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Intracellular oxidative stress and cytotoxicity in rat primary cortical neurons exposed to cholesterol secoaldehyde

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ABSTRACT

Cholesterol secoaldehyde (ChSeco or 3β-hydroxy-5-oxo-5,6-secocholestan-6-al) has been shown to induce Aβ aggregation and apoptosis in GT1-7 hypothalamic neurons. The present study was undertaken to evaluate the effects of ChSeco on rat primary cortical neuronal cells. ChSeco was cytotoxic at concentrations ranging from 5 to 20 μM, while cholesterol of comparable concentrations showed little or no toxicity. In ChSeco-exposed neuronal cells, there was an increased formation of intracellular peroxide or peroxide-like substance(s), the levels of which were comparable to those found in typical menadione exposures. There was a loss in the mitochondrial transmembrane potential, the extent of which was dependent on concentration of ChSeco employed. Pre-treatment with *N*-acetyl-L-cysteine (5 mM; 1 h) offered protection against the cytotoxicity and the generation of intracellular oxidants. Cytotoxicity of ChSeco was evidenced by the loss of axonal branches and also condensed apoptotic nuclei in these cells. Immunohistochemical analysis revealed a decreased intracellular Aβ42 staining proportional to the loss in the axonal out growth and dendritic branches. The observed decrease in Aβ42 has been suggested to be due to loss of integrity of dendrites and the plasma membrane, possibly resulting from increased production of reactive oxygen species.

Introduction

Cholesterol secoaldehyde (ChSeco or 3β-hydroxy-5-oxo-5,6-secocholestan-6-al) is a new class of oxysterol that can be formed *in vivo* due either to ozone-mediated oxidation of cholesterol [1,2] or Hock cleavage of cholesterol-5α-hydroperoxide [3], the latter being produced at the inflammatory sites from the reaction of singlet oxygen with cholesterol [4]. Although the precise mechanism of formation of this oxysterol is not yet clear, the occurrence of ChSeco has been demonstrated in the brain specimen of patients with Alzheimer's disease (AD) [5] and Lewy body dementia [6]. Recent reports suggest that ChSeco promotes misfolding of proteins [7] and elicits cytotoxic responses in a number of mammalian cell types including cultured human bronchial epithelial cells (16-HBE) [2,8], GT1-7 murine hypothalamic neurons [9], J774 murine macrophages and RAW 264.7 human macrophages [10,11], and H9c2 rat cardiomyoblasts [12,13]. Previous work from our laboratory has shown that ChSeco induces aggregation of Aβ *in vitro* and demonstrated an increased Aβ42 [9] secretion by GT1-7 hypothalamic neurons. Primary cells offer many advantages and are considered to be relevant due to their proximity to *in vivo* physiology. Therefore, in the present study, the cytotoxic effects of ChSeco were investigated in comparison with cholesterol in primary neuronal cultures derived from the rat cerebral cortex. The results demonstrate that ChSeco elicited cytotoxic responses via increased production of intracellular reactive oxygen species (ROS). The ChSeco-induced cytotoxicity was associated with loss of axonal outgrowth and dendritic branches. In addition, there was a decreased intracellular Aβ42 indicating an increased permeability resulting from the loss of integrity of dendrites, the plasma membrane, or both.

Materials and methods

Materials. Cholesterol secoaldehyde was synthesized by ozonation of cholesterol in mixed solvents of methanol and dichloromethane (1/1, v/v) followed by reduction of the peroxide mixtures with Zn/acetic acid [9,12]. *N*-Acetyl-L-cysteine (NAC), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), HEPES, 2-mercaptoethanol, phosphate buffered saline (PBS), poly-D-lysine hydrobromide (γ-irradiated; molecular weight: 70–150 kDa),

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Trizma base, and Triton X-100 were purchased from Sigma (St. Louis, MO). Alexa fluor 488 signal amplification kit for mouse antibodies, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), neurobasal medium, and glutamax were from Invitrogen (Carlsbad, CA); CellTiter 96 aqueous one (MTS/PMS) reagent from Promega (Madison, WI), Aβ42 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); chamber slides and multi-well (6- and 24-) culture plates from Corning (Acton, MA); and MitoCapture kit from Calbiochem (San Diego, CA). All other chemicals used in this study were of the high purity and obtained from standard sources. The multi-well plates were coated with poly-D-lysine (50 μg/ml) according to a protocol outlined by Genlantis (San Diego, CA).

Micro-surgically dissected cortical regions of day 18 embryonic Sprague/Dawley rat brain (Genlantis) were dissociated by digestion with papain (provided by the manufacturer). The neuronal cells harvested by centrifugation at 1100 rpm for 1 min were then re-suspended in the neurobasal medium containing a serum-free supplement (serum was avoided to prevent the growth of astrocytes).

Measurement of cell proliferation and viability. Neuronal cells in the complete neurobasal medium were seeded in 24-well plates (pre-coated with poly-D-lysine) at a cell density of 5×10^4/well and allowed to grow at 37 °C with 5% CO_2 and humidity for 48 h. At various time points, the cultures were examined for neuronal out growth and lack of astrocyte growth. At this stage the neuronal cells were treated with varying low concentrations of ChSeco (5–20 μM) and incubated for 24 h. In certain experiments, NAC (5 mM) was added to the neuronal cells 1 h before ChSeco treatment. At the end of experimental period the medium containing non-adherent dead cells was removed and 500 μl of the MTS/PMS reagent (diluted 1:25 with medium) was added per well. The formation of MTS formazan was measured at 490 nm over an incubation period of 3 h using a Biotek EL 800 microplate reader. Background absorbance of the wells that contained culture medium and ChSeco but without the neuronal cells was subtracted. The viability of untreated control cells (vehicle alone) was set to 100% and the viability in ChSeco-treated cultures was expressed as percentage change relative to the untreated controls. The experiments were repeated three times and the values of mean ± SD were calculated.

Evaluation of cellular morphology. Neuronal cells grown on chambered slides were stained with DAPI (10 μg/ml) after permeabilization with Triton X-100 (0.1 M). The nuclear morphology was visualized under a fluorescent microscope at an excitation wavelength of 350 nm. The cells were counted as either normal (i.e., when the nucleus is homogenously fluorescent) or apoptotic (i.e., when the nucleus is condensed and/or fragmented). A total of 100 cells were counted from each area and an average of three counts was used to calculate the percentage of apoptotic cells.

Measurement of intracellular oxidative stress. An increased oxidative stress and the associated expression of signaling pathway(s) play a crucial role in the cytotoxicity of oxysterols [14]. Therefore, we tested the possibility of increased oxidative stress in primary neurons exposed to ChSeco and un-oxidized cholesterol. For this, the neuronal cells grown in 24-well plates were preloaded with CM-H_2DCFDA (10 μM) in Krebs–Ringer–HEPES (KRH) buffer at 37 °C for 30 min [9]. The cells were then treated with ChSeco or cholesterol at concentrations of 1–15 μM. The time course of formation of intracellular 2,7-dichlorofluorescein (DCF) was measured over a period of 12 h using a Spectramax EM Gemini

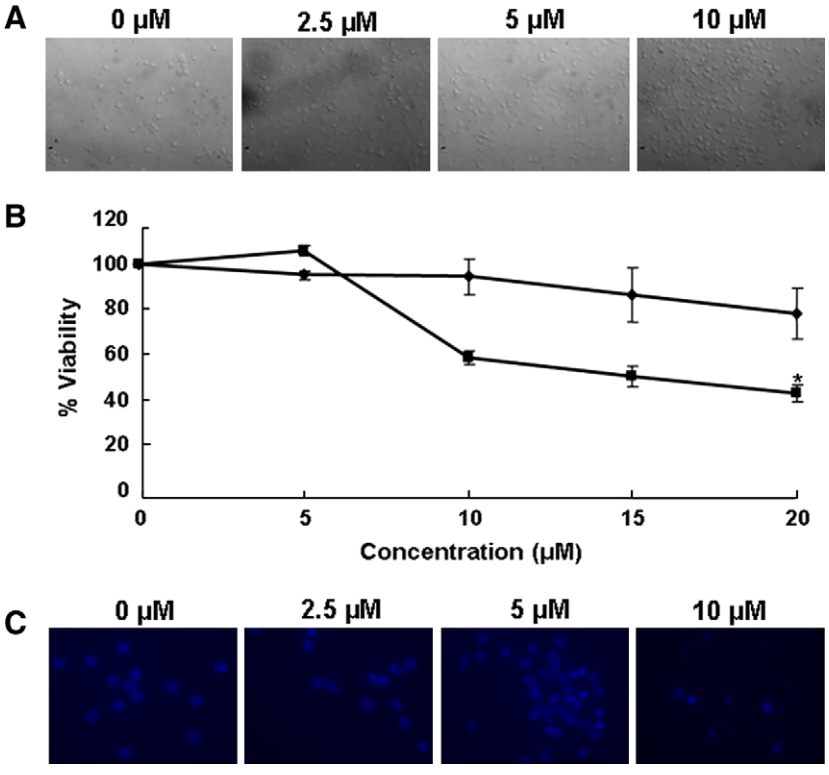

Fig. 1. (A) Loss of dendrite outgrowth, (B) decreased cell proliferation and viability, and (C) appearance of condensed nucleic in primary neuronal cells exposed to cholesterol and ChSeco. For assays of dendrite outgrowth and nuclear morphology, neuronal cells grown in chamber slides were exposed to 2.5–10 μM ChSeco or cholesterol for 24 h. The integrity of dendritic network was examined under phase contrast microscopy and the nuclear morphology assessed based on staining with DAPI performed on Triton X-100-permeabilized neuronal cells. For assays of cell proliferation and viability, neuronal cells were exposed to 5–20 μM (♦) cholesterol or (▲) ChSeco for 24 h. The metabolic activity of cells was based on reduction of MTS in a PMS-assisted reaction using a CellTiter 96 aqueous one kit. Other experimental details are as given in Materials and methods. Data presented are mean ± SD for three independent experiments. *Indicates $p < 0.05$ *vs.* untreated controls.

fluorescent microplate reader (excitation: 485 nm and emission: 538 nm). Neuronal cells treated with vehicle alone were used as negative control and menadione (25 μM)-exposed cells served as positive control. The background fluorescence generated by cells without treatment was subtracted at all time points. The results are expressed as increase in raw fluorescence over controls.

Measurement of mitochondrial transmembrane potential. Changes in the mitochondrial transmembrane potential ($\Delta\Psi m$) were assessed indirectly based on the distribution of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1), a cationic dye, in the mitochondrial and cytoplasmic fractions (MitoCapture kit) [13]. Briefly, neuronal cells grown in 24-well plates were exposed to ChSeco (2.5–10 μM) for 8 h. Followed by removal of the medium and washing with PBS, the cells were incubated with 10 μM JC-1 for 30 min at 37 °C. The unbound, extracellular dye was then removed by washing with PBS. A fresh incubation buffer (500 μl each), provided as part of the kit, was added to each well and the fluorescence was quantified at excitation and emission wavelengths of 488 and 590 nm, respectively, using a Spectramax Gemini EM spectrofluorimeter. The results were expressed as percentage decrease in $\Delta\Psi m$ compared to the untreated controls.

Immunocytochemistry of Aβ42. Primary neuronal cells grown on chamber slides (1×10^4 cells/well) were treated with cholesterol or ChSeco at concentrations of 1–5 μM for 72 h. Following washing with PBS, the cells were fixed using 3.7% formaldehyde (30 min) and permeabilized by incubation with 0.1 M Triton X-100 (5 min). The cells were then washed with PBS and blocked for 30 min at room temperature using PBS that also contained 6% (w/v) BSA. Following a brief washing with 1% (w/v) BSA in PBS, the primary mouse monoclonal antibody for Aβ42 (diluted 1:100 with 1% BSA in PBS) was added and incubated for 1 h at 4 °C. The antigen–antibody reaction was detected by Alexa flour 488 signal amplification kit for mouse antibodies as per manufacturer's instructions. DAPI was used as a counter stain for nucleus. The cells were observed under a fluorescent microscope using green/blue fluorescence.

Results

Cholesterol secoaldehyde elicits cytotoxic responses in rat primary cortical neurons

Fig. 1A shows the morphology of primary cortical neurons examined under phase contrast microscopy. The cells were on chamber slides. Since the cultures were grown on a serum-free medium, there was practically no astrocyte growth as expected. There was a loss of axonal growth and dendritic branches when neuronal cells were exposed to ChSeco. The lowest concentration of ChSeco employed in these assays was 2.5 μM and even at this concentration there was evidence of morphological changes. As the concentration of ChSeco increased to 10 μM, the loss was more profound and cell numbers started to decline, indicating that ChSeco is cytotoxic. In fact, an assay of the metabolic activity of ChSeco-exposed neuronal cells based on MTS reduction (Fig. 1B) confirmed a dose-dependent cytotoxicity of ChSeco over a concentration range of 5–20 μM. Un-oxidized cholesterol was non-cytotoxic at these concentrations. Staining with DAPI showed evidence for condensed and/or fragmented nuclei (Fig. 1C) typical of apoptosis. The percentage of cells with either condensed or fragmented nuclei increased with increase in the concentration of ChSeco employed in these cultures (data not shown), consistent with the measurements of cytotoxicity based on MTS reduction (Fig. 1B).

Evidence for increased intracellular generation of ROS in ChSeco exposures

There was a time- and dose-dependent increase in the intracellular formation of peroxide (possibly H_2O_2) or peroxide-like molecules in primary neuronal cells exposed to ChSeco (Fig. 2).

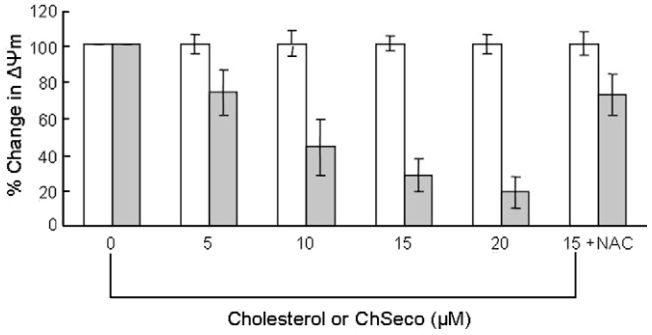

Fig. 3. Changes in mitochondrial transmembrane potential ($\Delta\Psi m$) in primary neuronal cells exposed to ChSeco. Neuronal cells were exposed to ChSeco or cholesterol at a final concentration of 5–20 μM for 8 h. The $\Delta\Psi m$ was measured using the MitoCapture kit as described in Materials and methods. Exposure of neuronal cells to NAC (5 mM; 1 h prior to oxysterol addition) protected neurons from loss of $\Delta\Psi m$. Results are expressed as percent change in $\Delta\Psi m$ compared to untreated controls. Data presented are mean ± SD of three separate experiments.

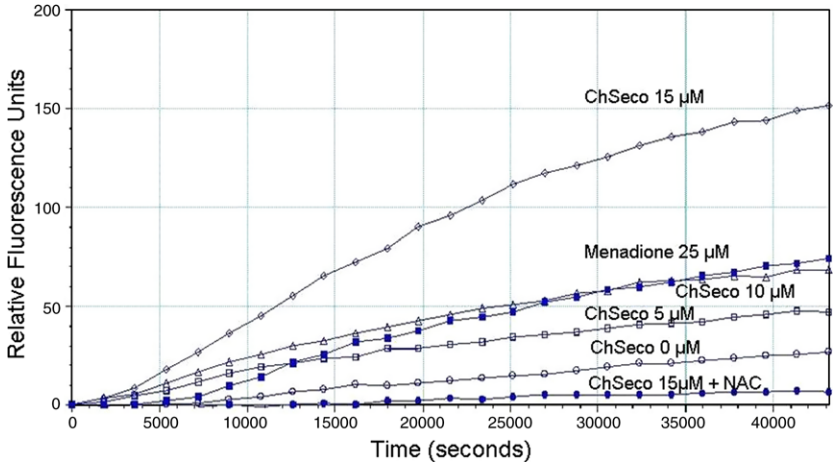

Fig. 2. Generation of intracellular ROS in primary neuronal cells exposed to ChSeco and the effect of supplementation with NAC. Neuronal cells preloaded with CM-H_2DCFDA were exposed to 5–15 μM ChSeco or cholesterol. The appearance of DCF fluorescence was monitored at excitation and emission wavelengths of 485 and 538 nm, respectively, as described in Materials and methods. Treatment with NAC (5 mM; 1 h) was performed prior to exposure to ChSeco or cholesterol. Menadione at a concentration of 25 μM was employed as a positive control.

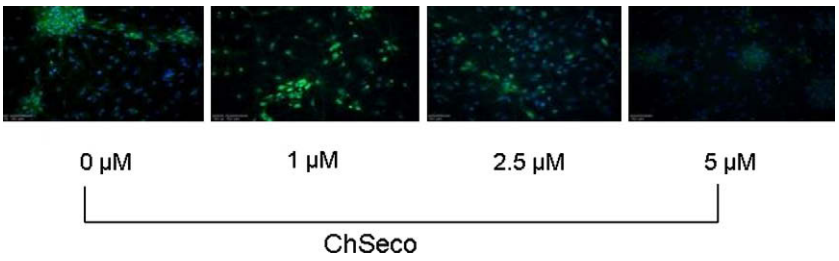

Fig. 4. Dose-dependent decrease in Aβ42 staining in the primary neuronal cells exposed to ChSeco. Neuronal cells grown on chamber slides were treated with of 0 (control), 2.5, and 5.0 μM ChSeco for 72 h. The antigen–antibody reaction was detected by Alexa flour 488 signal amplification kit for mouse antibodies as per manufacturer's instructions. DAPI was used as counter stain for nucleus and the cells examined under a fluorescent microscope using a green/blue filter.

Although the exact chemical nature of these ROS is not clear, it was interesting to note that the extent of their production in ChSeco exposures of 10 μM was comparable to those observed in exposures of menadione (25 μM; Fig. 2). At a concentration of 15 μM ChSeco, both rate and the overall yield of ROS were exceedingly larger than those observed with menadione (25 μM). Pre-incubation of neuronal cells with NAC (5 mM; 1 h) brought about a near total inhibition of the ChSeco-induced formation of ROS (Fig. 2).

Loss of mitochondrial transmembrane potential in primary neurons exposed to ChSeco

Consistent with the increase in ROS (Fig. 2), there was a decisive loss in the mitochondrial transmembrane potential in neuronal cells exposed to ChSeco (Fig. 3). The response was dose-dependent, and pre-incubation of neuronal cells with NAC (5 mM; 1 h) resulted in a substantial reversal of the loss of $\Delta\Psi$m. Un-oxidized cholesterol at concentrations comparable to those of ChSeco caused negligible change in $\Delta\Psi$m (Fig. 3).

Evidence for decreased intracellular Aβ42 in ChSeco-exposed neuronal cells

There was a loss of intracellular Aβ42 in neuronal cells exposed to ChSeco (Fig. 4). As can be seen, the staining of Aβ42 was observed mostly surrounding the nucleus and along the axonal regions of the control and ChSeco-treated cells. The staining was found reduced in ChSeco exposures and the extent of this decrease correlated with loss of axonal growth and dendritic branches (Fig. 1A and C) as well as general loss of neuronal cells (Fig. 1B). In general, in areas where cellular aggregates are present, there was more staining for Aβ42 (Fig. 4).

Discussion

Employing primary neuronal cells, the present study demonstrate for the first time that some of the early manifestations of cytotoxicity of ChSeco are the loss of dendritic branches and reduced axonal outgrowth. These structural changes are followed by the occurrence of condensed and fragmented nuclei, characteristic of apoptotic process. The cytotoxic effects described here may have a clinical implication, given the recent reports that decisive pathways other than those involving the environmental exposure [1,2] or the endogenous production of ozone [15] exist for the formation of ChSeco *in vivo* [3,4,15] and that ChSeco is present in brain samples of patients with AD [5] and Lewy body dementia [6]. The typical concentrations of ChSeco in brain specimens of AD patients (0.35–0.44 μM; [see Refs. [5] and [9]]), which are not very different from normal subjects [5], are about 10 times smaller than the lowest concentration of 2.5 μM used in these experiments. This does not undermine the significance of the role played by ChSeco in AD pathology if one considers factors such as the duration of exposure to ChSeco used in the present study (72 h), the slow and progressive nature of AD and other neurodegenerative diseases, the transient changes in ChSeco concentration *in vivo*, and the membranous sites where ChSeco could accumulate and form potential places for nucleation [9].

Impaired mitochondrial function has been suggested to be the major determinant in age-related pathology of AD [16,17]. In the present study, we observe intracellular peroxide or peroxide-like molecules (DCF fluorescence) were markedly elevated following exposure to ChSeco. The increase in the levels of peroxides are comparable to those observed in typical exposures to menadione, which, by far, is considered to be one of the most powerful inducers of ROS. Based on the observed loss of mitochondrial transmembrane potential and its partial reversal by NAC supplementation, it appears that mitochondria may be the primary site where these peroxides originate. It remains to be established, however, how the peroxides formed induce further signaling resulting in the loss of neuronal cells.

In a previous study, employing an immortalized cell line derived from mouse hypothalamous (GT1-7 neurons) we have shown that exposure to ChSeco results in the formation of amyloid foci, possibly aggregated Aβ, which enlarged in size and numbers with increase in the concentration of ChSeco. With primary neuronal cells, however, we do not see the development of such amyloid foci. Instead, there was a diffused staining of (possibly) monomer form of Aβ42 which decreased with increasing concentration of ChSeco. While an understanding of these differences require further experimentation, the lowered staining observed in the case of primary neuronal cells is thought to be a consequence of loss of Aβ42 due either to altered dendrite integrity, the plasma membrane permeability, or a combination of both. *In vivo*, if a similar loss of Aβ42 occurs, it could result in amyloid aggregation in the intercellular space as often seen in age-related AD pathology.

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