Metal-induced oxidative burst in isolated human neutrophils

Marisa Freitas a, José L.F.C. Lima a, Graça Porto b, Eduarda Fernandes a,⁎

a REQUIMTE, Departamento de Químico-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal
b Serviço de Hematologia Clínica, Hospital Geral de Santo António, Porto, Portugal

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Experimental evidence have been suggesting that the toxicity of metals may involve inflammatory processes, with subsequent sustained overproduction of pro-oxidant reactive species, leading to indirect toxic effects, namely genotoxicity. Neutrophils, as important mediators of the innate defence systems, may have a hitherto unknown role on these metal-induced adverse effects. Thus, the aim of the present study was to evaluate the putative activation of human neutrophils' oxidative burst by two groups of metals, the first group being able to undergo redox-cycling reactions (iron, copper, chromium and cobalt), whilst the primary route for the toxicity of the second group is not dependent on redox reactions (mercury and cadmium). The generation of reactive oxygen species (ROS) by metal-stimulated neutrophils was measured using the chemiluminometric probe luminol. Appropriate scavengers and metabolizing enzymes were subsequently used to identify the reactive species produced. The modulatory effects of metals on phorbol myristate acetate (PMA)-activated neutrophils were also studied. To evaluate the contribution of protein kinase C (PKC) on metal stimulatory effect, we used the specific inhibitor of PKC Gö6983. The obtained results showed that, in the present experimental conditions, only Cd (II) has the ability to stimulate the production of superoxide radical (O2•−), hydrogen peroxide (H2O2), and hypochlorous acid (HOCl) in isolated human neutrophils. The same metal showed a synergistic effect with PMA. It was also demonstrated that Cd (II) induces neutrophils' oxidative burst mainly via activation of PKC, precluding a significant contribution of other cellular pathways for ROS generation mediated by this metal. These observations indicate that the sustained activation of human neutrophils may contribute for the long term adverse effects on human health mediated by Cd (II).

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1. Introduction

Neutrophils are important factors in the first line of innate defence mechanisms of the organism. Upon activation, one of the most important mechanisms used by neutrophils to protect the organism against the invader is the production of an array of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1–5]. However, the sustained overproduction of reactive species or the impairment of antioxidant defences, may result in a pro-oxidative status of the cells known as oxidative stress, leading to detrimental effects to the host, namely alterations on the normal function of lipids, proteins or DNA [1,3,6]. Oxidative stress is also associated with a number of pathologies and physiological conditions, such as aging, arteriosclerosis and cancer [6–8].

There are several studies reporting the capacity of metals to induce the production of reactive species as well as the involvement of that production on their molecular mechanism of toxicity and carcinogenicity [9–11]. According to Valko et al. [12] potentially toxic metals could be divided into two groups. The first group, represented by iron, copper, chromium and cobalt, is able to undergo redox-cycling reactions, whilst the primary route for the toxicity of the second group, represented by mercury and cadmium, is not dependent on direct redox reactions. The cellular production of reactive species via stimulation by Cd [13–16], Cr [12,17,18], Co [19–21], Fe [12,22,23], Hg [24–26] and Cu [12,27] has already been reported. In neutrophils, relatively little is known about the potential of these metals to induce oxidative burst. Zhong et al. [28] found that neutrophils incubated with Cd did not generate hydrogen peroxide (H2O2). Ciapetti et al. [29] also reported that Cr and Co did not induce an oxidative response from neutrophils. In contrast, Ramafi et al. [21] observed that Co interacts pro-oxidatively with human neutrophils in vitro. Thus, the aim of the present study was to evaluate the putative activation human neutrophils' oxidative burst by metals, namely Fe (II), Fe (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), Hg (II) and Cd (II). The generation of ROS by metal-stimulated neutrophils was measured using the chemiluminometric probe luminol. Appropriate scavengers and metabolizing enzymes were subsequently used to identify the reactive species produced. The modulatory effects of metals on
phorbol myristate acetate (PMA)-activated neutrophils were also studied. To evaluate the contribution of protein kinase C (PKC) on metal stimulatory effect, we used the specific inhibitor of PKC Gö6983.

2. Materials and methods

2.1. Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, USA): luminol, tiron, N-nitro-o-arginine methyl ester (L-NAME), diphenyleneiodonium chloride (DPI), 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrolo-2,5-dione (Gö6983), catalase, iron chloride, iron chloride tetrahydrate, mercury dichloride, mercury chloride, copper chloride, and phorbol myristate acetate (PMA). Cobalt chloride, chromium potassium sulfate dodecahydrate and potassium chromate were obtained from Fluka Chemie GmbH (Steinheim, Germany). Tris(hydroxymethyl)-amino-methane (Tris-G) and mannitol was obtained from Riedel de Haën (Germany). 4-Aminobenzoyl hydrazide (ABAH) was purchased from Calbiochem (San Diego, CA, USA). Cadmium chloride was obtained from Merck (Darmstadt, Germany).

2.2. Methods

All determinations were performed in a microplate reader (Synergy HT, BIORAD), using chemilumimetric detection. Each study corresponds at least to 6 individual experiments and performed in triplicate in each experiment.

2.2.1. 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 K3EDTA. The isolation of human neutrophils was performed by the gradient density centrifugation method as previously reported [30]. Tris-G (Tris—25 mM, CaCl2—1.26 mM, KCl—5.37 mM, MgSO4—0.81 mM, NaCl—140 mM, and n-Glucose—5.55 mM) was the incubation media used, as previously recommended [31].

2.2.2. Cell viability

Cell viability was determined by the trypsin blue exclusion analysis. Neutrophils were incubated with Fe (II), Fe (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), Hg (II) and Cd (II) (0–1000 µM) for 1 h at 37 °C. Twenty µL of neutrophil suspension were added to an equal volume of 0.4% trypsin blue in a microtube and gently mixed. After 2 min on ice, neutrophil number and viability (viable cells excluding trypsin blue) were counted. Assays were performed in triplicate.

2.2.3. Measurement of human neutrophils’ oxidative burst using the luminol amplified chemiluminescence assay

The measurement of neutrophil’s oxidative burst was undertaken by chemiluminescence, by monitoring the oxidation of luminol by neutrophil-generated reactive species, according to a previously described procedure [30]. Luminol reacts with superoxide radical (O2•−), hydrogen peroxide (H2O2), hydroxyl radical (HO•), hypochlorous acid (HOCI), nitric oxide (NO) and peroxynitrite (ONO0•) generated by neutrophils to produce an excited aminophtalate anion that emits light when returning to ground state [1]. Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 200 µL): luminol (500 µM), Tris-G, Fe (II) (0–12 mM), Fe (III) (0–6 mM), Cu (I) (0–50 µM), Cu (II) (0–50 µM), Cr (III) (0–1000 µM), Cr (VI) (0–100 µM), Co (II) (0–100 µM), Hg (I) (0–1 µM), Hg (II) (0–1 µM) or Cd (II) (0–1000 µM) neutrophils (final suspension = 1 × 106 cells/mL). The reaction mixture was subjected to soft agitation and temperature of incubation of 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. In all experiments, this peak was observed at around 30 min. Effects are expressed as chemiluminescence arbitrary units/min.

2.2.4. Inhibitory effects of DPI, tiron, catalase, mannitol, ABAH and L-NAME on the oxidation of luminol by neutrophil-generated reactive species

The purpose of this evaluation was to use specific inhibitors of the enzymes responsible for the generation of reactive species namely DPI (NADPH oxidase inhibitor), L-NAME (nitric oxide synthase (NOS) inhibitor) and ABAH [myeloperoxidase (MPO) inhibitor], specific scavengers of reactive species, namely tiron (O2•− scavenger), and mannitol (HO• scavenger) and catalase (metabolizes H2O2). Reaction mixtures contained the following reagents at the indicated final concentrations (in a final volume of 200 µL): luminol (500 µM); one of the inhibitors of the enzymes responsible for the generation of reactive species [DPI (10 µM), ABAH (125 µM) or L-NAME (1000 µM)] or one of the scavengers of reactive species tiron (500 µM) or mannitol (5000 µM) or catalase (1000 U/mL), Cd (II) (1000 µM) and neutrophils (final suspension = 1 × 106 cells/mL). The reaction mixture was subjected to soft agitation and incubation temperature of 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. In all experiments, this peak was observed at around 30 min after starting the assay. Effects are expressed as chemiluminescence arbitrary units/min.

2.2.5. Evaluation of PKC contribution to the activation of human neutrophils by Cd (II)

The purpose of this study was to evaluate the contribution of PKC to the Cd (II) stimulatory effect, by using the PKC specific inhibitor Gö6983. Neutrophils (final suspension = 1 × 106 cells/mL) were pre-incubated with various concentrations of Gö6983 (62.5, 125 and 250 nM) for 5 min at 37 °C before Cd (II) (1000 µM) stimulation. After incubation, the following reagents were added at the indicated final concentrations (in a final volume of 200 µL): luminol (500 µM) and Cd (II) (1000 µM). The reaction mixture was subjected to soft agitation and incubation temperature of 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.

2.2.6. Evaluation of the influence of metals on the activation of neutrophils by PMA

The influence of metals on the activation of neutrophils by PMA was evaluated at the same conditions as above (2.2.3), except that neutrophils (final suspension = 1 × 106 cells/mL) were activated with PMA (16 nM) at 37 °C, just before the addition of Fe (II) (0–12 µM), Fe (III) (0–6 µM), Cu (I) (0–50 µM), Cu (II) (0–50 µM), Cr (III) (0–1000 µM), Cr (VI) (0–100 µM), Co (II) (0–100 µM), Hg (I) (0–1 µM), Hg (II) (0–1 µM) or Cd (II) (0–1000 µM). Luminol (500 µM) was used as probe.

3. Statistical analysis

Statistics were calculated using GraphPad Prism™ (version 5.0; GraphPad Software). Results are expressed as mean ± standard error of the mean (S.E.M.) (from at least six individual experiments, performed in triplicate in each experiment). Statistical comparison between groups was 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.
4. Results

4.1. Cell viability

Neutrophils' viability was tested by the trypan blue assay after exposure to the tested metals at different concentrations. Table 1 shows the concentrations of the tested metals that do not affect cell viability, after 1 h of exposure, comparing to the control (without metal), which were subsequently used in the oxidative burst experiments.

4.2. Metal-induced human neutrophils' oxidative burst

Fig. 1 shows that Cd (II) activated human neutrophils in a concentration-dependent manner, achieving significance at 1000 µM. In turn, the other tested metals, Fe (II), Fe (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), and Hg (II), had no effect on luminol amplification chemiluminescence, at the tested concentrations.

4.3. Inhibitory effects of DPI, tiron, mannitol, ABAH and L-NAME on the Cd (II)-induced oxidation of luminol by neutrophil-generated reactive species

DPI (NADPH oxidase inhibitor), tiron (O$_2^-$ scavenger), catalase (metabolizes H$_2$O$_2$) and ABAH (MPO inhibitor), totally inhibited neutrophils' oxidative burst induced by Cd (II) (1000 µM) (Fig. 2). A partial inhibition for mannitol (HO$_2^-$ scavenger) and L-NAME (NOS inhibitor) was also observed.

4.4. Contribution of PKC signalling to the activation of human neutrophils by Cd (II)

The PKC specific inhibitor Gö6983 inhibited the response of neutrophils to Cd (II) (Fig. 3) in a concentration-dependent manner, with the complete inhibition of luminol oxidation observed at 250 nM.

4.5. Modulatory effect of Cd (II) on the PMA-mediated stimulation of neutrophils' oxidative burst

Exposure to Cd (II) significantly increased ($p<0.01$) the PMA-mediated stimulation of neutrophils' oxidative burst (Fig. 4). At the concentration of 1000 µM, Cd (II) doubled PMA-mediated effect. The other metals tested had no effect on the activation of neutrophils by PMA, at the tested concentrations.

5. Discussion

In the present study, it was clearly shown that, from the tested metals [Fe (II), (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), Hg (II), and Cd (II)], only Cd (II) activated human neutrophils' oxidative burst, at non-toxic concentrations. In the present study, we choose luminol as a chemiluminimetric probe to study the effect of several metals in human neutrophils. This probe unspecifically detects O$_2^-$, H$_2$O$_2$, HO', HOClNO and ONOO$^-$ [1]. Thus, enzymatic inhibitors and specific reactive species scavengers were used to investigate the relative proportion of the reactive species that are produced upon activation of neutrophils by Cd (II). The main ROS that are produced during neutrophils' oxidative burst are NADPH-oxidase-generated O$_2^-$ (with subsequent formation of H$_2$O$_2$ and HO') [5] and MPO generated HOCl [32]. By using DPI (NADPH oxidase inhibitor), catalase (metabolizes H$_2$O$_2$) and ABAH (MPO inhibitor), we observed a total inhibition of neutrophils' activation by Cd (II). We also tested an O$_2^-$ scavenger, which caused a total inhibition of luminol oxidation, corroborating the results obtained with DPI. These results confirm that O$_2^-$ is formed in high amounts in Cd (II) stimulated neutrophils. The results obtained with ABAH, an inhibitor of MPO, allow the conclusion that HOCl is formed during the stimulation of neutrophils by this metal, and is the main responsible for the observed luminol chemiluminescence. We also used mannitol, a scavenger of HO', which caused some inhibition of neutrophil activation by Cd (II). The involvement of RNS was tested by using L-NAME. The obtained results show some inhibition of the signal provided by Cd (II). However, the results do not allow conclusions about the participation of RNS in neutrophil activation by Cd (II), since the RNS that may account for luminol oxidation is ONOO$^-$, which results from the rapid reaction of "NO and O$_2^-$". Thus, in the

![Fig. 1. Effect of Cd (II) (0–1000 µM) on human neutrophils' oxidative burst, as measured by luminol amplified chemiluminescence. *p<0.05 comparatively to blank assay. Values are given as mean±S.E.M. (n=6).](image)

![Fig. 2. Inhibitory effects of DPI, tiron, catalase, mannitol, ABAH and L-NAME on the oxidation of luminol by neutrophil-generated reactive species, when stimulated by Cd (II) (1000 µM). ***p<0.001, **p<0.01 and *p<0.05 comparatively to 1000 µM of Cd (II).](image)

<table>
<thead>
<tr>
<th>Metals</th>
<th>Concentration used (µM)*</th>
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<tbody>
<tr>
<td>Cd (II)</td>
<td>0–1000</td>
</tr>
<tr>
<td>Cr (III)</td>
<td>0–1000</td>
</tr>
<tr>
<td>Co (II)</td>
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<td>Cr (VI)</td>
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<td>Cu (I)</td>
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<td>Cu (II)</td>
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<td>Fe (II)</td>
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* Corresponds to a viability ≥98%.
The mechanism by which Cd (II) increased ROS formation is not clear. Because Cd (II) is not a Fenton metal constituting a redox-stable metal, indirect mechanisms for the generation of free radicals have been proposed [15]. It has been suggested that Cd (II) can replace Fe (II) and Cu (I) in various cytoplasmatic and membrane proteins leading to an increase in the amount of unbound free or chelated copper and iron ions which can then cause oxidative stress through Fenton reactions [10,15]. Another indirect mechanism by which Cd (II) induces the production of reactive species is related to the impairment of the cellular antioxidant systems. It has also been suggested that the mechanisms of Cd (II) toxicity involve the decrease of the activity and/or intracellular levels of antioxidants (such as catalase, glutathione peroxidase, and superoxide dismutase) leading to an increase in reactive species that can damage the surrounding cells [15,16]. Here, we provide further data, indicating that Cd (II) has the ability to activate and to enhance human neutrophils’ oxidative burst, at non-toxic concentrations.

The putative modulatory effect of Fe (II), Fe (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), Hg (II) and Cd (II) on the stimulation of neutrophils by PMA was evaluated. Interestingly only Cd (II) had a synergistic effect with PMA. Huber et al. [35] studied the effect of Cd (II) (0.1–100 µM) on sheep neutrophils stimulated by PMA and reported an immunosuppressive effect at all the tested concentrations. This result differs from our study, however, it is difficult to make evaluations since the assay conditions are different. Although luminol was used in both studies as probe, luminol concentration, neutrophil concentration and PMA concentration differ among the present study and Huber et al. [35] assay conditions. The use of human neutrophils or sheep neutrophils could also provide different responses of the cells to a stimulus.

The synergistic effect of Cd (II) with PMA suggests that both stimuli use the same mechanisms to promote neutrophils’ oxidative burst. Since PMA induces neutrophil respiratory burst by activating PKC, we studied the involvement of PKC on activation of human neutrophils by Cd (II). PKC contributes to NADPH oxidase activation [36]. Neutrophils express only five different isotypes of PKC: α [34], βI, βII, γ, and ζ [37] which are inhibited by Gö6983. The results obtained in this work showed that Gö6983 is a strong inhibitor of neutrophil activation by Cd (II). This finding indicates that Cd (II) induces neutrophils’ oxidative burst via activation of PKC, precluding a significant contribution of other cellular pathways for ROS generation mediated by this metal. Our results are in accordance with other studies that suggested that Cd (II) induced the production of reactive species via PKC [38,39]. Inclusively, Block et al. [40] reported that Cd (II) at low concentrations induces the production of inositol triphosphate and the release of intracellular calcium whose presence is relevant for the activation of PKC and consequent production of reactive species.

6. Conclusions

This study focused on assessing the putative activation of human neutrophils’ oxidative burst activated by several metals: Fe (II) and Fe (III), Cu (II), Cr (III), Cr (VI), Co (II), Hg (I), Hg (II) and Cd (II). In our experimental conditions, from all of the tested metals, at non-toxic concentrations, only Cd (II) stimulates human neutrophils to produce $\text{O}_2^-$, $\text{H}_2\text{O}_2$, and HOCl mainly via activation of PKC and NADPH oxidase, showing a major contribution of oxidative burst. These observations indicate that the sustained activation of human neutrophils may contribute for the long term adverse effects on human health mediated by Cd (II).

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