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Effects of cholesterol oxidation products on exocytosis

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ABSTRACT

Increase in levels of oxysterols or cholesterol oxidation products have been detected in brain areas undergoing neuroinflammation after excitotoxic injury, and the present study was carried out to elucidate possible effects of these products on exocytosis in rat pheochromocytoma-12 (PC12) cells. An increase in vesicle fusion with the cell membrane indicating exocytosis was observed by total internal reflection microscopy (TIRFM), and confirmed by capacitance measurements, after addition of 7 ketocholesterol, 24 hydroxycholesterol or cholesterol 5, 6 beta epoxide. 7 ketocholesterol induced exocytosis was attenuated by pretreatment with a disruptor of cholesterol-rich domains or "lipid rafts", methyl-β-cyclodextrin (MβCD) as demonstrated by capacitance and amperometry measurements of neurotransmitter release. Moreover, treatment of cells with thapsigargin to deplete intracellular calcium, or treatment of cells with lanthanum chloride to block calcium channels resulted in attenuation of 7 ketocholesterol induced exocytosis. Fura-2 imaging showed that 7 ketocholesterol induced rapid and sustained increases in intracellular calcium concentration, and that this effect was attenuated in cells that were pre-treated with MβCD, thapsigargin or lanthanum chloride. Together, the results suggest that neurotransmitter release triggered by 7 ketocholesterol is dependent on the integrity of cholesterol rich lipid domains on cellular membranes and a rise in intracellular calcium, either through release from internal stores or influx via calcium channels. Increased cholesterol oxidation product concentrations in brain areas undergoing neuroinflammation may enhance exocytosis and neurotransmitter release, thereby aggravating excitotoxicity.

Cholesterol is an important regulator of membrane lipid organization [13,23]. It influences the physical properties of membranes such as the ordering of phospholipids, membrane fluidity and elastic modulus, and enhances membrane fusion [8]. Cholesterol-rich domains or "lipid rafts" on the cell membrane cluster receptor molecules and recruit intracellular signaling molecules to the aggregated receptors [14]. Some domains contain soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins [4,10,11,32], and disruption of rafts by methyl-β-cyclodextrin (MβCD) reduces exocytosis [11,40,42].

Cholesterol can be converted to oxysterols or cholesterol oxidation products during hypercholesterolemia [28], atherosclerosis [2,26] and excitotoxicity [9,15,16,27,29]. These contain a second oxygen moiety as a carbonyl, hydroxyl, or epoxide group, and can be formed either by enzymatic action or direct oxidation of cholesterol. Enzymatic oxidation of cholesterol occurs along its iso-octyl side chain and seven alpha position of the sterol nucleus. Non-enzymatic oxidations occur at the 7-position on the sterol nucleus, and at the 5, 6 double bond [33]. The insertion of a polar oxygen moiety into the hydrophobic portion of the lipid bilayer is thermodynamically unstable [45]. Cholesterol oxidation products or oxysterols can be tilted with respect to the orientation of cholesterol in the membrane bilayer, such that both oxygen moieties are at the lipid water interface. This could disrupt ordered lipid domains on the cell membrane [21,22]. Cholesterol oxidation products formed by oxidative mechanisms are cytotoxic, and implicated in the pathophysiology of atherosclerosis [2,26] and neurodegenerative diseases [9,16,29].

The fusion of synaptic vesicles and cell membrane during exocytosis can be analyzed by total internal reflection fluorescence microscopy (TIRFM). The aqueous phase immediately adjacent to a glass interface is selectively illuminated in this technique, enabling direct, real time visualization of a pool of fluorescently labeled vesicles that are close to the cell membrane, and fusion events between the vesicles and cell membrane [1,39]. Exocytosis can also be measured by detecting changes in membrane capacitance of cells during voltage clamp conditions. An increase in membrane

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surface area is produced by fusion of vesicles with the cell membrane, resulting in increased capacitance of the cell [5,6,12,44]. In addition, release of catecholamines can be directly detected in rat pheochromocytoma-12 (PC12) cells by amperometry using carbon fiber electrodes [3,7].

Our recent studies showed an increase in levels of cholesterol and cholesterol oxidation products in the rat hippocampus after neuronal injury induced by the glutamate analog, kainate [15,16,27,29]. Cholesterol balances evoked and spontaneous neurotransmission by hindering spontaneous synaptic vesicle turnover but sustaining evoked exo-endocytosis [43,46,47] but thus far, little is known about whether cholesterol oxidation products could affect exocytosis. The present study was therefore carried out to elucidate possible effects of cholesterol oxidation products on exocytosis in PC12 cells.

The effect of external infusion of 2 μM of 24 hydroxycholesterol, 7 ketocholesterol, cholesterol 5, 6 alpha epoxide and cholesterol 5, 6 beta epoxide and 7 beta hydroxycholesterol (all from Sigma–Aldrich, St. Louis, MI, and diluted from 2 mM stock in 0.04% (v/v) ethanol vehicle) on vesicle fusion in PC12 cells was studied by TIRFM as previously described [47]. The concentration of cholesterol oxidation products used (2 μM, or 800 ng/ml) is below the range of concentrations of 7 ketocholesterol detected in the rat hippocampus after kainate excitotoxicity by gas chromatography/mass spectrometry (2500–3100 ng/g tissue) [16]. In brief, PC12 cells plated onto poly-L-lysine-coated glass coverslips and transfected with 2 μg of NPY-EGFP plasmid using FuGENE Transfection Reagent (FuGENE, Roche, USA), 1–2 days before the imaging experiments. The cells were then transferred to buffer solution containing (in mM): 150 NaCl, 5.4 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$, and 10 HEPES (pH 7.4) for TIRFM. The latter was carried out using a Zeiss Axiovert 200 inverted microscope. EGFP was excited by a 488 nm laser and the emission light collected at 520 nm. Time-lapse digital images were acquired at 1 or 0.2 Hz by a CCD camera with exposure time of 18 ms, and image stacks were analyzed using MetaMorph 6.3 software (Universal Imaging, Downingtown, PA). The number of subplasmalemmal vesicles was counted, and the means of 11–24 cells in each treatment plotted.

The effect of external infusion of 2 μM of 24 hydroxycholesterol, cholesterol 5, 6 alpha epoxide, cholesterol 5, 6 beta epoxide, 7 ketocholesterol and 7 beta hydroxycholesterol on PC12 cells was studied by capacitance measurements on PC12 cells under whole-cell voltage clamp conditions, using 3–7 MOhm pipettes as previously described [12,36,44]. Measurements were performed using an EPC-9 patch-clamp amplifier (HEKA Electronics, Germany) and the Lindau-Neher ('sine + dc') technique [12] implemented in Pulse software. A 1000 Hz, 50 mV peak-to-peak sinusoidal voltage stimuli was superimposed onto a DC holding potential of −70 mV. Capacitance values were recorded from each cell during the first 30 s after addition of cholesterol oxidation products, and divided by the value immediately before treatment, to yield a normalized value. This was to take into account slight differences in initial capacitances, between cells of different sizes. The cells appeared healthy and showed stable series resistance with no sudden changes of more than 10% during recording. Ten to twelve cells were recorded in each group. Possible changes in capacitance were analyzed by Student's *t*-test. $P < 0.05$ was considered significant.

Experiments were also carried out to explore possible factors that could affect exocytosis induced by 7 ketocholesterol: (1) Cells were pre-treated with MβCD to determine a possible role of cholesterol rich domains on the cell membrane in the effects of 7 ketocholesterol. MβCD disrupts the integrity of cholesterol rich domains or "lipid rafts" on the cell membrane [17,35,37]. PC12 cells were incubated with 10 mM MβCD (Sigma–Aldrich, St. Louis, MI) for 10 min at 37 °C, and washed twice with PBS before transfer to external solution and addition of 7 ketocholesterol. (2) Cells were pre-treated with thapsigargin to deplete intracellular calcium stores [25], and recorded in zero external calcium conditions, to determine a possible role of intracellular calcium ions in the effects of 7 ketocholesterol. PC12 cells were incubated with 1 μM thapsigargin for 15 min at room temperature and transferred to external solution containing EGTA (Sigma–Aldrich, St. Louis, MI) dissolved in (mM) 150 NaCl, 2.8 KCl, 10 EDTA, 1 $MgCl_2$ and 10 HEPES and 2 mg/ml glucose pH 7.2 (310 mOsm), before addition of 7 ketocholesterol. (3) Cells were pre-treated with thapsigargin and recorded in external solution containing calcium to determine a possible role of calcium release from internal stores, in the effects of 7 ketocholesterol. PC12 cells were incubated with 1 μM thapsigargin for 15 min at room temperature and transferred to external solution containing 10 mM calcium before addition of 7 ketocholesterol. (4) Cells were pre-treated with lanthanum chloride (Sigma–Aldrich, St. Louis, MI) to block calcium channels [34] and recorded in external solution containing calcium to determine a possible role of calcium influx, in the effects of 7 ketocholesterol. PC12 cells were incubated with 10 μM lanthanum chloride for 30 min at room temperature and transferred to external solution containing 10 mM calcium before addition of 7 ketocholesterol.

The quantal release of catecholamines from PC12 cells after 7 ketocholesterol treatment was also analyzed using carbon fiber electrodes as previously described [3]. The recordings were filtered at 1 kHz, digitized at 10 kHz, and analyzed using custom software. Ten to twelve cells were recorded in each group.

Intracellular calcium concentration of PC12 cells was analyzed after external infusion of 7 ketocholesterol as previously described [30,31]. Regions of interest corresponding to PC12 cells were selected, and the ratio of 340/380-nm excitation-emissions in these regions calculated to give an indication of intracellular calcium levels. Ten to twelve cells were recorded in each group.

An increase in the rate of membrane fusion indicating exocytosis was observed after addition of 24 hydroxycholesterol, 7 ketocholesterol, or cholesterol 5, 6 beta epoxide. In comparison, cholesterol 5, 6 alpha epoxide and 7 beta hydroxycholesterol were less effective in inducing vesicle fusion (Fig. 1A and B).

Significant increase in membrane capacitance (Fig. 1C), indicating exocytosis was detected after external infusion of 24 hydroxycholesterol, 7 ketocholesterol, or cholesterol 5, 6 beta epoxide (Fig. 1D). In contrast, non-significant increase in capacitance was observed after addition of cholesterol 5, 6 alpha epoxide (data not shown), 7 beta hydroxycholesterol or ethanol (vehicle control) (Fig. 1D).

Pre-incubation of cells with MβCD resulted in no significant increase in capacitance after addition of 7 ketocholesterol. Cells that were pre-treated with thapsigargin and recorded in zero external calcium conditions, and cells pre-treated with thapsigargin or lanthanum chloride and recorded in external solution containing calcium, showed no increase in capacitance after addition of 7 ketocholesterol (Fig. 1E).

External infusion of 7 ketocholesterol in the external solution resulted in an immediate increase in catecholamine release indicated by significant increase in number of spikes from PC12 cells. Pre-incubation of cells with MβCD resulted in significantly fewer spikes after addition of 7 ketocholesterol, compared to treatment with 7 ketocholesterol alone. Cells that were pre-treated with thapsigargin and recorded in zero external calcium conditions, or cells that were pre-treated with thapsigargin or lanthanum chloride and recorded in external solution containing calcium, also showed significantly fewer spikes after addition of 7 ketocholesterol, compared to 7 ketocholesterol alone (Fig. 2A–G).

External infusion of 7 ketocholesterol in the external solution resulted in an immediate and sustained increase in intracellular calcium in PC12 cells (Fig. 2H). This increase was not observed in cells that had been pre-incubated with MβCD, or pre-treated with