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HOCl causes necrotic cell death in human monocyte derived macrophages through calcium dependent calpain activation

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ABSTRACT

The abundance of dead macrophages in close proximity to HOCl-modified proteins in advanced atherosclerotic plaques implicates HOCl in the killing of macrophages and the formation of the necrotic core region. The mechanism of HOCl mediated death of macrophages was unknown, so using human monocyte derived macrophages (HMDM) we here have shown that HOCl causes a rapid necrotic cell death characterized by loss of MTT reduction, cellular ATP and cell lysis without caspase-3 activation in HMDM cells. The HOCl causes a rise in cytosolic calcium level *via* the plasma membrane L- and T-type calcium channels and endoplasmic reticulum RyR channel. Blocking of the calcium channels or the addition of calpain inhibitors prevents the HOCl mediated loss of mitochondrial potential, lysosome failure and HMDM cell death. Blocking MPT-pore formation with cyclosporin A also prevents the loss of mitochondrial membrane potential, lysosomal destabilization and HMDM cell death. Blocking the calcium mitochondrial uniporter with ruthenium red also blocks the loss of mitochondrial potential but only at high concentrations. HOCl appears to cause HMDM cell death through destabilization of cytosolic calcium control resulting in the failure of both the mitochondria and lysosomes.

1. Introduction

Hypochlorous acid (HOCl) is a potent oxidant secreted by activated phagocytic cells at sites of inflammation. HOCl is generated by the enzyme myeloperoxidase (MPO), which catalyzes the oxidation of chloride ions by hydrogen peroxide. HOCl plays a central role in the killing of microorganisms at sites of inflammation [1–2] due to its high reactivity with a range of biomolecules including nucleotides [3], ATP [4], lipids [5], amino acid side residues [6], ascorbate [7], glutathione [8] and protein thiols [9]. This wide range of reactivity has also implicated HOCl as a possible cause of injury to the surrounding host cells and tissues during inflammation [10].

Phagocytic macrophages are the predominant cell type within atherosclerotic plaques. The death of lipid loaded macrophages has been implicated in the growth of the necrotic core of advanced atherosclerotic plaques [11]. MPO and 3-chlorotyrosine, a specific marker of HOCl oxidation of proteins, have been found within atherosclerotic plaques rich in dead macrophages [12–13]. Macrophages have also been reported to generate MPO, but not at the levels recorded for neutrophils [13]. HOCl appears to be a significant oxidant to which macrophages are exposed to and may be one of the causes of macrophage cell death within atherosclerotic plaques.

High concentrations of HOCl kill cells by generally oxidizing and lysing the cells. At the lower, more physiological concentration generated by neutrophils (20–400 μM) [14], HOCl attacks specific sites on and within the cells triggering a variety of cell death mechanisms depending on the cell type. HOCl damage to neuronal cells causes a rise in cytosolic calcium ion level triggering calpain activation, lysosome rupture and cell death [15]. In endothelial cells, HOCl triggers apoptosis through a rapidly decreased endothelial Bcl-2 and cytochrome c release [16] and caspase-3 activation [17]. With HEPG2 and human fetal liver cells, HOCl causes mitochondrial permeability transition (MPT) pore formation triggering caspase-3 activation through cytochrome c release [18]. The mechanism by which HOCl triggers cell death in macrophages is not known.

Macrophage cell death has been described by both necrotic and apoptotic mechanisms [19]. We have previously shown that HOCl causes large amounts of protein oxidation in U937 cells leading to cell death [20]. Treatment of the monocyte like U937 cells with aqueous peroxyl radicals causes caspase inactivation, glutathione loss and necrosis [21]. Oxidized low density lipoproteins cause caspase-independent

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; A23187, 4-bromo-calcium ionophore A23187; ANT, adenine nucleotide translocator; EBSS, Earle's balanced salt solution; fluo-3-AM ester, fluo-3-acetoxymethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOCl, hypochlorous acid; HMDM, human monocyte derived macrophages; HPLC, high performance liquid chromatography; MBB, monobromobimane; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; MPO, myeloperoxidase; MPT, mitochondrial permeability transition; PBS, phosphate buffered saline; PI, propidium iodide; RyR, ryanodine receptor; TMRM, tetramethylrhodamine methyl ester

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cell death in human monocyte derived macrophages (HMDM) [22–24] while nitric oxide donors induce caspase activation in J774 mouse macrophage cell line by inducing hydrogen peroxide formation [25]. In this study, we show that HOCl induces cell necrosis in HMDM cells by triggering a rise in cytosolic calcium level, MPT-mediated mitochondrial membrane potential ($\Delta\Psi_m$) loss, calpain activation and lysosome destabilization. These results provide a novel insight into the mechanisms of HOCl-mediated cell death at inflammatory sites.

2. Materials and methods

2.1. Materials

All tissue culture plastic ware was supplied from Greiner Bio-one (Germany) through Raylab New Zealand Ltd. except for the 12-well adherent cell culture plates, which were supplied by Nunc (Kamstrupvej, Denmark) through In Vitro, New Zealand. Reagents and chemicals were of analytical grade or better and obtained from BDH Chemicals New Zealand Ltd. or Sigma Chemical Company (USA). All solutions were prepared using ion-exchanged ultra-filtered water, which was produced using a NANOpure ultrapure water system from Barnstead/Thermolyne (IA/USA). Granulocyte-macrophage colony stimulating factor (GM-CSF) was a generous gift from the Haematology group of the Christchurch Hospital. Molecular weight marker was provided by Fermentas International Inc. (Canada). Complete, Mini protease inhibitor cocktail tablet was supplied from Roche (Germany). Calpeptin, SJA6017, cyclosporin A, fluo-3-acetoxymethyl (AM) ester and tetramethylrhodamine methyl ester (TMRM) were obtained from Merck (Germany). Acridine orange was supplied from Invitrogen (USA). The Bicinchoninic acid protein determination kit for protein quantification was supplied from Pierce Biotechnology Inc. (USA).

2.2. Human monocyte-derived macrophage cell culture

Human monocyte derived macrophages (HMDM) were prepared using unlinked blood donated by hemochromatosis patients at the NZ Blood Bank (Riccarton Road, Christchurch) under ethics approval CTY/98/07/069 from the New Zealand Upper South B Ethics Committee. Monocytes were isolated from human blood by density gradient centrifugation in the presence of Lymphoprep™ (Axis-Shield PoC AS (Oslo, Norway)) as described by the manufacturer, and washed four times in sterile phosphate buffered saline (PBS) containing 150 mM sodium chloride and 10 mM sodium dihydrogen orthophosphate pH 7.4 [26]. After 40 hour incubation in serum free RPMI-1640 in non-adherent plates at 37 °C, 5% CO_2, cells were plated into 12-well adherent plates from Nunc at a cell density of 5×10^6 cells/well in RPMI-1640 media supplemented with 10% heat-inactivated human serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were also plated on 22×22 mm cover slips (Marienfeld) at 5×10^6 cells/cover slip, which were placed in 6-well suspension culture plates at 1 cover slip/well. Prior to use, the cover slips were sterilized by immersion in 96% ethanol under aseptic conditions followed by exposure UV light for 15 min. The plates were left undisturbed at room temperature for 1 h, allowing the cells to attach to the cover slip, before adding one extra ml of the RPMI-1640 media with human serum into each well. GM-CSF at 3 μg/ml was added to the media on the first day after plating but not added during subsequent media changes, which occurred every three days. The monocytes were incubated at 37 °C, 5% CO_2 and differentiated into adherent macrophages over 7 to 14 days.

2.3. Cell treatment

HOCl (Clorogene Supplies, Petone) concentration was determined by absorbance at 292 nm, pH 12 using the extinction coefficient 350 M^{-1} cm^{-1}[27]. HOCl was diluted to final concentrations in sterile Earle's balanced salt solution (EBSS) (Invitrogen, USA) just before addition to cells. When required, the EBSS was supplemented with 0.68 mM $CaCl_2$ to give the same concentration as in RPMI-1640 media. This media is referred to as calcium-supplemented EBSS while the straight EBSS buffer without $CaCl_2$ supplementation will be referred to as calcium-free EBSS.

For the majority of experiments HMDM cells were incubated at 37 °C with HOCl for 10 min in either calcium-supplemented EBSS or calcium-free EBSS. For some experiments cells were pre-incubated with different calcium metabolism modifiers. After treatment, the cells were washed three times with PBS and incubated with the RPMI-1640 media with human serum for the time periods stated.

2.4. Cellular metabolic activity

HMDM cell viability was measured by the reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [28]. Intracellular ATP levels were measured by reverse phase ion pair-high performance liquid chromatography (HPLC) [29] using a Shimadzu 10A series HPLC with a SPD-M20A UV/Vis detector. For there to be sufficient ATP to be detected, 3 wells of 5×10^6 cells/well HMDM cells were combined together by scraping with a total of 125 μl cold 0.07 M perchloric acid. After the sonication for 1 min and incubation on ice for 30 min, the cell lysates was centrifuged at 20,800 *g* for 5 min at 4 °C to remove proteins. The resulting supernatant was neutralized with 12.5 μl of 2 M potassium carbonate. After centrifugation, 40 μl supernatant was injected onto a reverse phase 5 μm ODS (C18), 250×4.6 mm, 5 μm column (Phenomenex) and ATP was detected by absorbance at 254 nm with comparison to standards of known concentration. ATP level in each sample was normalized for protein content.

Intracellular glutathione was measured using a light sensitive and cell-permeable fluorescent dye monobromobimane (MBB) that binds to thiol groups, especially glutathione [30]. The resulting fluorescent glutathione-MBB adducts were detected by HPLC analysis.

2.5. Caspase-3 detection by Western blot analysis

HMDM cells after HOCl treatment were collected using ice cold lysis buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1× protease inhibitor cocktail, pH 7.4) and incubated on ice for 30 min. After protein analysis of the cell lysates and protein precipitation with ice cold acetone, 90 μg protein samples were dissolved in cracker buffer (0.125 M Tris–HCl (pH 6.8), 1% ($^w/_v$) sodium dodecyl sulfate, 20% ($^w/_v$) glycerol, 0.1% ($^w/_v$) bromophenol blue, 2% ($^v/_v$) β-mercaptoethanol and 0.5 mM EDTA). The protein samples were separated in a 4–12% gradient polyacrylamide gel and transferred to a 0.45 μm-pore size nitrocellulose membrane (Invitrogen, USA). Immunodetections were performed using the primary antibody mouse monoclonal IgG2a and the secondary antibody goat anti-mouse IgM HRP-conjugate (Santa Cruz Biotechnology Inc., USA). Equal loading of proteins was confirmed by re-probing the nitrocellulose membrane with mouse monoclonal antibodies against β-actin (Sigma Chemical Company, USA) and peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences, England). Signals were detected using Supersignal West Dura chemiluminescence (Pierce Biotechnology Inc., USA) and exposure to Syngene Chemigenius-2 bioimaging system using Genesnap software (Global, NZ). Caspase-3 enzymatic activation was measured by monitoring the change in fluorescence (λ_{ex} 370 nm/λ_{em} 440 nm) during the hydrolysis of the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-Amido-4-methyl-coumarin (Ac-DEVD-AMC) [31].

2.6. Fluorescence microscopy measurements

All microscopy investigation was carried out using HMDM cells grown on cover slips placed directly into the tissue culture well. The cells were viewed using a Zeiss AxioImager.M1 epifluorescent