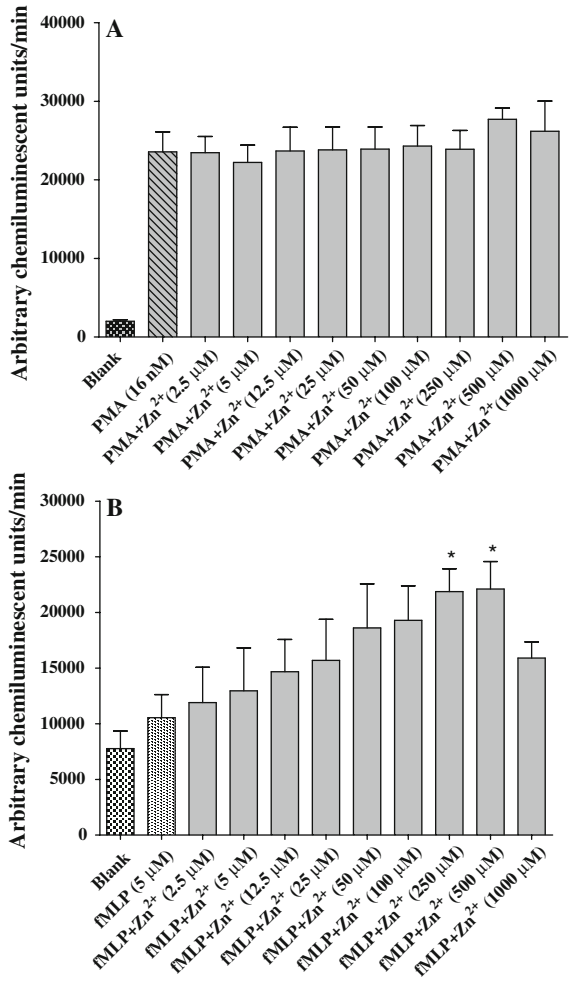

Fig. 5 The effect of zinc on neutrophils' oxidative burst, stimulated by PMA (16 nM) (**a**) or fLMP (5 μM) (**b**), measured by luminol and using Tris-G as incubation media. * $P < 0.05$ compared to blank assay. Values are given as mean ± SEM ($n \geq 6$)

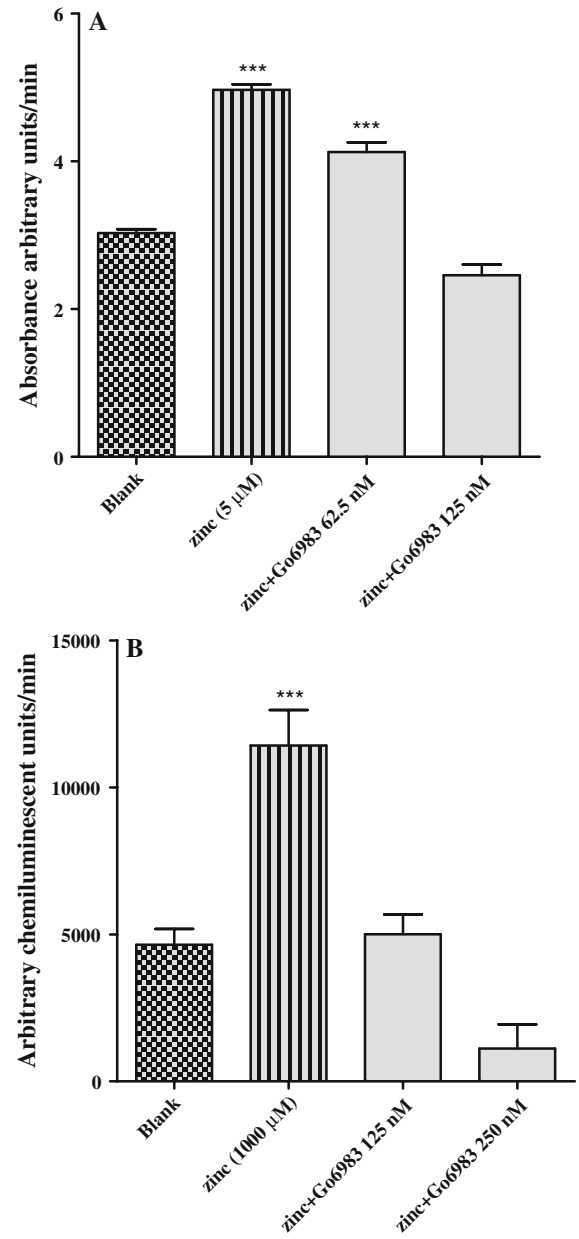

Fig. 6 The inhibitory effect of Gö6983 on human neutrophils'oxidative burst: **a** Induced by zinc (1,000 μM) and using luminol as detection method; **b** Induced by zinc (5 μM) and using cytochrome c as detection method. *** $P < 0.001$ compared to the blank assay. Values are given as mean ± SEM ($n \geq 6$)

Discussion

The results obtained in the present study clearly demonstrate that zinc activates human neutrophils' oxidative burst in vitro. Previous studies provided contradictory results about the effect of zinc on NADPH oxidase activation and consequently on production of reactive species, with some authors indicating that zinc induces neutrophils' oxidative burst (Benoni et al. 1998; Lindahl et al. 1998; Takeyama et al. 1995) while in turn, others argued that zinc inhibits $O_2^{\bullet -}$ production (DeCoursey et al. 2003; Henderson et al. 1988).

It was shown that the ability of zinc to activate human neutrophils in vitro depends on the presence or absence of phosphate in the buffer used in the assay system. Indeed, when phosphate was used in the incubation media (PBS and HBSS), zinc was not

able to induce neutrophils' oxidative burst at any of the concentrations tested (0–1,000 μM). The detection of zinc-induced oxidative burst was only possible when neutrophils were tested in Tris-G, since this incubation media is phosphate-free. When different concentrations of phosphates were added to Tris-G, the activation of neutrophils by zinc decreased in a phosphate-concentration dependent manner (graphical data not shown). Collier (1979) quantified free Zn^{2+} concentration in different buffers. The absorbance change at 620 nm is linear with Zn^{2+} concentration. Phosphate buffers had an Abs = 0.072 and Tris buffer an Abs = 0.585 revealing that despite of Tris-binding some of free Zn^{2+}, phosphate buffers had a much stronger binding effect on zinc when compared with Tris, reducing its effective concentrations. This interference could be somehow foreseen, though it is often ignored by those studying the biological effects of metals. More recently, it was clearly demonstrated that phosphate buffers deeply interfere with the biological activity of several other metals in vitro (Frasco et al. 2005).

Using Tris-G for testing neutrophils, zinc concentrations as low as 5 μM were shown to stimulate neutrophils' oxidative burst. Human plasma free levels of free zinc have been reported to lie around 12–16 μM, for a total body content of zinc around 2–4 g (Rink and Gabriel 2001). Though most of plasma free zinc will be complexed by plasma phosphates, it may reach sufficient concentrations to stimulate neutrophils in case of hypophosphatemia, which is characteristic of certain subgroups of patients, such as those who are hospitalized (2.2–3.1%) or admitted to intensive care units (28.85–33.9%), and those with sepsis (65–80%), chronic alcoholism (2.5–30.4%), major trauma (75%), and chronic obstructive pulmonary disease (21.5%; Brunelli and Goldfarb 2007). In addition, plasma levels of free zinc may increase significantly in specific groups of risk, as it happens with industrial workers from petroleum refineries and diesel distribution, smelters, and chemical plants (Dawson et al. 2000; Muzyka et al. 2002).

The use of different detecting probes to measure zinc-activated oxidative burst also provided different results, which proved to be complementary and determinant for a better understanding of its mechanism of action. The results obtained in the luminol amplified chemiluminescence assay revealed that zinc stimulates neutrophils' oxidative burst from 100 to 1,000 μM ($P < 0.001$). Importantly, the NADPH oxidase inhibitor DPI completely prevented the zinc-activated oxidative burst, indicating the essential role of this enzyme in the observed formation of reactive species. Since the studied reactive species are formed downstream of NADPH oxidase activation, and contribute to luminol chemiluminescence, these results, together with those obtained in the cytochrome c assay system, at lower concentrations, allows the conclusion that zinc activates human neutrophils' oxidative burst in vitro. Zinc had no effect on neutrophils previously stimulated with PMA, which suggests common activating mechanisms. In contrast, when fMLP was used, zinc tendentially increases the signal, suggesting different activating mechanisms. By using cytochrome c, the colorimetric signal increased when neutrophils were challenged with zinc at 2.5–12.5 μM. In contrast, zinc, from 100 to 1,000 μM, decreased the colorimetric signal in the assay system. Interestingly, these results are negatively correlated to those obtained for luminol amplified chemiluminescence, but are in agreement with those obtained by Hasegawa et al. (2000) (Hasegawa et al. 2000), who studied the effects of zinc on lucigenin and luminol-dependent chemiluminescence of human neutrophils stimulated by PMA. These authors reported that zinc, at the concentrations window of 10–1,000 μM, lead to a decrease of the lucigenin-dependent chemiluminescence response, while that of luminol-dependent chemiluminescence increased. That divergent response between cytochrome c and luminol could be explained by with the wider capacity of luminol to detect the different reactive species formed, independently to the interconversion among them. The decrease of the cytochrome c-mediated colorimetric signal when zinc concentrations are increased, could reflect a zinc-dependent faster conversion of $O_2^{\bullet -}$ into the other related reactive species. This radical is quickly converted to H_2O_2 and O_2, either spontaneously or by the enzyme SOD. Considering that, under the present experimental conditions, SOD is not present in the phagosome, this catalysis is probably not enzyme-related. The discrepancy of responses among luminol and cytochrome c emphasizes the importance of the use of different methodologies to detect oxidative burst, in order to avoid erroneous conclusions. Indeed, if only cytochrome c was used the results would indicate, like in other reports

(DeCoursey et al. 2003), that zinc decreases $O_2^{\bullet-}$ production in human neutrophils.

The enzymatic complex NADPH oxidase is considered electrogenic because, when activated, transfers electrons from intracellular NADPH to molecular oxygen, producing $O_2^{\bullet-}$ in the extracellular or intraphagosomal space (Cross and Segal 2004). This charge movement must be compensated to prevent an extreme depolarization that would directly inactivate NADPH oxidase. It is generally assumed that a proton efflux compensates the charge movement induced by electron translocation (DeCoursey et al. 2003; Henderson et al. 1988). However, Ahluwalia et al. (Ahluwalia et al. 2004) have reported that the foremost ionic conductance in PMA-stimulated human neutrophils and eosinophils is a K^+ conductance through large-condutance Ca^{2+} activated K^+ (BK) channels. Neverthless, Femling et al. (2006) and Essin et al. (2007) have recently found no evidence that BK channel inhibition affected $O_2^{\bullet-}$ production by stimulated neutrophils or compromised their killing function. The assumption that the charge induced by electron translocation, through the NADPH oxidase, is largely compensated by proton fluxes is supported by the fact that both zinc and cadmium, known as proton blockers, were also referred to inhibit $O_2^{\bullet-}$ production (Cross and Segal 2004). However, the concentrations of zinc needed to inhibit $O_2^{\bullet-}$ production were higher than those required to block proton channels, raising the possibility that these metal directly inhibits NADPH oxidase or affect other processes (DeCoursey et al. 2003). The results obtained in the present study with luminol and the complete inhibition of oxidative burst by DPI (NADPH oxidase inhibitor) of neutrophils activated by 1,000 μM of zinc, allows the conclusion that zinc (up to 1,000 μM) does not inhibit NADPH oxidase. Results depicted in Figs. 1 and 2 clearly show that the concentrations of zinc that decrease the signal of cytochrome c originated an increased of the chemiluminescence signal, indicating that higher concentrations of zinc can accelerate the dismutation of $O_2^{\bullet-}$. Ahluwalia et al. (2004) studied the interference of zinc in the xanthine-xanthine oxidase system and revealed that 3 mM of zinc induced the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2. Once again, Femling et al. (2006) disagree with that hypothesis. The later authors assessed H_2O_2 production, and observed a progressive and profound reduction of H_2O_2 production. At 1,000 μM, zinc inhibited H_2O_2 release by ~90%. Thus, these authors concluded that the inhibition of H_2O_2 release by zinc was due to reduction of NADPH oxidase activity via inhibition of the proton current. We also performed a study to measure H_2O_2 in zinc-stimulated neutrophils. Our results corroborate those obtained by Femling et al. (2006) since higher concentrations of zinc, from 50 to 1,000 μM, decreased the signal. In contrast, the fluorescence signal tends to be higher with zinc concentrations from 2.5 to 25 μM. However, our conclusions differ from those of Femling et al. (2006), since it seems that higher concentrations of zinc promote the conversion of $O_2^{\bullet-}$ into other reactive species beyond H_2O_2. According to Irato and Albergoni (2005) a greater availability of zinc leads to decrease in the catalase activity. We speculate that this inhibition of catalase augmented H_2O_2 levels, which then may be rapidly transformed in other reactive species. As such, we performed a study with APF, a fluorescent probe that reacts with $HO^{\cdot}$, HOCl and $ONOO^-$. To distinguish which species are involved on the measured oxidative burst, we used a MPO inhibitor- ABAH, a NOS inhibitor-L-NAME and a specific scavenger of $HO^{\cdot}$-mannitol. Only the inhibitor of MPO decreased the signal down to the blank assay (without added zinc), indicating that zinc promotes the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2, which in turn is used by MPO to generate HOCl. These results are in agreement with Hasegawa et al. (2000) who studied the effect of zinc on human neutrophils stimulated by PMA using luminol and an inhibitor of MPO. The authors observed that the MPO inhibitor prevented PMA-induced luminol amplified chemiluminescence response in a zinc concentration-dependent manner, suggesting that HOCl is involved in the process. Also, Takeyama et al. (1995) reported that the addition of zinc, at 1,000 μM, increased the intracellular MPO activity.

Protein kinase C (PKC) can be considered a major mediator in the activation of the NADPH oxidase (Dekker et al. 2000). This enzyme constitutes the primary source of oxidizing agents used by neutrophils to kill invading microorganisms (Freitas et al. 2009a). Neutrophils express only five different PKC isotypes: α, βI, βII, δ, and ζ, which are inhibited by Gö6983 (Remijsen et al. 2006). PKC contain zinc finger structures that are important for its enzymatic activity (Beyersmann and Haase 2001). For that

reason, zinc could have regulatory effects on PKC activity. According to Csermely et al. (1988), zinc activates PKC in T-lymphocytes and causes a translocation to the plasma membrane, a central event in the activation of PKC. Zinc-induced NADPH oxidase activation has already been described (Benoni et al. 1998; Kauppinen et al. 2008; Noh and Koh 2000; Suh et al. 2008; Takeyama et al. 1995). Inclusively, Noh and Koh (2000) reported that zinc induces and activates NADPH oxidase in cortical neurons and astrocytes in a PKC-dependent manner. Our results are in agreement with those obtained by Noh and Koh (2000) since the PKC specific inhibitor Gö6983 decreased the response of neutrophils to zinc in a concentration dependent manner. This finding indicates that zinc induces oxidative burst in neutrophils via activation of PKC, precluding a significant contribution of other cellular pathways for ROS generation mediated by this metal.

In conclusion, in the present study we demonstrated that zinc activates human neutrophils' oxidative burst in vitro, and that the use of phosphates to buffer the incubation media precludes this effect, probably due to the formation of phosphate–zinc complexes. Thus, the use of a phosphate-free incubation media is recommended for evaluating the influence of metals on neutrophils' oxidative burst. In addition, the use of different methods to detect reactive species is essential to avoid erroneous conclusions, as demonstrated in the present study. It was clearly demonstrated that lower concentrations of zinc (5–12.5 μM) activate NADPH oxidase, mainly via PKC, leading to the formation of $O_2^{\bullet -}$. Higher concentrations of zinc results on a rapid dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2, which in turn is used by MPO to generate HOCl.

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References

Ahluwalia J, Tinker A, Clapp LH, Duchen MR, Abramov AY, Pope S, Nobles M, Segal AW (2004) The large-conductance Ca^{2+}-activated K^+ channel is essential for innate immunity. Nature 427:853–858

Babior BM (1999) NADPH oxidase: an update. Blood 93:1464–1476

Benoni G, Cuzzolin L, Marrella M, Crivellente F, Adami A, Bertaso C, Velo G, Milanino R (1998) Neutrophil behavior following exposure to in vivo or in vitro zinc in normal and acutely-inflamed rats: studies on lysozyme secretion, superoxide anion release and platelet adhesion. Inflammation 22:175–189

Beyersmann D, Haase H (2001) Functions of zinc in signalling, proliferation and differentiation of mammalian cells. Biometals 14:331–341

Brunelli SM, Goldfarb S (2007) Hypophosphatemia: clinical consequences and management. J Am Soc Nephrol 18: 1999–2003

Collier HB (1979) Binding of Zn^{2+} by buffers. Clin Chem 25:495–496

Costa D, Gomes A, Lima JL, Fernandes E (2008) Singlet oxygen scavenging activity of non-steroidal anti-inflammatory drugs. Redox Rep 13:153–160

Cross AR, Segal AW (2004) The NADPH oxidase of professional phagocytes–prototype of the NOX electron transport chain systems. Biochem Biophys Acta 1657:1–22

Csermely P, Szamel M, Resch K, Somogyi J (1988) Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. J Biol Chem 263:6487–6490

Dawson EB, Evans DR, Harris WA, Powell LC (2000) Seminal plasma trace metal levels in industrial workers. Biol Trace Elem Res 74:97–105

DeCoursey TE, Morgan D, Cherny VV (2003) The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. Nature 422:531–534

Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, Segal AW (2000) Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. Biochem J 347:285–289

Denicola A, Radi R (2005) Peroxynitrite and drug-dependent toxicity. Toxicology 208:273–288

Essin K, Salanova B, Kettritz R, Sausbier M, Luft FC, Kraus D, Bohn E, Autenrieth IB, Peschel A, Ruth P, Gollasch M (2007) Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity. Am J Physiol Cell Physiol 293:C45–C54

Femling JK, Cherny VV, Morgan D, Rada B, Davis AP, Czirjak G, Enyedi P, England SK, Moreland JG, Ligeti E, Nauseef WM, DeCoursey TE (2006) The antibacterial activity of human neutrophils and eosinophils requires proton channels but not BK channels. J Gen Physiol 127: 659–672

Fosmire GJ (1990) Zinc toxicity. Am J Clin Nutr 51:225–227

Frasco MF, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10:360–375

Freitas M, Porto G, Lima JL, Fernandes E (2008) Isolation and activation of human neutrophils in vitro. The importance of the anticoagulant used during blood collection. Clin Biochem 41:570–575

Freitas M, Lima JL, Fernandes E (2009a) Optical probes for detection and quantification of neutrophils' oxidative burst. A review. Anal Chim Acta 649:8–23

Freitas M, Porto G, Lima JL, Fernandes E (2009b) Optimization of experimental settings for the analysis of human neutrophils oxidative burst in vitro. Talanta 78:1476–1483

Hampton MB, Kettle AJ, Winterbourn CC (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92:3007–3017

Hasegawa H, Suzuki K, Nakaji S, Sugawara K (2000) Effects of zinc on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity in vitro. Luminescence 15:321–327

Henderson LM, Chappell JB, Jones OT (1988) Superoxide generation by the electrogenic NADPH oxidase of human neutrophils is limited by the movement of a compensating charge. Biochem J 255:285–290

Irato P, Albergoni V (2005) Interaction between copper and zinc in metal accumulation in rats with particular reference to the synthesis of induced-metallothionein. Chem Biol Interact 155:155–164

Kauppinen TM, Higashi Y, Suh SW, Escartin C, Nagasawa K, Swanson RA (2008) Zinc triggers microglial activation. J Neurosci 28:5827–5835

Lindahl M, Leanderson P, Tagesson C (1998) Novel aspect on metal fume fever: zinc stimulates oxygen radical formation in human neutrophils. Hum Exp Toxicol 17:105–110

Maret W, Sandstead HH (2006) Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20:3–18

Miyamoto S, Ronsein GE, Prado FM, Uemi M, Correa TC, Toma IN, Bertolucci A, Oliveira MC, Motta FD, Medeiros MH, Mascio PD (2007) Biological hydroperoxides and singlet molecular oxygen generation. IUBMB Life 59:322–331

Mladenka P, Simunek T, Hubl M, Hrdina R (2006) The role of reactive oxygen and nitrogen species in cellular iron metabolism. Free Rad Res 40:263–272

Muzyka V, Bogovski S, Viitak A, Veidebaum T (2002) Alterations of heme metabolism in lymphocytes and metal content in blood plasma as markers of diesel fuels effects on human organism. Sci Total Environ 286: 73–81

Noh KM, Koh JY (2000) Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci 20:RC111

Parkin G (2004) Synthetic analogues relevant to the structure and function of zinc enzymes. Chem Rev 104:699–767

Remijsen QF, Fontayne A, Verdonck F, Clynen E, Schoofs L, Willems J (2006) The antimicrobial peptide parabutoporin competes with p47(phox) as a PKC-substrate and inhibits NADPH oxidase in human neutrophils. FEBS Lett 580:6206–6210

Rink L, Gabriel P (2001) Extracellular and immunological actions of zinc. Biometals 14:367–383

Stefanidou M, Maravelias C, Dona A, Spiliopoulou C (2006) Zinc: a multipurpose trace element. Arch Toxicol 80: 1–9

Suh SW, Hamby AM, Gum ET, Shin BS, Won SJ, Sheline CT, Chan PH, Swanson RA (2008) Sequential release of nitric oxide, zinc, and superoxide in hypoglycemic neuronal death. J Cereb Blood Flow Metab 28:1697–1706

Takeyama Y, Ogino K, Segawa H, Kobayashi H, Uda T, Houbara T (1995) Effects of zinc on production of active oxygen species by rat neutrophils. Pharmacol Toxicol 76:50–55

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000) Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80:617–653

Zhang XG (1996) Corrosion and electrochemistry of zinc. Plenum Press, New York