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Forum News & Views

Do Mitochondriotropic Antioxidants Prevent Chlorinative Stress-Induced Mitochondrial and Cellular Injury?

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ABSTRACT

Reactive chlorine species such as hypochlorous acid (HOCl) are cytotoxic oxidants generated by activated neutrophils at the sites of chronic inflammation. Since mitochondria are key mediators of apoptosis and necrosis, we hypothesized that mitochondriotropic antioxidants could limit HOCl-mediated intracellular oxidative injury to human fetal liver cells, preserve mitochondrial function, and prevent cell death. In this current study, we show that recently developed mitochondria-targeted antioxidants (MitoQ and SS31) significantly protected against HOCl-induced mitochondrial damage and cell death at concentrations ≥25 n*M*. Our study highlights the potential application of mitochondria-specific targeted antioxidants for the prevention of cellular dysfunction and cell death under conditions of chlorinative stress, as occurs during chronic inflammation. *Antioxid. Redox Signal.* 10, 641–650.

GENERATION OF REACTIVE CHLORINE SPECIES *IN VIVO*

At sites of chronic inflammation, neutrophils secrete the enzyme myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) to catalyze the formation of the cytotoxic reactive chlorine species (RCS), hypochlorous acid (HOCl). Up to 80% of the H_2O_2 generated by activated neutrophils is used to form 20–400 μM HOCl an hour (13, 19). Evidence for chlorinative stress is apparent *in vivo* from the detection of chlorinated protein tyrosine residues in the form of a biomarker, 3-chlorotyrosine (reviewed in Ref. 27). Chlorinated tyrosine has been observed in a number of inflammatory tissues including septal and periseptal hepatocytes and Kupffer cells of cirrhotic human liver (6, 10) and in the hepatocytes and parenchymal cells in animal models of endotoxemia (10). HOCl is cytotoxic and its addition to cells leads to the oxidation of many critical biomolecules, including plasma membrane lipids, proteins, and DNA (27), resulting in cell growth arrest (24) or cell death (17, 25, 26), although the mechanisms for accounting for cell death are ill-defined.

MITOCHONDRIA ARE INTRACELLULAR TARGETS FOR REACTIVE CHLORINE SPECIES

One particular intracellular target for HOCl is mitochondria (44). A key mediator in regulating cell death by apoptosis or necrosis, as well as cell survival, is the mitochondrial permeability transition (MPT) (reviewed in Ref. 2). MPT is a critical

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event that occurs during cell death, either as a result of apoptosis or necrosis, and results in the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) through opening of high conductance permeability transition pores in the mitochondrial inner membrane. This phenomenon allows the nonselective diffusion of solutes (<1,500 Da) across the membrane, resulting in organelle swelling, membrane rupture, and inhibition by cyclosporine A (CSA) which blocks the peptidyl-prolyl isomerase (PPIase) activity of matrix cyclophilin D (CyD) required for the formation of an ANT/CyD protein complex required for MPT activation (2). The intracellular redox environment is a critical MPT regulatory factor 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 (ROS) by respiratory complex III (3–5). HOCl readily depletes intracellular GSH in human cells including hepatic cells (25, 26), creating favorable intracellular conditions for MPT activation (2). Indeed, the addition of HOCl to isolated rat liver mitochondria results in mitochondrial swelling and cytochrome *c* release, processes inhibited by CSA and indicative of MPT induction. In whole hepatic cells, the addition of HOCl resulted in CSA-inhibitable collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome *c* release resulting in cell death (26).

STRATEGIES TO PREVENT OXIDANT-INDUCED MITOCHONDRIAL DYSFUNCTION

Strategies aimed at preventing oxidant-induced cellular injury and cell death at sites of chronic inflammation are currently being sought. A recent ground breaking approach has been the development of novel synthetic antioxidants which selectively target the mitochondria (15, 20, 23). The most notable of these compounds are MitoQ, and more recently, SS31. MitoQ consists of a mixture of the lipophilic antioxidants ubiquinone and ubiquinol covalently attached to lipophillic triphenylphosphonium (TPP^+) cations, resulting in mitoubiquinone and mitoubiquinol, respectively. Mitoquinone and mitoquinol are readily oxidized by bovine heart mitochondria membranes analogous to endogenous ubiquinone, suggesting redox cycling of the drugs (20, 23). Due to the large negative mitochondrial $\Delta\Psi_m$ generated by the proton gradient across the mitochondrial inner membrane, MitoQ has been shown to selectively target the matrix face of the inner mitochondrial membrane where it accumulates in a potential-dependent manner (20). *In vitro*, MitoQ, but not TPP^+ (18). exhibits potent antioxidant properties in that it inhibits H_2O_2-mediated endothelial cell (8, 9, 12) and Jurkatt cell apoptosis (24), scavenges $O_2^{\cdot -}$ and peroxynitrite ($ONOO^-$), as well as prevents *t*-butylhydroperoxide-mediated MPT in isolated bovine heart mitochondria (14), although the effects of MitoQ on HOCl-mediated processes and cellular injury have yet to be examined.

Even though MitoQ elicits highly potent antioxidant and cell protective properties, its uptake and mitochondrial targeting are strongly dependent on cells having 'normal' mitochondrial $\Delta\Psi_m$. Pretreatment of cells with FCCP (carbonyl cyanide (4-trifluoromethoxy)phenylhydrazone) to depolarize the mitochondrial $\Delta\Psi_m$ resulted in substantially less mitochondrial accumulation (9). Since 'normal' mitochondria may be lacking in human diseases containing an impaired mitochondrial component, the utility of mitochondrial potential-dependent delivery based strategies (*i.e.*, using TPP^+) for mitochondrial targeting could be limited, but it has prompted investigations into additional processes for mitochondrial selectivity. One such approach is the use of novel small peptides such as SS31 that contains mitochondrial sequence motifs to encourage mitochondrial selectivity (23). SS31 is a novel cell-permeable small peptide consisting of alternating aromatic and basic amino residues with a dimethyltyrosine moiety (Dmt) apparently providing the antioxidant function (SS31; D-Arg-Dmt-Lys-Phe-NH_2) (23). SS31 is water soluble and resistant to aminopeptidases by virtue of a D-Arg at the first position, and C-terminus degradation is prevented by C-terminus amidation. In contrast to MitoQ, SS31 accumulates in the inner mitochondrial membrane in a mitochondrial $\Delta\Psi_m$ -independent manner resulting in significant scavenging of peroxynitrite, lipid hydroperoxides and H_2O_2 with EC_{50} in the n*M* and sub-n*M* range (23).

Therefore, in the present article, we used cultured human fetal liver (HFL) cells to investigate the effects of mitochondria-targeted antioxidants (SS31 and MitoQ) as well as nontargeted antioxidants decylubiquinone (DecUb) and ascorbate (Asc) on chlorinative stress-mediated mitochondrial $\Delta\Psi_m$ collapse and cell death induced by HOCl. Our study further highlights the potential application of mitochondria-specific targeted antioxidants for the prevention of cellular dysfunction and cell death under conditions of oxidative and chlorinative stress.

HOCl INDUCED LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL IS INHIBITED BY MITOCHONDRIA-TARGETED BUT NOT NONTARGETED ANTIOXIDANTS

In order to select appropriate conditions for examining any potential inhibitory effects of mitochondria-targeted and nontargeted antioxidants on HOCl-induced loss of $\Delta\Psi_m$, we initially performed concentration- and time-dependent experiments. Figure 1A shows that exposure of HFL cells to HOCl caused a time- and concentration-dependent decrease in mitochondrial $\Delta\Psi_m$ measured using TMRM and flow cytometry. HOCl-induced loss of mitochondrial $\Delta\Psi_m$ was rapid with substantial mitochondrial $\Delta\Psi_m$ lost after 5 min exposure to $\geq$30 μM HOCl and a maximal effect observed after 40 min. Therefore, based on these preliminary investigations, we performed subsequent experiments using a final HOCl concentration of 30 μM for 30 min. Figure 1B shows flow cytometric and confocal microscope representations of screening experiments performed using an initial 200 n*M* concentration of antioxidant. Mitochondria-targeted antioxidants SS31 (but not the nonscavenging control, SS20) and MitoQ (but not its targeted, nonscavenging control TPMP) exerted substantial and significant inhibition of HOCl-induced loss of $\Delta\Psi_m$ compared to nontargeted antioxidants decylubiquinone (DecUb) or ascorbate (Asc). Figure 1C shows concentration-dependent inhibition of

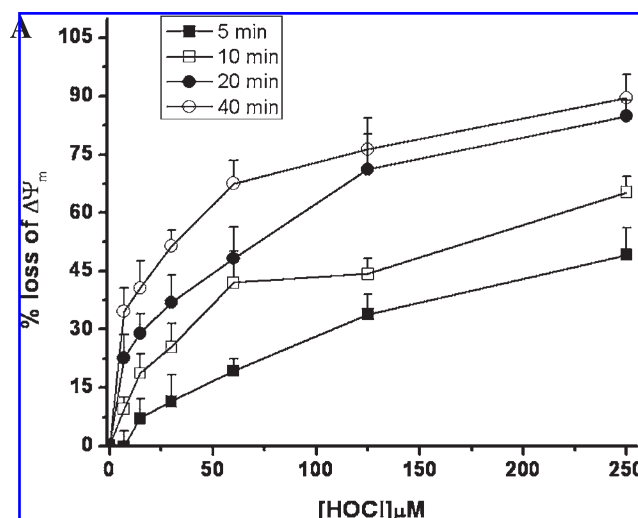
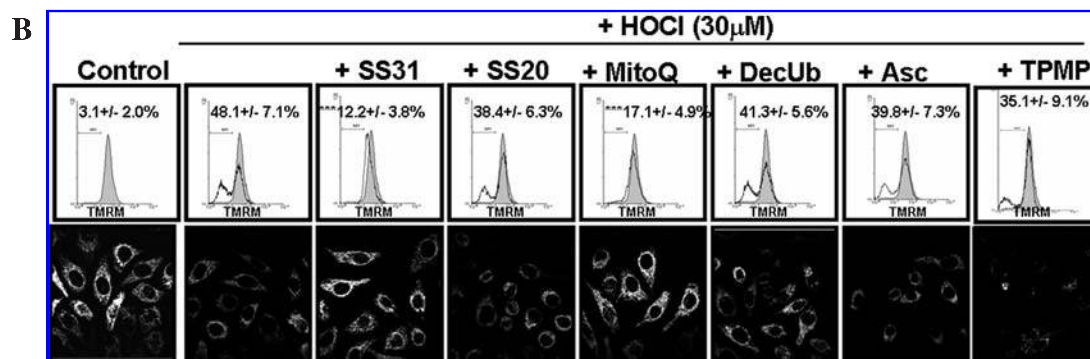
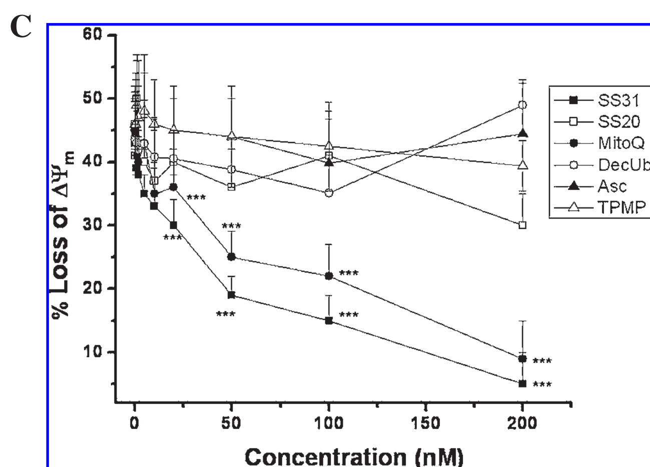
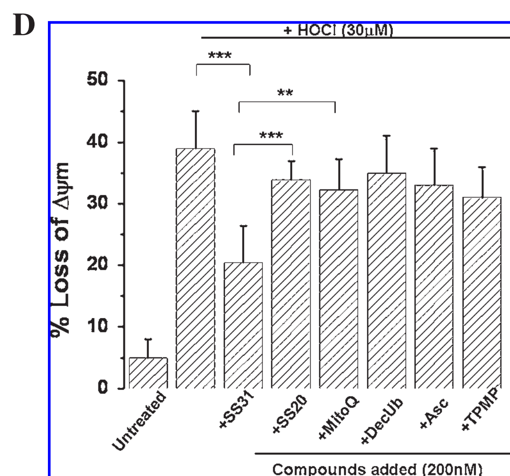


FIG. 1. HOCl-induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) and the effect of mitochondria targeted and nontargeted antioxidants. HFL cells were incubated with serum-free DMEM containing TMRM (50 n*M*) for 1 h prior to the addition of increasing concentrations of HOCl in EBSS. HOCl-induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) was then measured at the times stated by flow cytometry. (**A**) HOCl induced a time and concentration decrease in $\Delta\Psi_m$. (**B** and **C**) Inhibition of HOCl-induced loss of $\Delta\Psi_m$ by mitochondria-targeted and other antioxidants. Cells were initially incubated in DMEM containing 200 n*M* of antioxidants as an initial screen prior to the addition of HOCl (60 μM, 20 min) (**B**) and $\Delta\Psi_m$ determined by flow cytometry and confocal microscopy. (**C**) Concentration-dependent inhibition of HOCl-induced loss of $\Delta\Psi_m$ determined by flow cytometry. (**D**) Effect of mitochondria targeted and nontargeted antioxidants on $\Delta\Psi_m$ added 30 min after HOCl treatment. Experiments were conducted as described in Materials and Methods and data are expressed as mean ± standard deviation of 6 or more separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.01$ compared to cells exposed to HOCl without drug treatment (**B**, **C**).







HOCl-induced loss of $\Delta\Psi_m$ in HFL cells and that significant inhibition was observed with SS31 and MitoQ when added at concentrations ≥25 n*M*. In contrast, the nontargeted antioxidants DecUb and Asc, as well as the nonscavenging SS20 peptide and TPMP, were ineffective at the concentrations used (Fig. 1C). Since MitoQ is reported to accumulate in mitochondria in a potentiometric manner (20) but SS31 is not (23), the effects of adding antioxidants to cells 30 min after HOCl addition were examined (*i.e.*, after significant loss of $\Delta\Psi_m$. Figure 1D shows significant reversal of HOCl-mediated loss of $\Delta\Psi_m$ was observed with SS31 which was significantly greater than equimolar MitoQ.

HOCl-INDUCED SUPEROXIDE FORMATION IS INHIBITED BY MITOCHONDRIA-TARGETED BUT NOT NONTARGETED ANTIOXIDANTS

The addition of HOCl (30 μM) to HFL cells for 30 min led to the substantial formation of mitochondrial intracellular $O_2^{\cdot -}$ measured using DHE (Fig. 2A). Further analysis shows significant production observed after 30 min with 7 μM HOCl (Fig. 2B). Since treatment of HFL cells with 30 μM HOCl for 30 min gave ~50% of the maximum response (Fig. 2B), these conditions were chosen for subsequent experiments with mitochondria-targeted and nontargeted antioxidants. Figures 2C and D show the effect of 200 nM antioxidants on inhibition of HOCl-induced mitochondrial $O_2^{\cdot -}$ formation by confocal mi-

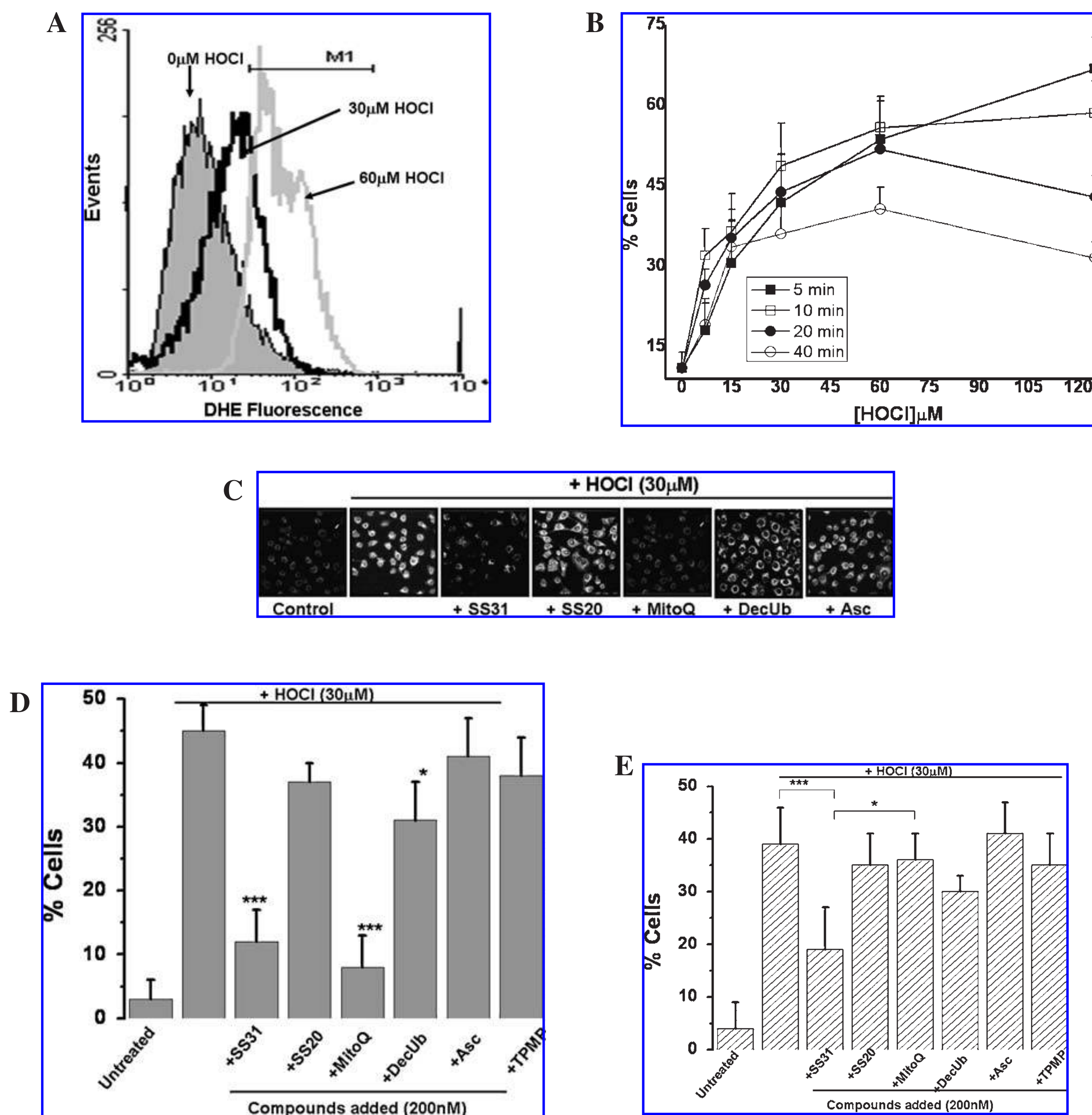


FIG. 2. HOCl-induced formation of mitochondrial superoxide formation and the effect of mitochondria targeted and nontargeted antioxidants. HFL cells were incubated with serum-free DMEM containing 5 μM dihydroethidium (DHE) for 30 min and EBSS containing HOCl added. (**A** and **B**) HOCl induced a time- and concentration-dependent increase in mitochondrial superoxide formation assessed using DHE and quantified by flow cytometry. (**C** and **D**) Effects of mitochondria targeted and nontargeted antioxidants on HOCl-induced superoxide formation detected using confocal microscopy (**C**) and quantified by flow cytometry (**D**). (**E**) Effect of mitochondria targeted and nontargeted antioxidants on mitochondrial $O_2^{\cdot -}$ formation when added 30 min after HOCl treatment. Experiments were conducted as described in Materials and Methods and data are expressed as mean ± standard deviation of 6 or more separate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$* compared to cells exposed to HOCl without drug treatment. '% cells' refers to the % of cells with increased DHE fluorescence measured by flow cytometry.

croscopy and flow cytometry, respectively. SS31 and MitoQ (200 n*M*) induced substantial and significant inhibition of HOCl-induced $O_2^{\cdot-}$ formation, whereas nontargeted antioxidants DecUb and Asc or nonscavenging controls SS20 and TPMP were ineffective under our experimental conditions. Figure 2E shows SS31 significantly reduced HOCl-mediated $O_2^{\cdot-}$ production when added to HFL cells for 1 h after HOCl addition; an effect significantly greater than MitoQ. In contrast, under the same conditions DecUb, SS20, TPMP, or Asc did not significantly reduce $O_2^{\cdot-}$ levels detected.

MITOCHONDRIA-TARGETED ANTIOXIDANTS PREVENT HOCl-INDUCED CELL DEATH

Figures 3 and 4 show that the addition of 30 μM HOCl to HFL cells induced substantial and significant cell death measured using several parameters; cellular viability using MTT (Fig. 3A) and LDH release (Fig. 3B), but that incubation of cells with SS31 or MitoQ significantly prevented loss of cell

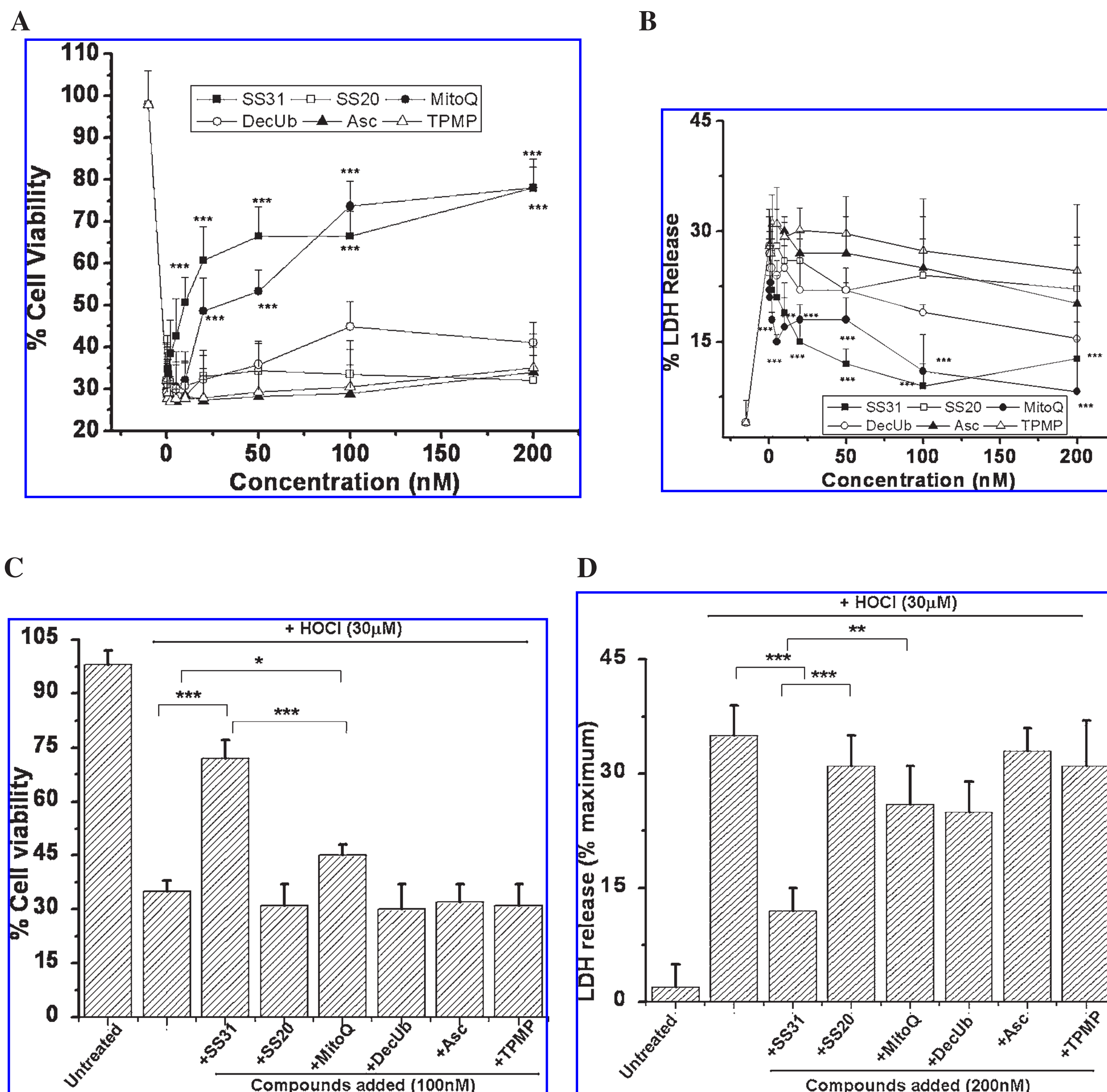


FIG. 3. HOCl-induced cell death and the effect of mitochondria targeted and nontargeted antioxidants. HFL cells were treated with antioxidants for 1 h prior to the addition of HOCl (**A**, **B**) or added cells were treated with HOCl for 30 min prior to the addition of anitoxidants (**C**, **D**). Cell death was then subsequently analyzed 18 h after HOCl addition by MTT assay (**A**) and LDH release assay (**B**). Effect of mitochondria targeted and nontargeted antioxidants on cell death by MTT assay (**C**) or LDH release assay (**D**) when added 30 min after HOCl treatment. Experiments were conducted as described in Materials and Methods, and data are expressed as mean ± standard deviation of 6 or more separate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$* compared to cells exposed to HOCl without drug treatment.

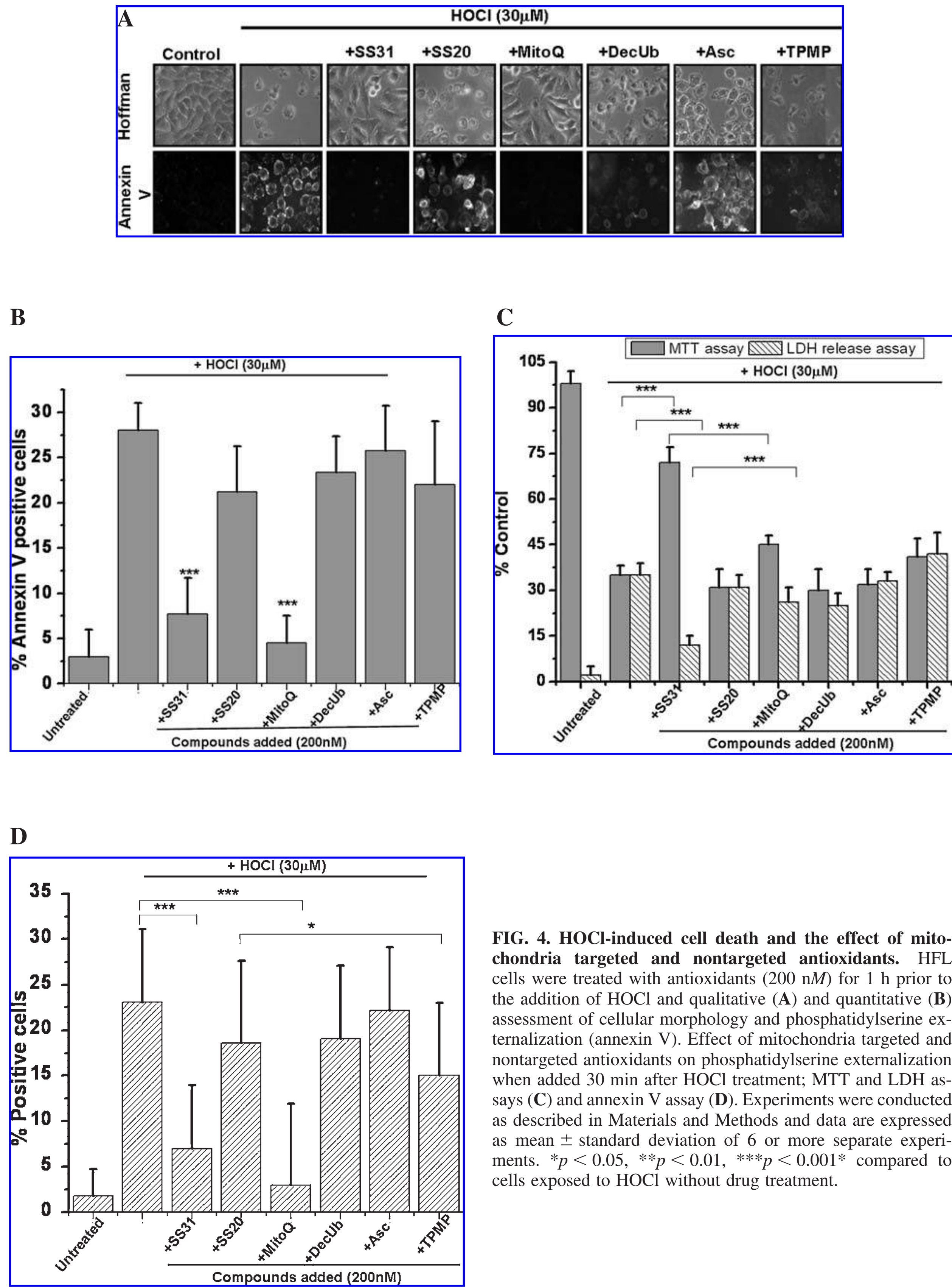


FIG. 4. HOCl-induced cell death and the effect of mitochondria targeted and nontargeted antioxidants. HFL cells were treated with antioxidants (200 nM) for 1 h prior to the addition of HOCl and qualitative (**A**) and quantitative (**B**) assessment of cellular morphology and phosphatidylserine externalization (annexin V). Effect of mitochondria targeted and nontargeted antioxidants on phosphatidylserine externalization when added 30 min after HOCl treatment; MTT and LDH assays (**C**) and annexin V assay (**D**). Experiments were conducted as described in Materials and Methods and data are expressed as mean ± standard deviation of 6 or more separate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$* compared to cells exposed to HOCl without drug treatment.

viability (Fig. 3A) and LDH release (Fig. 3B) in a concentration-dependent manner. In sharp contrast, the nonscavenging peptide control for SS31 (SS20), TPMP and nontargeted antioxidants DecUb and Asc were markedly less effective at preventing HOCl-induced cellular injury (Fig. 3A and B). SS31, but not SS20, MitoQ or nontargeted antioxidants, significantly inhibited loss of viability measured using the MTT and LDH assays, respectively, when added 30 min after HOCl addition (Fig. 3C and D). Since HOCl has previously been shown to induce apoptotic cell death in HFL cells (26), we investigated the ability of mitochondrially-targeted and nontargeted antioxidants to inhibit phosphatidylserine externalization (PS) and cell body shrinkage. Figure 4 shows that MitoQ and SS31 prevented HOCl-induced cell body shrinkage (Fig. 4A) and significantly inhibited HOCl-induced PS externalization (Fig. 4B), suggesting that SS31 and MitoQ prevented HOCl-induced apoptotic cell death in HFL cells. Furthermore, we also observed that adding SS31, but not MitoQ, to cells for 1 h after pretreatment of cells with HOCl resulted in significant increase in cell viability (Fig. 4C) and significant decrease in annexin V-positive cells (Fig. 4D).

IMPLICATIONS

Although HOCl, produced at sites of chronic inflammation, results in depletion of endogenous antioxidant defenses and the oxidation of numerous biomolecules (46), the protective effects of antioxidants against HOCl-mediated cell injury and death have only been investigated using nontargeted antioxidants, often added at relatively high concentrations [i.e, ascorbate (17)], presumably because the mechanisms of HOCl-induced cytotoxicity are relatively unexplored. We recently showed that exposure of hepatic cells to HOCl at concentrations within the reported range generated by activated neutrophils (20–400 μM) (13, 19) induced substantial loss of mitochondrial $\Delta\Psi_m$, loss of intracellular ATP, as well as mitochondrial cytochrome *c* release and cell death (26). In isolated rat liver mitochondria, HOCl induced large amplitude mitochondrial swelling and cytochrome *c* release, indicative of MPT induction. HOCl also rapidly depleted intracellular GSH (25, 26), inducing the formation of intracellular oxidants and creating intracellular redox changes advantageous for MPT activation. These studies strongly implicated mitochondria as a viable and important target in HOCl-mediated toxicity and that antioxidants targeted to the mitochondria were likely to offer significant cytoprotection. With these observations in mind, we sought to investigate this hypothesis using the novel mitochondrially targeted antioxidants SS31 and MitoQ and compared their effectiveness against nontargeted antioxidant compounds.

In our current study, both MitoQ and SS31 were potent inhibitors of HOCl-mediated loss of mitochondrial $\Delta\Psi_m$, mitochondrial $O_2^{\cdot-}$ production, and prevented HOCl-induced cell death when added to cells at concentrations $\leq$200 nM. In sharp contrast, neither the nonscavenging mitochondria-targeted controls SS20 or TPMP for SS31 and MitoQ, respectively, nor the nonselective antioxidants Asc or DecUb significantly inhibited HOCl-induced cytotoxicity in any assay examined. These findings are in agreement with other oxidant stress-induced cell injury studies. For example, *in vitro* cell culture studies have shown SS31 (but not SS20) to inhibit H_2O_2-induced oxidant $O_2^{\cdot-}$ formation and MPT in isolated rat liver mitochondria (23), inhibit *tert*-butylhydroperoxide-induced ROS formation, mitochondrial depolarization, and cell death in N_2A and SH-SY5Y neuronal cells (29, 30). In Caco-2 cells, SS31 but not SS20, prevented 3-propionic acid-induced loss of mitochondrial $\Delta\Psi_m$ (30). Similarly, MitoQ has been reported to inhibit H_2O_2-induced and lipid hydroperoxide-induced apoptosis of endothelial (10) and Jurkat cells (18) when used at concentrations as low as 1 μM as well as inhibit oxidant-induced cell death in fibroblasts from Friedrich's ataxia patients (16).

The effectiveness of mitochondrial-targeted antioxidants at protecting against oxidant-induced cellular injury has also been illustrated outside simple *in vitro* assay systems. For example, MitoQ has been shown to improve cardiac function after ischemia-reperfusion in rat hearts (1) and SS31 (but not the nonscavenging but mitochondrial-targeted peptide SS20) prevented myocardial stunning and reduced infarct size (23) when administered either preischemia (23), upon reperfusion (28, 30), or after acute ischemia in guinea pig and rat hearts. Furthermore, SS31 is also reported to reduce infarct volume and hemispheric swelling, and reduce GSH depletion when administered after the occlusion of the middle cerebral artery in a murine model of cerebral ischemia (7), suggesting SS31 can minimize oxidative ischemic damage by preserving GSH levels. SS31 also reduces lipid peroxidation and oxidative cellular damage in a G93A transgenic familial murine model of amyotrophic lateral sclerosis (21), further highlighting the potential for mitochondrially-targeted antioxidants in preventing oxidative injury *in vivo*.

Interestingly, with each assay examined DecUb, a compound we previously showed to prevent redox-dependent and cytochrome bc_1-mediated MPT after intracellular GSH depletion (4), exhibited small but statistically significant protection against HOCl-induced loss of mitochondrial $\Delta\Psi_m$ (Fig. 1), $O_2^{\cdot-}$ formation (Fig. 2), and cell death (Figs. 3 and 4). Although the addition of HOCl to hepatic cells results in the rapid loss of intracellular GSH, the extent and rate of loss is not as great as that induced by DEM treatment, suggesting that intracellular oxidants in addition to mitochondrial $O_2^{\cdot-}$ were induced by HOCl. Indeed, from our study, it is uncertain whether SS31 and MitoQ exerted their cytoprotective effects *via* scavenging of intracellular HOCl or interaction with species derived from the reaction of HOCl with intracellular constituents resulting in the formation of chloramines, chlorhydrins, protein carbonyl moieties (11), or additional intracellular oxidants. The exposure of cells to HOCl also causes cellular lipid peroxidation (25) and lipid peroxides such as hydroxynonenal and malondialdehyde induce collapse of ψ_m and apoptotic cell death. Given that SS31 (23) and MitoQ (20) inhibit lipid peroxidation and scavenge lipid peroxyl radicals, as well as prevent peroxide-induced cell death, it is possible that some of the observed phenomena were due at least in part to species derived from the reaction of HOCl with intracellular or membrane constituents and organelle lipids. Therefore, it is likely that the effectiveness of SS31 and MitoQ could be due, at least in part, to an interaction with these HOCl-derived intermediates.

Although the TPP^+ moiety of MitoQ allows it to accumulate in the mitochondrial matrix in a membrane-dependent man-

ner, its accumulation is severely reduced after mitochondrial depolarization, suggesting it will not enter the mitochondria to exert protective effects in cells previously stressed with oxidants. Since many human diseases with an oxidative stress component manifest their pathology after considerable cellular damage, cell death, and presumably mitochondrial dysfunction, it is probable that MitoQ would be beneficial in preventing oxidant-induced mitochondrial injury, rather than in treating mitochondrial injury. In sharp contrast, SS31 loading into the mitochondria is not dependent on mitochondrial membrane potential, and mitochondrial loading is not substantially impeded by FCCP pretreatment (23). This prompted us to investigate whether SS31 could reverse HOCl-mediated $O_2^{\cdot -}$ formation and loss of mitochondrial $\Delta\Psi_m$. In agreement with previous findings using FCCP, the addition of SS31 but not MitoQ, DecUb, or Asc after HOCl treatment resulted in a modest but statistically significant increase in mitochondrial $\Delta\Psi_m$ (Fig. 1), reduction of $O_2^{\cdot -}$ levels (Fig. 2), and protection against cell death (Figs. 3 and 4). Therefore, these data suggest that SS31 could be more effective than MitoQ at treating mitochondrial dysfunction induced by oxidants. Indeed, in animal models of ischemia-reperfusion injury, SS31 has been shown to protect against oxidative damage when administered both before initiation of ischemia (23) and when administered upon reperfusion (47, 50), whereas MitoQ has been shown to be extremely potent when administered during reperfusion (30).

It is unlikely that at the concentrations used that SS31 and MitoQ directly scavenged HOCl since control experiments performed using antioxidants pretreated with HOCl prior to the addition of cells did not result in significant inhibition of either HOCl-induced loss of mitochondrial $\Delta\Psi_m$, $O_2^{\cdot -}$ formation, or cell death. It is more likely that SS31 and MitoQ exerted their cytoprotective effect *via* additional mechanisms. Our study showed for the first time that HOCl induced mitochondrial $O_2^{\cdot -}$ formation, although the mechanisms responsible for this remain to be elucidated. Since SS31 and MitoQ have been shown to efficiently scavenge mitochondrial-derived $O_2^{\cdot -}$ to prevent redox-dependent induction of MPT and cell death, it is possible that the protective effect of these mitochondria-targeted antioxidants was due to scavenging HOCl-induced mitochondrial $O_2^{\cdot -}$ (Fig. 2). However, HOCl also induces lipid peroxidation in cultured cells (25) as well as formation of secondary intracellular oxidants (11) and lipid peroxides (*i.e.*, 4-hydroxynonenal and malondialdehyde) are also known to induce loss of mitochondrial $\Delta\Psi_m$ and cell death (2). Since MitoQ and SS31 potently scavenge peroxides and protect against *t*-butylhydroperoxide-mediated MPT and cell death, it is also possible that SS31 and MitoQ prevented HOCl-mediated peroxide toxicity. Although the precise species or oxidant is responsible for the cytoprotection observed merits further study, our data clearly show that mitochondrially-targeted antioxidants were effective at n*M* concentrations against μM concentrations of added HOCl.

CONCLUDING REMARKS

In summary, we have shown that mitochondrially-targeted antioxidants are potent inhibitors of HOCl-mediated mitochondrial dysfunction and cell death in HFL cells. HOCl-induced loss of $\Delta\Psi_m$ and cell death was potently inhibited by mitochondria-targeted antioxidants SS31 and MitoQ added at n*M* concentrations. Our data suggest that targeting mitochondria with synthetic antioxidants may represent a viable approach to preserve cell function and prevent cell death in pathologies associated with increased production of RCS, such as liver cirrhosis, hepatitis, and other chronic inflammatory conditions. However, the precise mechanism(s) of HOCl-induced mitochondrial dysfunction and cell death remain to be elucidated. Selective mitochondria-targeted antioxidants may provide a unique opportunity to study the pathways of RCS-induced cellular dysfunction and death.

APPENDIX

1. Materials

MitoQ was a generous gift from Professor M. Murphy (MRC Dunn Human Nutrition Unit, Cambridge, England). SS31 and SS20 were prepared by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Montreal, Quebec, Canada) using solid phase synthesis as described in Ref. 22. Tetramethylamonium methyl ester (TMRM) and dihydroethidium were obtained from Molecular Probes (Eugene, OR). All cell culture flasks and microplates were obtained from Greiner. LDH kit (CytoTox 96) was purchased from Promega Corporation (Madison, WI). Annexin V kit (APOAF), methyltriphenylphosphonium (TPMP) bromide (used as a targeted, nonantioxidant control for MitoQ), Earle's Balanced Salt Solution (EBSS), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Throughout this article, we use the term "hypochlorous acid" (pKa = 7.46) to refer to the ~50% ionized mixture of HOCl and OCl^- species that exists at physiological pH.

2. Equipment

Gemini XS fluorescence and SpectraMax 190 microplate readers (Molecular Devices, Sunnyvale, CA) were used for fluorescence and UV-visible measurements, respectively. Flow cytometric analyses were carried out using a flow cytometer (Epics Elite ESP model, Coulter, Miami, FL) (26). 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) (2, 44, 45). Laser confocal microscopy was performed with a Zeiss LSM 510 confocal microscope (44, 45) and cells were maintained at 37°C using a Zeiss (Jurong, Singapore) Incubator S with a TempControl 31-2 digital monitor. CO_2 levels were maintained at 5% using a Zeiss CTI 3700 digital controller and cells viewed using a C-Apochromat 63x/1.2W water objective (26).

3. Cell culture and exposure of cells to hypochlorous acid (HOCl)

Human fetal liver cells (HFL) were a generous gift from Professor Wong Kim Ping (National University of Singapore) and were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin as described (26). The concentration of HOCl was quantified immediately before use spectrophotometrically at 290 nm (pH 12, $\varepsilon = 350\ M^{-1}\ cm^{-1}$). HOCl was diluted in ice-cold EBSS to a concentration of 10 m*M* and stored on ice for no longer than 1 min (26). To expose the cells to HOCl, cells were washed twice with PBS and once with EBSS warmed to 37°C. Fresh EBSS was then added, followed by oxidant addition as described (26). The addition of HOCl did not significantly alter the pH of the reaction mixture.

All incubations involving cell culture experiments were conducted in a cell culture incubator at 37°C under 5% CO_2.

4. Assessment of cell death and measurement of apoptotic indices

Metabolic activity was estimated using 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and data are expressed as % of untreated cells (26). Leakage of lactate dehydrogenase (LDH) into the culture media was measured at 340 nm using a commercially available kit (Sigma, LD-50) and data are expressed as a % LDH activity from cells lysed with 0.1% Triton X for 30 min at 37°C (26). Analysis of extracellular facing plasma membrane phosphatidylserine residues was performed by fluorescence microscopy using an Annexin V kit (Sigma) (26).

5. Determination of mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial superoxide ($O_2^{\cdot-}$) formation

Mitochondrial membrane potential ($\Delta\Psi_m$) in whole cells was measured using tetramethylrhodamine methyl ester (TMRM) as previously described (3, 4, 26). Briefly, cells were incubated with serum free DMEM 30 min containing 50 n*M* TMRM in the cell culture incubator prior to HOCl addition in warm (37°C) EBSS. Cells were then either analyzed by confocal microscopy using a Zeiss LSM 510 confocal microscope or flow cytometry using a Coulter Epics Elite ESP flow cytometer. To determine mitochondrial $O_2^{\cdot-}$ formation, we employed the fluorescent probe dihydroethidium (DHE), and cells were analyzed by flow cytometry and confocal microscopy as described above.

6. Statistical analysis

Data are expressed as mean ± standard deviation of the mean (SD) of separate experiments ($n \geq 6$) performed on separate days using freshly prepared reagents. Where significance testing was performed, ANOVA was used ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) and concentration-dependent effects investigated with post hoc Dunnett's test using SPSS 12.0 software.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Asc, ascorbate; DecUb, decylubiquinone; $\Delta\Psi_m$, mitochondrial membrane potential; Ca^{2+}, calcium; DHE, dihydroethidium; EBSS, Earle's buffered salt solution; HFL, human fetal liver; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; MPT, mitochondria permeability transition; PBS, phosphate-buffered saline; PS, phosphatidylserine; RCS, reactive chlorine species; SS20, Phe-D-Arg-Phe-Lys-NH_2; SS31, D-Arg-2′6′-dimethyltyrosine-Lys-Phe-NH_2; $O_2^{\cdot-}$, superoxide; TMRM, tetramethylrhodamine methyl ester; TPMP, methyltriphenylphosphonium.

REFERENCES

1. Adlam VJ, Harrison JC, Porteous CM, James AM, Smith RA, Murphy MP, and Sammut IA. Targeting an antioxidant to mitochondria decreases cardiac ischaemia-reperfusion injury. *FASEB J* 19: 1088–1095, 2005.
2. Armstrong, JS. Mitochondrial membrane permeabilization: the sine qua non for cell death. *Bioessays* 28: 253–260, 2006.
3. Armstrong JS, Yang H, Duan W, and Whiteman, M. Cytochrome bc_1 regulates the mitochondrial permeability transition by two distinct pathways. *J Biol Chem* 279: 50420–50428, 2004.
4. Armstrong JS and Jones DP. Glutathione depletion enforces the mitochondrial permeability transition and causes cell death in Bcl-2 overexpressing HL60 cells. *FASEB J* 16: 1263–1265, 2002.
5. Armstrong JS, Whiteman M, Rose P, and Jones DP. The coenzyme Q_{10} analog decyl-ubiquinone inhibits the redox-activated mitochondrial permeability transition: role of mitochondrial respiratory complex III. *J Biol Chem* 278: 49079–49084, 2003.
6. Brown KE, Brunt EM, and Heinicke JW. Immunohistochemical detection of myeloperoxidase and its oxidation products in Kupffer cells of human liver. *Am J Pathol* 159: 2081–2088, 2001.
7. Cho S, Szeto HH, Kim E, Kim H, Tolhurst AT, and Pinto JT. A novel cell permeable antioxidant peptide, SS31, attenuates ischemic brain injury by down-regulating CD36. *J Biol Chem* 282: 4634–4642, 2007.
8. Dhanasekaran A, Kotamraju S, Karunakaran C, Kalivendi SV, Thomas S, Joseph J, and Kalyanaraman B. Mitochondria superoxide dismutase mimetic inhibits peroxide-induced oxidative damage and apoptosis: role of mitochondrial superoxide. *Free Radic Biol Med* 39: 567–583,2005.
9. Dhanasekaran A, Kotamraju S, Kalivendi SV, Matsunaga T, Shang T, Keszler A, Joseph J, and Kalyanaraman B. Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. *J Biol Chem* 279: 37575–37587, 2004.
10. Gujral JS, Farhood A, Bajt ML, and Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. *Hepatology* 38: 3553–3563, 2003.
11. Hawkins CL, Pattison DI, and Davies MJ. Hypochlorite-induced oxidation of amino acids, peptides and proteins. *Amino Acids* 25: 259–274, 2003.
12. Hughes G, Murphy MP, and Ledgerwood EC. Mitochondrial reactive oxygen species regulate the temporal activation of nuclear factor kappaB to modulate tumour necrosis factor-induced apoptosis: evidence from mitochondria-targeted antioxidants. *Biochem J* 389: 83–89, 2005.
13. Hussien M, Delecata RJ, and Carey PD. Neutrophil hypochlorous acid production is impaired in multiple organ failure patients with candidaemia; reversal with antifungal agents *Inflamm Res* 51: 213–217, 2002.
14. James AM, Cocheme HM, Smith RA, and Murphy MP. Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species. Implications for the use of exogenous ubiquinones as therapies and experimental tools. *J Biol Chem* 280: 21295–21312, 2005.
15. James AM, Cocheme HM, and Murphy MP. Mitochondria-targeted redox probes as tools in the study of oxidative damage and ageing. *Mech Ageing Dev* 126: 982–986, 2005.
16. Jauslin ML, Meier T, Smith RA, and Murphy MP. Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants. *FASEB J* 17: 1972–1974, 2003.
17. Jenner AM, Ruiz EJ, Dunster C, Halliwell B, Mann GE, and Siow RCM. Vitamin C protects against hypochlorous acid- induced glutathione depletion and DNA base and protein damage in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 22: 574–580, 2002.
18. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, and Murphy MP. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem* 276: 4588–4596, 2001.

19. King CC, Jefferson MM, and Thomas EL. Secretion and inactivation of myeloperoxidase by isolated neutrophils. *J Leuk Biol* 61: 293–302; 1997.
20. Murphy MP and Smith RA. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol* 47: 629–656. 2007.
21. Petri S, Kiaei M, Damiano M, Hiller A, Wille E, Manfredi G, Calingasan NY, Szeto HH, and Beal MF. Cell-permeable peptide antioxidants as a novel therapeutic approach in a mouse model of amyotrophic lateral sclerosis. *J Neurochem* 98: 1141–1148, 2006.
22. Schiller PW, Nguyen TM, Berezowska I, Dupuis S, Weltrowska G, Chung NN, and Lemieux C. Synthesis and *in vitro* opioid activity profiles of DALDA analogues. *Eur J Med Chem* 3535: 895–901, 2000.
23. Szeto HH. Cell-permeable, mitochondrial-targeted, peptide antioxidants. *AAPS J* 8: E277–283, 2006.
24. Vissers MC, Pullar JM, and Hampton MB. Hypochlorous acid causes caspase activation and apoptosis or growth arrest in human endothelial cells. *Biochem J* 344: 443–449, 1999.
25. Whiteman M, Rose P, Siau JL, and Halliwell B. Nitrite-mediated protection against hypochlorous acid-induced chondrocyte toxicity: a novel cytoprotective role of nitric oxide in the inflamed joint? *Arthritis Rheum* 48: 3140–3150, 2003.
26. Whiteman M, Rose P, Siau JL, Cheung NS, Tan GS, Halliwell B, and Armstrong JS. Hypochlorous acid-mediated mitochondrial dysfunction and apoptosis in human hepatoma HepG2 and human fetal liver cells: role of mitochondrial permeability transition. *Free Radic Biol Med* 38: 1571–1584, 2005.
27. Winterbourn CC. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid. *Toxicology* 181–182: 223–227, 2002.
28. Wu D, Soong Y, Zhao GM, and Szeto HH. A highly potent peptide analgesic that protects against ischaemia-reperfusion-induced myocardial stunning. *Am J Physiol Heart Circ Physiol* 283: H783–791, 2002.
29. Zhao K, Luo G, Giannelli S, and Szeto HH. Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Biochem Pharmacol* 70: 1796–1806, 2005.
30. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, and Szeto HH. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. *J Biol Chem* 279: 34682–34690, 2004.

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2. David I. Pattison, Michael J. Davies, Clare L. Hawkins. 2012. Reactions and reactivity of myeloperoxidase-derived oxidants: Differential biological effects of hypochlorous and hypothiocyanous acids. *Free Radical Research* **46**:8, 975-995. [CrossRef]
3. Dao-Fu Dai , Tony Chen , Simon C. Johnson , Hazel Szeto , Peter S. Rabinovitch . 2012. Cardiac Aging: From Molecular Mechanisms to Significance in Human Health and Disease. *Antioxidants & Redox Signaling* **16**:12, 1492-1526. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
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5. Dao-Fu Dai, Tony Chen, Hazel Szeto, Madeline Nieves-Cintrón, Vassily Kutyavin, Luis F. Santana, Peter S. Rabinovitch. 2011. Mitochondrial Targeted Antioxidant Peptide Ameliorates Hypertensive Cardiomyopathy. *Journal of the American College of Cardiology* **58**:1, 73-82. [CrossRef]
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