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Cytotoxic effects of oxysterols produced during ozonolysis of cholesterol in murine GT1-7 hypothalamic neurons

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Abstract

Ozone present in the photochemical smog or generated at the inflammatory sites is known to oxidize cholesterol and its 3-acyl esters. The oxidation results in the formation of multiple "ozone-specific" oxysterols, some of which are known to cause abnormalities in the metabolism of cholesterol and exert cytotoxicity. The ozone-specific oxysterols have been shown to favor the formation of atherosclerotic plaques and amyloid fibrils involving pro-oxidant processes. In the present communication, cultured murine GT1-7 hypothalamic neurons were studied in the context of cholesterol metabolism, formation of reactive oxygen species, intracellular Ca^{2+} levels and cytotoxicity using two most commonly occurring cholesterol ozonolysis products, 3β- hydroxy-5-oxo-5,6-secocholestan-6-al (ChSeco) and 5β, 6β-epoxy-cholesterol (ChEpo). It was found that ChSeco elicited cytotoxicity at lower concentration ($IC_{50} = 21 \pm 2.4\,\mu M$) than did ChEpo ($IC_{50} = 43 \pm 3.7\,\mu M$). When tested at their IC_{50} concentrations in GT1-7 cells, both ChSeco and ChEpo resulted in the generation of ROS, the magnitude of which was comparable. *N*-acetyl-L-cysteine and Trolox attenuated the cytotoxic effects of ChSeco and ChEpo. The intracellular Ca^{2+} levels were not altered by either ChSeco or ChEpo. Methyl-β-cyclodextrins, which cause depletion of cellular cholesterol, prevented ChSeco- but not ChEpo-induced cytotoxicity. The cell death caused by ChEpo, but not ChSeco, was prevented by exogenous cholesterol. Although oxidative stress plays a significant role, the results of the present study indicate differences in the pathways of cell death induced by ChSeco and ChEpo in murine GT1-7 hypothalamic neurons.

Keywords: *Cholesterol, cytotoxicity, neuronal cells, oxysterols, ozone, reactive oxygen species*

Introduction

Cholesterol (cholest-5-en-3β-ol) is an unsaturated neutral lipid ubiquitously present in mammalian tissues. Cholesterol is susceptible to oxidation by several oxidants including radical initiators, hypochlorous acid, nitrogen oxides, peroxynitrite, singlet oxygen and metal ions such as copper and iron [1–4] resulting in the formation of oxysterols. It has been shown that, in participating solvents containing water, ozone can oxidize cholesterol at the carbon–carbon double bond resulting in the formation of aldehydes (3β-hydroxy-5-oxo-5,6-secocholestan-6-al or ChSeco), epoxides (5β,6β-epoxycholesterol (ChEpo) and hydroperoxides (5-hydroperoxy-*B*-homo-6-oxa-cholestane- 3β,7α-diol) [5]. ChSeco and ChEpo have been shown to be formed endogenously in rats exposed to 0.3 ppm of ozone, providing evidence for the formation of ozone specific oxysterols *in vivo* [6]. Although only ChSeco is considered to be a biologically relevant marker for ozone exposure [7], ChEpo is also formed in ozone-mediated oxidations

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presumably due to the secondary reactions of carbonyl oxide intermediates (e.g. 3β-hydroxy-5-oxo-5,6-seco-cholestan-6-carbonyl oxide) with cholesterol [5,8,9] or by other auto-oxidation reactions [10]. In general, oxysterols are more lipophilic than cholesterol and can easily cross cell membranes and the blood–brain barrier [11] and this, in turn, may contribute to the relaying of toxic effects of ozone to extra-pulmonary tissues including the brain.

Ozone-specific oxysterols are shown to favor the formation of atherosclerotic plaques [12] and amyloid fibrils [13]. Also, increasing evidence suggests that cholesterol ozonolysis products cause abnormalities in the metabolism of cholesterol and related molecules and thereby contribute to cytotoxicity [5]. ChSeco is known to promote macrophage foam cell formation [12] and cause apoptotic cell death in cardiomyocytes [14]. ChSeco and ChEpo are toxic to cells of the respiratory system such as the bronchial epithelial cells [5]. Recently, we have shown that cholesterol ozonation products are also toxic to cells of neural origin such as GT1-7 hypothalamic neurons [15].

In the present study, the cytotoxic effects of ChSeco and ChEpo, the two major ozonation products were examined in cultured murine GT1-7 hypothalamic neurons. Since oxidative stress and intracellular Ca^{2+} are involved in many pathological changes in cells of the nervous system, we have extended this line of research to analyze the potential role of reactive oxygen species (ROS) and the effects of antioxidants against ChSeco- and ChEpo-induced toxicity. In addition, we have examined the effect of ChSeco and ChEpo on intracellular Ca^{2+} levels. Finally, we have examined the cytotoxic effects of ChSeco and ChEpo following the modulation of cellular cholesterol levels. It was observed that the two oxysterols elicit comparable cytotoxic responses but differ markedly in their responses to modulation of cholesterol levels. Like most other oxysterols, ChSeco and ChEpo mediate cytotoxicity through regulation of ROS but do not involve changes in intracellular Ca^{2+}.

Materials and methods

Murine hypothalamic GT1-7 neurons and reagents

Immortalized murine GT1-7 hypothalamic neurons (obtained from Dr Pam Mellon, University of California at San Diego, CA, USA) were grown in DMEM containing 10% FBS at 37 ± 0.5°C in 5% CO_2. During incubations with ChSeco and ChEpo, cells were maintained in DMEM containing 2% FBS. Reagents for tissue cultures, ChEpo and other chemicals were purchased from Sigma unless otherwise stated.

ChSeco was synthesized by ozonation of cholesterol followed by reduction of the product mixture with Zn/acetic acid [7]. The purity of the final product was tested by reversed phase HPLC and the chemical identity confirmed by GC/MS/EI [14].

Stock solutions of ChSeco, ChEpo, cholesterol and Trolox were prepared in ethanol at concentrations of 10 mM each and, where necessary, stored at −20°C until use. Methyl-β-cyclodextrin was dissolved in water at a concentration of 40 mg/ml, and stored at −20°C. Stock solutions of *N*-acetyl-L-cysteine (NAC; 500 mM) were prepared in PBS, and added to the culture medium at a final concentration of 5 mM. In appropriate experiments, supplementation of culture medium with NAC, cholesterol, methyl-β-cyclodextrin and Trolox were done 30 min prior to addition of ChSeco or ChEpo. Control cultures received vehicle alone (ethanol/PBS) in every experiment.

Neuronal cell survival

The neuronal cells were incubated with varying concentrations of ChSeco and ChEpo for 24 h. Relative cell viability and cytotoxicity were assessed based on MTS reduction and LDH content of viable cells. MTS reduction assay is based on reduction of a water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) by viable cells in a phenazine methosulfate (PMS)-assisted reaction (CellTiter 96 AQueous; Promega). The neuronal cells typically were incubated with MTS for 2–4 h at 37°C in 5% CO_2 and the MTS formazan formed was determined by measuring the absorbance at 490 nm using a microplate reader (Biotek EL 800). Results are expressed as a percentage of untreated controls (mean ± SEM).

Measurement of LDH content in intact cells is an indirect measure of cell viability. LDH activity was measured based on the enzymatic conversion of resazurin into a fluorescent resorufin product using the CytoTox-ONE homogeneous membrane integrity assay kit (Promega). After incubation with various concentrations of ChSeco and ChEpo for 24 h, the culture medium was removed and cells were washed twice with PBS. Cells in each well were lysed by adding 100 μl of 0.2% Triton X-100 and then the lysate was incubated with an equal volume of CytoTox-ONE reagent for 10 min and the fluorescence was recorded at an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Spectramax EM Gemini; Molecular Devices). The background fluorescence was subtracted and the percent viability expressed relative to control.

Measurement of intracellular ROS

The ROS was measured based on the hydrolysis and subsequent oxidation of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes). Briefly, the neuronal

cells grown in 24-well plates were incubated with 10 μM DCFH-DA in Krebs-Ringer-HEPES (KRH) buffer (131 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 6 mM glucose, 20 mM HEPES and pH 7.4) at 37°C for 30 min. After washing with KRH buffer twice, the cells were treated with varying concentrations of ChSeco or ChEpo. The time course of appearance of 2′,7′-dichlorofluorescein (DCF), which is fluorescent, was measured at excitation and emission wavelengths of 485 and 538 nm (respectively) using a fluorescent microplate reader (Spectramax EM Gemini; Molecular Devices). Cells containing vehicle, but no ChSeco or ChEpo were carried through the entire protocol. The background fluorescence of the control wells was subtracted at all time points. The results are expressed as raw fluorescence increase over controls.

Measurement of intracellular Ca^{2+} levels

A kinetic study of the increase in intracellular Ca^{2+} was conducted as follows. The neuronal cells were first loaded with 10 μM Fura-2/AM (Molecular Probes) for 30 min at 37°C. The cells were washed free of extracellular Fura-2/AM, resuspended in KRH buffer, and kept at room temperature (25 ± 1°C) for 15 min. The increase in fluorescence was recorded over a 6 h period following the addition of ChSeco or ChEpo. A fluorescence microplate reader (Spectramax EM Gemini; Molecular Devices) set at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm was used to monitor the changes in fluorescence.

Statistical analysis

Values of IC_{50} were calculated by fitting the log concentration-response curves by standard nonlinear regression analysis using Prism 4.0 (GraphPad Software). The results are expressed as mean ± SEM of three or more independent determinations. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni test to determine the significance of difference, and $p < 0.05$ between groups was considered significant.

Results

Cytotoxic effects of ChSeco and ChEpo in murine GT1-7 hypothalamic neurons

Experiments were performed to determine the relative cytotoxic potential of ChSeco and ChEpo in GT1-7 hypothalamic neurons. The neuronal cells exposed to ChSeco showed a dose-dependent loss of viability with an IC_{50} of 21 ± 2.4 μM (Figure 1(A)) in MTS reduction assay and 22 ± 0.9 μM (Figure 1(B)) based on the LDH content. On the contrary, ChEpo tested

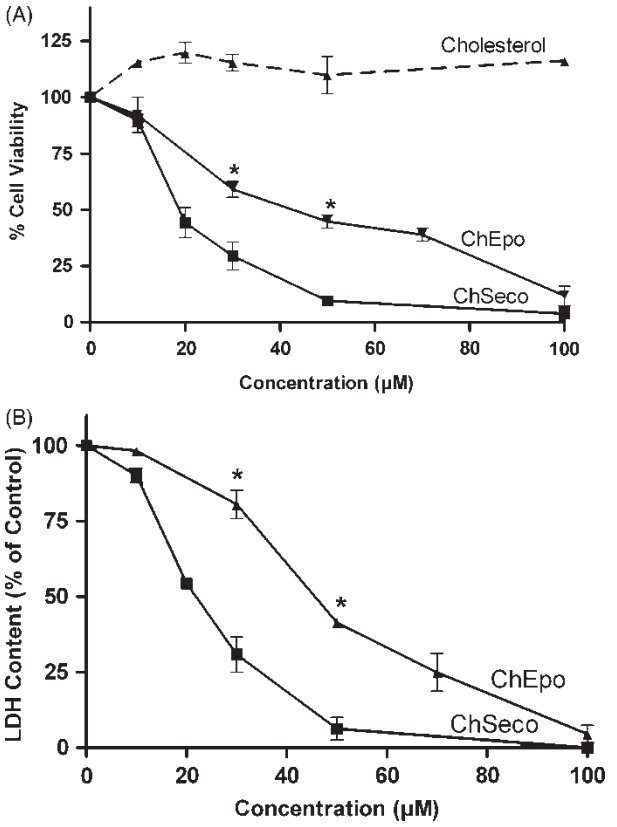

Figure 1. Effect of ChSeco and ChEpo on the viability of GT1-7 hypothalamic neurons. Neuronal cells were treated with various concentrations of ChSeco and ChEpo for 24 h. The cell viability after each treatment was determined by (A) MTS reduction assay using a CellTiter 96 AQueous kit and (B) measurement of LDH content using CytoTox-ONE homogeneous membrane integrity assay kit. Neuronal cells treated with cholesterol were used as negative control. Data presented are mean ± SEM for four independent experiments. *Indicates $p < 0.05$ vs. corresponding ChSeco treatments.

at various comparable concentrations showed less cytotoxicity; the IC_{50} was determined to be 43 ± 3.7 μM (Figure 1(A)) in MTS reduction assay and 42 ± 1.7 μM (Figure 1(B)) based on the LDH content. Controls that did not have either ChSeco or ChEpo but contained cholesterol showed no decrease in cell viability. These results demonstrate that the GT1-7 cells are more sensitive to ChSeco cytotoxicity than to ChEpo.

ChSeco and ChEpo induce generation of reactive oxygen species

Exposure of neuronal cells to 20 μM ChSeco or 50 μM ChEpo caused significant formation of ROS measured over a period of 6 h (Figure 2). We chose to study the ChSeco and ChEpo at concentrations close to their respective IC_{50} values for the relative of comparison of cellular responses to the two ozone-specific cholesterol oxidation products. The induction of ROS was seen as early as 1 h following the

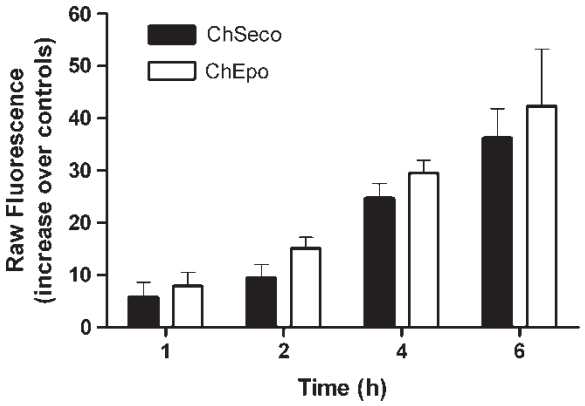

Figure 2. Effect of ChSeco and ChEpo on the generation of ROS in GT1-7 hypothalamic neurons. Following exposure to 20 μM ChSeco or 50 μM ChEpo, the intracellular production of ROS was monitored fluorimetrically for a period of 6 h using a microplate reader based on the hydrolysis and subsequent oxidation of CM-H_2DCFDA to DCF. The basal fluorescence generated by controls was subtracted at all points. The results are expressed as raw fluorescence increase over controls. Data represent the mean ± SEM for four independent experiments. No significant difference between treatments at each time point.

incubation with ChSeco or ChEpo. The yields of ROS measured as increase in raw fluorescence over controls at various time points of 1, 2, 4 and 6 h were not significantly different in neuronal cells exposed to ChSeco and ChEpo.

Effect of antioxidants on ChSeco- and ChEpo-induced cytotoxicity

The effect of the radical scavengers, NAC and Trolox on cell viability was tested on cells treated with ChSeco and ChEpo. Co-incubation with 500 μM Trolox increased the cell viability significantly, whereas 5 mM NAC offered complete protection at all concentrations of ChSeco (Figure 3(A)) and ChEpo (Figure 3(B)).

Intracellular Ca^{2+} in ChSeco- and ChEpo-mediated cytotoxicity

Intracellular Ca^{2+} concentrations as determined by measurement of FURA 2/AM fluorescence were not increased after exposure to 10–20 μM ChSeco or 40–50μM ChEpo. At high concentrations of ChSeco (100 μM) and ChEpo (200 μM), there was an increase in fluorescence, but at these high concentrations the cell membranes were compromised (as assessed by Trypan blue dye exclusion) resulting in Ca^{2+} influx from extracellular medium.

Cytoprotective effects of methyl-β-cyclodextrin and cholesterol on neuronal cells exposed to ChSeco and ChEpo

Experiments were performed to determine the effects of addition of methyl-β-cyclodextrin (MβC) and

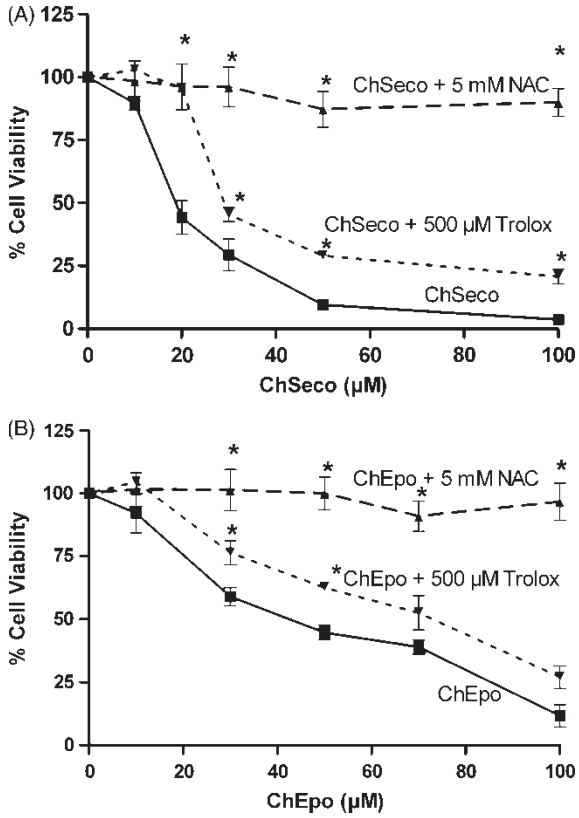

Figure 3. Protective effect of antioxidants on GT1-7 hypothalamic neurons exposed to ChSeco and ChEpo. The neuronal cells were treated with different concentrations of (A) ChSeco and (B) ChEpo for 24 h in presence and absence of antioxidants, NAC (5 mM) and Trolox (500 μM). The cell viability was measured using a CellTiter 96 AQueous kit. The data on cell viability for treatments involving only ChSeco or ChEpo were the same as shown in Figure 1. These data were presented here for ready reference and comparison. *Indicates $p < 0.05$ vs. corresponding ChSeco or ChEpo treatments.

cholesterol on cytotoxicity induced by ChEpo (Figure 4) and ChSeco (Figure 5) in GT1-7 hypothalamic neurons. Pretreatment with MβC (200 μg/ml) potentiated the cytotoxic effects of ChEpo (Figure 4(B)). On the other hand, supplementation of cells with cholesterol offered protection against the cytotoxic effects of ChEpo. The cytoprotective effect of cholesterol was dose-dependent protection with the maximum protection observed at 100 μM (Figure 4(A)).

With regard to ChSeco-induced toxicity, pretreatment with MβC at concentrations of 10–200 μg/ml offered protection against the cytotoxicity induced by 20 μM ChSeco (Figure 5). The effect was dose-dependent eliciting a maximum protection at 200 μg/ml. As can be seen, at MβC concentration of 200 μg/ml, there was a near complete reversal of the cytotoxicity induced by ChSeco. In all these assays, MβC by itself was without any effect on cell viability. Supplementation with cholesterol at concentrations of 50 μM did not show any effects on the cytotoxic effects of ChSeco (data not shown).

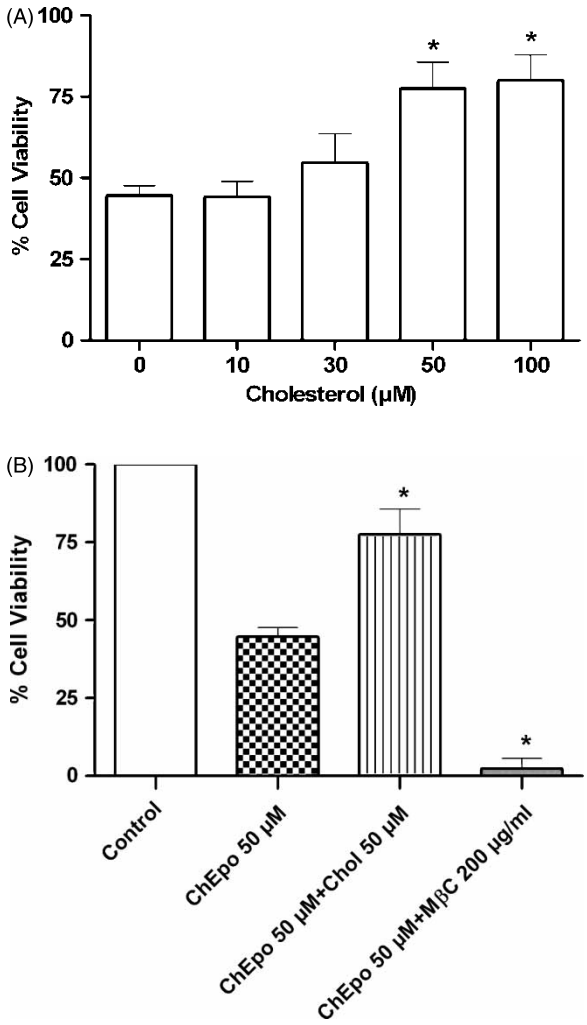

Figure 4. Effect of supplementation of cholesterol on ChEpo-induced cytotoxicity in GT1-7 hypothalamic neurons. (A) Inhibition of ChEpo (50 μM)-induced cell death by varying low concentrations of cholesterol (10–100 μM). (B) Pretreatment with MβC (200 μg/ml) potentiated the cell death induced by ChEpo (50 μM). Cell viability was measured 24 h after the exposure to ChEpo by MTS reduction assay. *Indicates $p < 0.05$ vs. ChEpo treated controls. The abbreviation Chol refers to cholesterol.

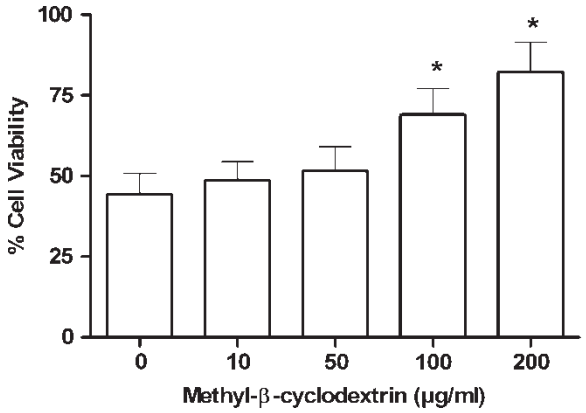

Figure 5. Effect of methyl-β-cyclodextrin on ChSeco-induced toxicity in GT1-7 hypothalamic neurons. Neuronal cells were incubated with ChSeco (20 μM) together with MβC (10-200 μg/ml) for 24 h. The cell viability following these treatments was determined using a CellTiter 96 AQueous kit. *Indicates $p < 0.05$ vs. ChSeco treated controls.

Discussion

In the present study, we show that addition of ChSeco and ChEpo to GT1-7 hypothalamic neurons leads to cell death in 24 h; ChSeco is more potent than ChEpo. Several investigators have shown that oxysterols inhibit DNA synthesis in replicating cells [16–18]. This however may not be contributing to the toxicity observed in hypothalamic neurons as they are post-mitotic. The other possible mechanism could be disruption of intracellular cytoskeletal structures such as vimentin and actin as reported by Palladini et al. [19,20] in studies of oxysterols with endothelial cells. Given the large surface area of dendrites and axons in neuronal cells, the disturbance of membrane function or cytoskeleton could play a significant role in mediating cytotoxic effects of ChSeco and ChEpo in neuronal cells. It is pertinent to mention that ChSeco has been shown to promote the formation of amyloid β-peptide [15], the putative causative agent of Alzheimer's disease. We therefore believe that either amyloid accumulation or a process resulting thereafter, like increased generation of ROS, may also be a contributing factor in the observed cytotoxicity. The concentration of ChSeco used here is 1-2 orders of magnitude higher than the concentrations (0.35–0.44 μM) observed *in vivo* in human brains [13]. However, it is important to note that the concentration of ChSeco of 0.35–0.44 μM in the brain homogenates does not reflect the actual concentration at the sites of toxicity. If one assumes that the cytotoxic responses are due to their incorporation into the plasma membrane or other membrane sites, then the ChSeco concentration at these sites could be 10–100 times higher than 0.33–0.44 μM. This comes close to the solution concentrations of 1–50 μM that were employed in the present study. Further, it is to be noted that the observed effects of ChSeco are seen following an acute exposure of 24 h.

We do find a marked increase in ROS production in neuronal cells exposed to ChSeco and ChEpo. Although the results presented represent the yields of ROS at IC_{50} concentrations, ROS were also formed at lower cytotoxic concentrations of ChSeco and ChEpo. The extent of ROS production is about the same when the two ozone-specific oxysterols are tested at their respective IC_{50} concentrations, meaning that there exists a quantitative relationship between the ROS production and cytotoxicity. The importance of ROS is confirmed further by the observation that NAC and trolox attenuate the cytotoxic effects of ChSeco and ChEpo. The reasons for not finding complete reversal of cytotoxicity by Trolox are not clear. It is possible that the scavenging of oxy radicals by NAC is more wide-spread when compared to trolox which primarily

scavenges peroxyl radicals. These results are in accordance with several other studies that reported a role for ROS in cytotoxic effects of oxysterols in macrophages [21], monocytes [22], smooth muscle cells [23], stromal cells [24] and ovarian granulosea cells [25]. Understandably, ROS is one of the early events in cell death induced by oxysterols [26]. When there is oxidative stress, mitochondria are generally involved in the cell death processes. For example, oxidative stress causes opening of the mitochondrial permeability transition pore (MPTP) [27,28], which in turn, results in the release of various mediators of cell death such as cytochrome *c*, AIF and Smac/DIABLO. ChSeco was found to cause loss of mitochondrial transmembrane potential followed by activation of caspase-3 in GT1-7 neuronal cells [15] and H9c2 cardiomyocytes [unpublished data]. In contrast, ChEpo was shown to have no effect on mitochondrial membrane potential or cytochrome *c* release [29]. Nevertheless, the precise role of mitochondria in mediating the cytotoxicity of oxysterols needs further investigation.

It is intriguing to note that the two ozone-specific oxysterols induce ROS similarly but the neuronal cells respond differently when pretreated with MβC. At the cellular level, MβC is known to promote efflux of cholesterol and, possibly, oxysterols from cells [30–32]. We find when neuronal cells were treated with MβC, the ChSeco-induced cytotoxicity was reversed. This would mean that either ChSeco or cholesterol accumulates in neuronal cells similar to what was described in cultured L cells [18] and macrophages [12,33]. Exogenously added oxysterols, when incorporated, cause a condensing effect in the cell membrane [34]. This process probably was reversed by addition of MβC, thereby resulting in reversal of cytotoxicity.

With regard to ChEpo, we find that the cytotoxic effects were reversed by supplementation with exogenous cholesterol. This is in line with the observations of Pulfer and Murphy [5] who showed that ChEpo inhibited cholesterol synthesis and contributed to increased cytotoxicity in bronchial epithelial cells. The hypocholesterolemic effects resulting from ChEpo were probably more pronounced when neuronal cells were co-incubated with MβC. This would have culminated in additional cytotoxic effects of ChEpo.

Calcium plays an important role in cell death induced by certain oxysterols [35]. However, in the present study, we did not find changes in intracellular Ca^{2+} in neuronal cells exposed to ChSeco and ChEpo. This suggests that Ca^{2+} levels do not play a significant role in the ChSeco- and ChEpo-induced toxicity in GT1-7 hypothalamic neurons.

In summary, the two oxysterols ChSeco and ChEpo elicit comparable cytotoxic responses in murine GT1-7 hypothalamic neurons but differ markedly in their responses to modulation of cholesterol levels. Like most other oxysterols, they mediate cytotoxicity through regulation of ROS but do not involve changes in intracellular Ca^{2+}.

General implications

Oxysterols represent a class of potent regulatory molecules with remarkably diverse, important biological actions. Although oxysterols have been extensively studied for their pivotal role in atherosclerosis, much less is known about the potential effects in the nervous system. Increasing evidence indicates the formation of and role for ChSeco, an ozone specific oxysterol in atherosclerosis and Alzheimer's disease. The origin as well as the metabolic fate of ozone-specific oxysterols is largely unknown and a topic of intense investigation. As the transport system for oxysterols is efficient [11], it is expected that these oxysterols readily redistribute in different parts of the body [36] promoting tissue-specific pathologies such as Alzheimer's disease and atherosclerosis. The concentrations of oxysterols, in particular ChSeco, used in this study are not too high compared to what are detected in human brains and arterial plaque. For example, the concentration ChSeco in human brain samples is 0.35–0.44 μM [13], and in arterial plaque it goes up to 60 μM [12]. The neurotoxic potential of oxysterols, especially, ChSeco raises a number of questions concerning their possible role in neurodegenerative diseases. Translation of these *in vitro* studies into *in vivo* models of neurodegeneration may provide a better understanding of these disease processes.

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