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Free Radical Biology & Medicine 38 (2005) 1571–1584

www.elsevier.com/locate/freeradbiomed

Original Contribution

Hypochlorous acid-mediated mitochondrial dysfunction and apoptosis in human hepatoma HepG2 and human fetal liver cells: Role of mitochondrial permeability transition

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Received 30 September 2004; revised 14 January 2005; accepted 18 February 2005
Available online 19 March 2005

Abstract

Liver cirrhosis is often preceded by overt signs of hepatitis, including parenchymal cell inflammation and infiltration of polymorphonuclear (PMN) leukocytes. Activated PMNs release both reactive oxygen species and reactive halogen species, including hypochlorous acid (HOCl), which are known to be significantly cytotoxic due to their oxidizing potential. Because the role of mitochondria in the hepatotoxicity attributed to HOCl has not been elucidated, we investigated the effects of HOCl on mitochondrial function in the human hepatoma HepG2 cell line, human fetal liver cells, and isolated rat liver mitochondria. We show here that HOCl induced mitochondrial dysfunction, and apoptosis was dependent on the induction of the mitochondrial permeability transition (MPT), because HOCl induced mitochondrial swelling and collapse of the mitochondrial membrane potential with the concomitant release of cytochrome *c*. These biochemical events were inhibited by the classical MPT inhibitor cyclosporin A (CSA). Cell death induced by HOCl exhibited several classical hallmarks of apoptosis, including annexin V labeling, caspase activation, chromatin condensation, and cell body shrinkage. The induction of apoptosis by HOCl was further supported by the finding that CSA and caspase inhibitors prevented cell death. For the first time, these results show that HOCl activates the MPT, which leads to the induction of apoptosis and provides a novel insight into the mechanisms of HOCl-mediated cell death at sites of chronic inflammation.

Keywords: Hypochlorous acid; Inflammation; Cell toxicity; Liver; Apoptosis; Free radicals

At sites of chronic inflammation, neutrophils secrete hydrogen peroxide (H_2O_2) and the enzyme myeloperoxidase, which catalyzes the formation of hypochlorous acid (HOCl) (Eq. (1)).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-. \quad (1)$$

Up to 80% of the H_2O_2 generated by activated neutrophils is used to form 20–400 μM HOCl an hour [1–5]. Throughout this paper we use the term "hypochlorous acid" (pK_a 7.46) to refer to the approximately 50% ionized mixture of HOCl and OCl^- species that exists at physiological pH [6].

There is extensive evidence for a pathophysiological role for HOCl in vivo (reviewed in [7,8]), including the demonstration of chlorinated protein tyrosine residues, including 3-chlorotyrosine, a proposed biomarker for

Abbreviations: ANT, adenine nucleotide translocase; BKA, bongkrekic acid; CSA, cyclosporin A; CyD, cyclophilin D; $\Delta\Psi_m$, mitochondrial membrane potential; EBSS, Earle's buffered salt solution; GC-MS, gas chromatography–mass spectrometry; GSH, reduced glutathione; HFL, human fetal liver; HPLC, high-pressure liquid chromatography; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; TCA, trichloroacetic acid; TMRM, tetramethylrhodamine methyl ester; MPT, mitochondria permeability transition; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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doi:10.1016/j.freeradbiomed.2005.02.030

reactive chlorine species [9–19]. Immunochemical, as well HPLC and GC-MS, techniques have identified 3-chlorotyrosine and HOCl-modified proteins in a number of inflammatory tissues [7,9–15], including septal and periseptal hepatocytes and Kupffer cells of cirrhotic human liver [16,17], as well as in the hepatocytes and parenchymal cells in murine models of endotoxemia [17]. This may involve polymorphonuclear cell activation [16,17] and the production of excess HOCl, because the formation of 3-chlorotyrosine has been observed in intracellular proteins of *Staphylococcus aureus* and human neutrophils during phagocytosis [18].

Exposure of cells to HOCl results in the oxidation of many critical biomolecules, including plasma membrane lipids, proteins, DNA, and small molecules, including ascorbate, nucleotides, sulfhydryls, and thioethers (reviewed in [19]). Although HOCl is known to deplete intracellular ATP stores [20–22] and induce cell death or cell growth arrest involving caspases [23,24] and stress kinases [21,25], the role of mitochondria in HOCl-mediated cell death pathways and the mechanisms accounting for caspase activation are currently unknown. Because mitochondria are key players in both apoptotic and necrotic forms of cell death (reviewed in [26–28]), it is important to determine their role during HOCl-mediated cell death. A crucial event occurring in mitochondria when a cell dies, whether it occurs as a result of apoptosis or necrosis, is collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), which can be mediated by the opening of high-conductance membrane permeability transition pores (MPT) in the mitochondrial inner membrane, which allow the nonselective diffusion of solutes (<1500 Da) across the membrane with resulting organelle swelling and membrane rupture [26,29]. MPT activation can either result in the release of cytochrome *c* from the mitochondrial inner membrane with the activation of caspases and the promotion of apoptotic cell death or lead to a drastic loss of ATP production and necrosis [26–29]. Factors involved in MPT regulation are of considerable importance in cell biology because the MPT is central to the cell death process. The intracellular redox environment is a critical MPT regulatory factor [30,31], which is in part controlled by glutathione (GSH). Intracellular depletion of GSH creates an increasingly oxidized environment in the cytosol and mitochondria due to increased production of mitochondrial reactive oxygen species by respiratory complex III [32]. Because HOCl is known to deplete intracellular GSH [19–22], this reactive chlorine species could potentially create favorable conditions for activation of the MPT during HOCl-induced cytotoxicity.

In the present paper we show that HOCl induces mitochondrial swelling, loss of the $\Delta\Psi_m$, and release of cytochrome *c* in isolated mitochondria and in mitochondria in situ. These events resulted in apoptotic cell death as determined by annexin V labeling and caspase-3 activation. Our results show that HOCl activates the MPT, leading to apoptosis in HepG2 cells and human fetal liver (HFL) cells and provide a novel insight into the mechanisms of HOCl-mediated cell death at sites of chronic inflammation.

Materials and methods

Materials

Propidium iodide (PI), tetramethylammonium methyl ester (TMRM), and rhodamine 123 were obtained from Molecular Probes (Eugene, OR, USA). Caspase inhibitors Z-VAD-FMK and Ac-DEVD-CHO, caspase-3/7 substrate Ac-DEVD-AMC, and bongkrekic acid (BKA) were obtained from Calbiochem (San Diego, CA, USA). All cell culture flasks and microplates were obtained from Greiner. Cytochrome *c* ELISA was purchased from Chemicon (No. APT200; Temecula, CA, USA). LDH kit (CytoTox 96) was purchased from Promega Corp. (Madison, WI, USA). The APO-ONE homogeneous (No. G7792) caspase-3/7 assay was purchased from Promega. Cyclosporin A (CSA), annexin V kit (APOAF), Earle's balanced salt solution (EBSS), trypsin–EDTA solution, firefly lantern extract, and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Equipment

Gemini XS fluorescence, LMax luminescence, and SpectraMax 190 microplate readers (Molecular Devices, Sunnyvale, CA, USA) were used for fluorescence, luminescence, and UV–visible measurements, respectively. Flow cytometric analyses were carried using a Coulter Epics Elite ESP flow cytometer (Miami, FL, USA) [33,34]. At least 10,000 cells were analyzed from each group and the data analyzed using WinMDI 2.7 software (Scripps Research Institute; La Jolla, CA, USA) [33–36]. Laser confocal microscopy was performed with a Zeiss LSM 510 confocal microscope [33–36]. Cells were maintained at 37°C using a Zeiss Incubator S with a TempControl 31-2 digital monitor and CO_2 levels maintained at 5% using a Zeiss CTI 3700 digital controller. Cells were viewed using a C-Apochromat 63×/1.2-W water objective. The red fluorescence of TMRM was excited with the 543-nm lines of an argon–krypton laser. Fluorescence was split by a 560 nm emission dichroic filter and collected by separate photomultipliers through 515–565 nm band-pass and 590 nm long-pass barrier filters with the following settings held constant: PMT 600, gain 3.5%, and offset 2.5%.

Cell culture and exposure of cells to hypochlorous acid

Human HepG2 hepatoma cells were obtained from the American Tissue Culture Collection (Gaithersburg, MA, USA) and grown in minimum essential medium (MEM)