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Estimation of antioxidant capacity against pathophysiologically relevant oxidants using Pyrogallol Red

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ABSTRACT

Peroxynitrite and hypochlorite are oxidants relevant in many pathological situations. We propose a simple spectrophotometric assay to determine antioxidant capacity against hypochlorite and peroxynitrite based on protection against Pyrogallol Red decolorization. The assay can be performed on a microplate and requires minute amounts of material. Standard antioxidants show different reactivities for both oxidants. Antioxidant capacity of blood plasma (anticoagulated with EDTA) of healthy persons was found to be 559 ± 49 μmol/l and 11.6 ± 1.2 mmol/l of ascorbic acid equivalents for peroxynitrite and hypochlorite, respectively.

Introduction

The "Total antioxidant capacity" assays have been widely used in several field of research including analysis of food and beverages, analysis of plant material and analysis of body fluids, especially in pathologies [1–3]. The concept of measurements of "total antioxidant capacity" has been lately criticized. It has been pointed out correctly that neither the term "total" nor the term "capacity" are justified since (i) the assays measure non-enzymatic antioxidant activity while the antioxidant defense of the body is mainly enzymatic, and (ii) the samples assayed are removed from their biological context, which is characterized by enzymatic maintenance of the steady state of antioxidant concentrations [4]. While this criticism is justified, one could nevertheless notice that there are reactive oxygen species occurring *in vitro* which are not dealt with by enzymes, such as peroxyl radicals, peroxynitrite and hypochlorite. Their formation is dependent on, or contributed by enzymes (microsomal lipid peroxidation, formation of hypochlorite by myeloperoxidase) and no specific enzymatic means of their detoxification is known, although peroxyredoxins, heme proteins and glutathione peroxidases may be of considerable importance in the detoxification of peroxynitrite [5,6]. In some situations it is of importance to know how the damage by these species can be counteracted by components of the body, especially blood plasma and other extracellular fluids. It is of course, true that assays allowing for such estimates are made *in vitro*, on samples withdrawn from their biological environment. Nevertheless, they provide an estimate of the ability of a biological fluid to withstand acute exposure to an oxidant.

With this arguments in mind, we propose a simple assay allowing to evaluate the antioxidant capacity of biological fluids against two oxidants of physiological and pathological relevance for which no enzymatic defense is known, peroxynitrite and hypochlorite.

Materials and methods

All reagents were from Sigma/Aldrich (Poznań, Poland). Peroxynitrite was synthesized from sodium azide and ozone according to the method of Pryor et al. [7] with small modifications [8]. Blood, anticoagulated with EDTA, was collected from the antecubital vein of 22 healthy volunteers of both sexes, centrifuged (5000*g*, 15 min, 4 °C) and supernatant was used at once for the measurement (kept on ice until putting on a plate). All subjects gave informed consent for participating in the research. The study has been approved by the local ethics committee of the Medical University of Łódź.

Pyrogallol Red (to obtain 50 μM final) in 100 mM phosphate buffer, pH 7.4, was mixed in the presence or absence of antioxidants (ascorbate, Trolox, uric acid, glutathione or bovine serum albumin; BSA) with 100 μM HOCl or $ONOO^-$ to the final volume of 200 μl on 96-well plate. If blood plasma was tested, various volumes of diluted plasma was added to the reaction mixture keeping the final volume constant. Addition of plasma did not affect the pH value of the samples. Peroxynitrite and hypochlorite were added on a shaker. After incubation at 23 °C the absorbance of the

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Effect of phosphatidylcholine chlorohydrins on human erythrocytes

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ABSTRACT

Hypochlorite generated *in vivo* under pathological conditions is a known oxidant and chlorinating agent, able to react with proteins and lipids, which affects the stability of biological membranes. Reaction with unsaturated fatty acyl chains in glycerophospholipids such as phosphatidylcholine results in the formation of chlorohydrins. The aim of this study was to determine the effects of chlorohydrins formed by the reaction of hypochlorite with 1-stearoyl-2-oleoyl-, 1-stearoyl-2-linoleoyl-, and 1-stearoyl-2-arachidonylphosphatidylcholine on biophysical properties of bilayers and their effects on human erythrocytes. Using electrospray mass spectrometry we observed complete conversion of the lipids into chlorohydrins, which resulted in a decrease in the rotational correlation time and an increase in the order parameter of liposomes. Unilamellar chlorohydrin liposomes had a lower permeation coefficient for calcein than liposomes made of parent lipids. Flow cytometry demonstrated fast incorporation of uni and multilamellar chlorohydrin liposomes labeled with NBD-phosphatidylethanolamine into erythrocytes. This effect was accompanied by changes in erythrocyte shape (echinocyte formation) and aggregation. Similar but less pronounced effects were noticed for parent lipids only after longer incubation. Chlorohydrins showed also a stronger hemolytic action, proportional to the lipid:erythrocyte ratio. These results are important for understanding the effects of HOCl on mammalian cells, such as might occur in inflammatory pathology.

1. Introduction

Hypochlorous acid (HOCl) is an oxidizing and chlorinating agent produced by the enzyme system myeloperoxidase-H_2O_2-chloride in phagocytic cells. Its primary importance is thought to act as an antimicrobial agent, as it has been shown to cause killing of a variety of bacterial and fungal species, but excessive or inappropriate production can also cause host tissue damage, and has been implicated in the pathology of inflammatory diseases such as atherosclerosis, renal and neurodegenerative diseases (Gallin et al., 1988; Pullar et al., 2000; Malle et al., 2003; Yap et al., 2007). Although under normal physiological conditions the tissue concentrations of HOCl are likely to be very low, concentrations in the high micromolar or even low millimolar range have been reported in some pathological situations (Katrantzis et al., 1991; Guo et al., 1996).

HOCl reacts readily with a range of biological molecules, including lysine, tryptophan, cysteine and methionine sidechains in proteins, antioxidants, and lipids such as cholesterol, plasmalogens and unsaturated fatty acyl chains (Albert et al., 2001; Spickett et al., 2001; Malle et al., 2006). Electrophilic addition of HOCl to the double bond in cholesterol or in oleic or linoleic acids can yield chlorohydrins (α,β-chlorohydroxy compounds) (Winterbourn et al., 1992; Heinecke et al., 1994). The mechanism of chlorohydrin formation with a variety of phospholipids has been investigated and shown to involve initial electrophilic attack by Cl^+ followed by the addition of hydroxide from water (Spalteholz et al., 2004). Although it has been reported that the rate of reaction of HOCl *in vitro* with double bonds in fatty acyl analogues is slow compared to its reaction with thiols, amines and aromatic rings (Malle et al., 2006), chlorohydrins have been detected in LDL and cultured cells oxidized with MPO–H_2O_2–Cl^- or HOCl (Carr et al., 1996; Jerlich et al., 2000; Spickett et al., 2001) and also in atherosclerotic lesions (Messner et al., 2008), demonstrating that they can be formed even in the presence of substantial amounts of proteins. Moreover, the extent of chlorohydrin formation from palmitoyllinoleoylphos-

Abbreviations: A.m.u., atomic mass unit; *m*/*z*, mass-to-charge ratio; SAPC, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; SLPC, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine; NBD-PE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) ammonium salt (NBD-phosphatidylethanolamine); HOCl, hypochlorite; MPO, myeloperoxidase.

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Chloric acid(I) affects antioxidant defense of lung epitelial cells

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ABSTRACT

Generation of chloric acid(I) and reactive oxygen and nitrogen species by activated phagocytes is associated with the course of many inflammatory-related lung diseases. Thus, we studied the effects of HOCl on the redox state of A549 cells as well as on the activity of enzymes involved in cell protection against oxidants. Additionally, we determined the ability of plasma antioxidants to prevent the HOCl-induced cytotoxicity to lung epithelial A549 cells. Cell treatment with HOCl at concentrations above 50 μM for 1 h resulted in the loss of cell viability. The decrease of GSH concentration and antioxidant capacity of cell extracts was accompanied by an increase of the level of GSSG and the rate of generation of ROS and peroxyl radicals. Hyperpolarization of the mitochondrial membrane was also observed. HOCl at concentrations of 50 μM significantly decreased the activity of all antioxidant enzymes studied in A549 cells. All antioxidants employed protected cells against the action of HOCl, with the efficiency decreasing as follows: albumin > GSH > uric acid > ascorbate > Trolox. HOCl was found to affect the redox state of A549 by oxidation of GSH, inactivation of antioxidant enzymes and increase of ROS generation.

1. Introduction

Lung inflammation is a key feature of many diseases of the lower respiratory tract and occurs as a consequence of inhalation of allergens and environmental pollutants, microbial colonisation and cigarette smoking (Dworski et al., 1999; Walser et al., 2008; Lambrecht and Hammad, 2010). In most cases lung inflammation is accompanied by accumulation of phagocytes, known to produce and release antimicrobial agents (Balamayooran et al., 2010; Cornwell et al., 2010). Among them, reactive oxygen (ROS) and nitrogen species (RNS) play a pivotal role. Furthermore, in the course of many diseases the presence of myeloperoxidase was demonstrated in lung tissues affected by inflammation (Williams and Jose, 2001; Aratani et al., 2006; Ichinose, 2009; Thomson et al., 2010; Harwood et al., 2011). The enzyme is a hemoprotein catalyzing oxidation of chloride ion by hydrogen peroxide to chloric acid(I) (But et al., 2003; Malle et al., 2007). This compound, half dissociated at near neutral pH (pK_a ~7.54), has a relatively high redox potential E^0_{HOCl/Cl^-} ~1.482 V) so it behaves as an oxidant in biological systems (Switzer et al., 2006). HOCl was also demonstrated to be a chlorinating agent able to introduce Cl atom to other molecules (Carr et al., 1996; Hawkins and Davies, 1999; Chapman et al., 2000; Jerlich et al., 2000; Auchere et al., 2001). At the cellular level, HOCl was shown to be a cytotoxic agent, inducing apoptosis or direct cell necrosis, depending on the concentration. Previous papers concerning the effects of HOCl or the myeloperoxidase/H_2O_2/Cl^- system on the lung cells *in vitro* and lung tissue *in vivo* demonstrated increased levels of carbonyl groups, 3-chlorotyrosine, lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals) and decrease of intracellular glutathione and sulfhydryl groups (Lamb et al., 1999; Hammerschmidt et al., 2002; Buss et al., 2003; Venglarik et al., 2003). HOCl was shown to be mutagenic for lung epithelial adenocarcinoma A549 cells (Gungor et al., 2010). Moreover, nucleotide excision repair (NER) was effectively inhibited in these cells (Gungor et al., 2007). The effects of HOCl on A549 cells *in vitro* included mitochondrial potential dissipation followed by cell growth arrest and induction of apoptosis (Robaszkiewicz et al., 2010).

Because generation of HOCl is usually accompanied by formation of reactive oxygen and nitrogen species, the efficient antioxidant barrier made up by low molecular weight antioxidants and enzymes neutralizing these harmful oxidants is extremely important in determination of the cellular effects of HOCl action. Apart from the intracellular antioxidant defence, human lung is additionally protected by the lung surfactant system containing high levels of glutathione, ascorbate, uric acid and alpha-tocopherol, antioxidants demonstrating HOCl, ROS and RNS scavenging capacity (van der Vliet et al., 1999; Robaszkiewicz and Bartosz, 2009; Robaszkiewicz and Bartosz, 2010).

Having in mind all these findings, we decided to study the effects of HOCl on the redox state of A549 cells as well as on the

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N-Chloroamino acids mediate the action of hypochlorite on A549 lung cancer cells in culture

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ABSTRACT

Hypochlorous acid, a chlorinating and oxidative agent, has been reported to be implicated in many pathologies. Its markers were found under inflammatory conditions and, at least some of it reveals biological activity. Thus, in this paper we examined whether N-chloroamino acids may act as mediators of the action of hypochlorous acid in cell culture. N-Chloroamino acids were found to possess lower oxidative capacity than $HOCl/OCl^-$ just after addition to the growth medium. However, all the chlorocompounds studied were cytotoxic to A549 cells, induced a dose-dependent increase in the G_0/G_1 fraction with simultaneous reduction in the G_2/M fraction, collapse of the mitochondrial potential and caspase-dependent apoptosis. The content of cellular thiols decreased after 1-h incubation with the chlorocompounds studied.

Although amino acids act as scavengers of hypochlorite in plasma, the chlorinated products formed stay reactive and the pattern of their action on cells *in vitro* is similar to that of hypochlorite.

1. Introduction

The increasing interest in the role of reactive chlorite species (RCS) has been observed recently. Numerous evidences indicate that excessive production of hypochlorous acid/hypochlorite may occur in the course of many diseases (atherosclerosis, chronic inflammation, some cancers and many others) (Podrez et al., 2000; Hoy et al., 2002; Son et al., 2005; Güngör et al., 2007). It is generally thought that senescence is a proinflammatory state involving, i.e., increased activation of granulocytes (Meydani and Wu, 2007; Marchalant et al., 2008). Therefore, hypochlorous acid, one of the compounds produced by activated granulocytes, may contribute to macromolecular damage underlying ageing. The experiments employing the anti-HOP (HOCl-oxidised proteins) antibodies proved the presence of epitopes modified by $HOCl/OCl^-$ in atherosclerotic lesions and kidney diseases (Hazell et al., 1996; Malle et al., 1997). Nowadays, chlorinated products like 3-chlorotyrosine and chlorohydrins seem to be the most specific markers of $HOCl/OCl^-$ action (Winterbourn, 2002). However, the variety of mechanisms of $HOCl/OCl^-$ action gives rise to the unspecific pattern of oxidised products like protein carbonyls (Chapman et al., 2002), glutathione disulphide and protein–glutathione mixed disulphides, sulphonamides (Winterbourn and Brennan, 1997), formylkynurenine, dityrosine, etc. (Robaszkiewicz et al., 2008a).

Simultaneously, the reactivity of $HOCl/OCl^-$ modified products was reported by other authors. For example, fatty acid and phospholipid chlorohydrins were found to bring about a decrease of ATP concentration and some of them to cause an increase of caspase 3 activity in U937 cells (Dever et al., 2006), taurine chloramine to induce damage in mitochondria and hence to trigger apoptosis in human B lymphoma cells (Klamt and Shacter, 2005), monochloramine and oxidised retinoblastoma protein to cause cell growth arrest of Jurkat cells (Hosako et al., 2004). In our previous papers we proved that chlorinated amino acids can oxidise erythrocyte membrane proteins and cause oxidative events inside erythrocytes with efficiency similar to that of $HOCl/OCl^-$ or even higher in some cases (Robaszkiewicz et al., 2008a,b). Therefore, amino acids, the potential targets for $HOCl/OCl^-$, need to be considered not only as scavengers, but as mediators of $HOCl/OCl^-$ as well. Up to now, N-chlorohistamine and N-chlorotaurine have been investigated intensively, because they are likely to be formed at the site of inflammation (taurine concentration inside neutrophils ~20 mM, while that of histamine in mast cells ~100 mM (Fukuda et al., 1982; Pattison and Davies, 2006)). Because of the presence of amino

Abbreviations: Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; AMC, 7-amido-4-methylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, etylenediaminetetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4); PBSG, PBS with 1% glucose; SDS, sodium dodecyl sulfate.

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Research paper

Detection of 3-chlorinated tyrosine residues in human cells by flow cytometry

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ABSTRACT

Hypochlorite is a strong oxidant, generated under pathological conditions, with the potency to introduce chlorine atom into a number of molecules. 3-Chloro- and 3,5-dichlorotyrosine are documented to be generated by this oxidant and their elevated levels were found in many diseases. Thus, we decided to check the possibility of use of FITC-conjugated antibodies for flow cytometric detection of 3-chlorotyrosine residues in human cells (A549, MCF-7, HUVEC-ST) exposed to the action of hypochlorite. Additionally, we compared the effects of chlorohydrins and *N*-chloroamino acids as chlorine donors. Cell fixation and permeabilization was followed by incubation with rabbit polyclonal anti-3-chlorotyrosine primary antibody and subsequent staining with goat anti-rabbit FITC-labeled secondary antibody. For antibody isotypic control, normal rabbit IgG was employed. Hypochlorite appeared to be the most efficient from the chlorocompounds analyzed in chlorotyrozine generation in all cell lines. Statistically significant increase of fluorescence corresponding to the level of 3-chlorotyrosine residues was found in cells treated with hypochlorite even at non-toxic concentrations (<5 μM). This effect was not observed in cells exposed to the action of chlorinated amino acids or chlorohydrins. The use of anti-3-chlorotyrosine antibodies in conjunction with fluorophore-conjugated secondary antibodies analysis allows for detection of 3-chlorotyrosine residues by flow cytometry in cells treated with low doses of hypochlorite.

1. Introduction

Hypochlorite, a pathologically relevant oxidant, has been documented to contribute to the initiation and the course of some diseases (van der Veen et al., 2009). The fundamental role of this compound was intended to protect the host organism against pathogens, but under some conditions (e.g. inflammation) the concentration of HOCl may exceed locally 200 μM, leading to host tissue injury (Pullar et al., 2000). Hypochlorite is generated in the reaction between H_2O_2 and Cl^- catalyzed by myeloperoxidase, mainly in activated neutrophils. Protein thiols and amine groups, and low molecular weight antioxidants are the preferred targets of hypochlorite in the serum, but most of products of these reaction are also formed by action of other oxidants as well (Winterbourn and Kettle, 2000; Pattison and Davies, 2001). In contrast, chlorine-substituted molecules appear to be specific markers of this oxidant. Nowadays, the presence of 3-chlorotyrosine residues is attributed to the enhanced activity of myeloperoxidase and used as an indicator of HOCl production in biological systems (Hazen and Heinecke, 1997; La Rocca et al., 2009). Since chlorination reactions are a few orders of magnitude slower than oxidation, free chlorotyrosine or chlorine-containing tyrosine residues in proteins are less abundant in biological material than products of HOCl-induced oxidation. Therefore, the possibility of detection of 3-chlorotyrosine detection is limited to the most sensitive methods. Up to now, the content of 3-chlorotyrosine was frequently estimated using HPLC and mass spectrometry, while antibodies against 3-chlorotyrosine were succesfully adopted in immunocyto- and immunohistochemistry for visualization of HOCl-modified tyrosine residues in cells and tissues (Hazen

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