RESEARCH ARTICLE

Increase in Cholesterol and Cholesterol Oxidation Products, and Role of Cholesterol Oxidation Products in Kainate-induced Neuronal Injury

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Little is known about changes in sterols, in particular cholesterol, and cholesterol oxidation products (COPs) in oxidative injury in neural tissues. We have therefore examined changes in cholesterol and COPs using a model of excitotoxic injury. Intracerebroventricular injections of kainate in rats resulted in an increase in immunoreactivity to cholesterol in the affected CA fields of the hippocampus. The increase was confirmed by increased filipin staining of cholesterol in adjacent sections from the same animals, and in hippocampal slice or neuronal cultures after kainate treatment. In neuronal cultures, addition of lovastatin, an inhibitor of cholesterol synthesis, attenuated the increased filipin staining after kainate treatment, indicating that the increase in cholesterol could involve increased cholesterol synthesis. Furthermore, gas chromatographic mass spectrometric (GC/MS) analysis of cholesterol and COPs in kainate-injected rat brain showed a marked increase in cholesterol and COPs including 7-ketocholesterol, 3 days after kainate treatment. The addition of some COPs, including 7-ketocholesterol and cholesterol epoxides to hippocampal slices resulted in neuronal injury as reflected by decreased staining of a neuronal marker in the affected CA fields. The ability of these COPs to produce neuronal injury was attenuated by glutathione, suggesting that oxidative mechanisms are involved in neuronal injury induced by these products. These results, together with GC/MS results that showed significant increase in 7-ketocholesterol at 3 days post-kainate injury suggest that 7-ketocholesterol may be a factor in aggravating oxidative damage to neurons, after the initial stages of kainate-induced neuronal injury.

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Introduction

Cholesterol is essential for neuronal viability and for neuronal and glial cell function in the brain (6). It not only affects the physicochemical properties of neural membranes but also regulates the activities of membrane-bound enzymes, receptors and ion channels. Cholesterol has also been shown to modulate the stability of microtubules in cultured neurons, as well as neuronal functions such as endocytosis and antigen expression (3, 9, 17, 31).

There is considerable turnover of cholesterol in neurons and glia during brain growth, neuronal repair and remodeling (6). Disturbances in cholesterol homeostasis have been implicated in the neurodegenerative disorder Niemann-Pick type C disease (32). Abnormalities in cholesterol metabolism have also been reported to play a role in the pathophysiology of Alzheimer's disease (AD) (8). Clinical and epidemiological data have indicated that patients with elevated cholesterol have increased susceptibility to AD (35). The incidence of AD is higher in western countries suggesting a possible link with the consumption of high-fat and high-caloric diets (20). Changes in the cholesterol content of intracellular membranes alter the trafficking of membrane proteins and cholesterol have been shown to modulate the processing of amyloid precursor protein (APP) as well as the cellular generation of β-amyloid (Aβ) peptide (4, 13, 16). However, the precise mechanism(s) linking disturbances in cholesterol metabolism to the pathophysiology of neurodegenerative disorders are not fully understood.

Cholesterol is susceptible to oxidation leading to the formation of cholesterol oxidation products (COPs). More than 80 COPs have been identified so far, including 7-ketocholesterol, 6-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholesterol 5α, 6α-epoxide, cholesterol 5β, 6β-epoxide, 25-hydroxycholesterol and cholestanetriol (for review, see 41). In recent years, COPs, have drawn much attention mainly because of their potential health implications. Numerous studies have shown that COPs possess biological effects such as mutagenicity (37), angiotoxicity (7, 34), carcinogenicity (27), cytotoxicity (18) and cell membrane

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damaging properties (15, 18, 34, 38). COPs are potent inhibitors of de novo cholesterol biosynthesis (1, 2). Furthermore, COPs have been implicated in atherosclerosis (19, 33, 34).

It is becoming increasingly evident that COPs can modify neuronal membrane function. They not only alter calcium influx but also inhibit the Na^+, Ca^{2+}-exchanger and Ca^{2+}, Mg^{2+} ATPase activity (21). COPs are potent and highly stereospecific modulators of calcium channels and are known to increase fluidity of brain synaptic membranes (28, 43). Among the COPs, 7-ketocholesterol, 24-hydroxy and 25- hydroxycholesterols have attracted considerable attention. The concentration of 24S-hydroxycholesterol (cerebrosterol) is 30 to 1500-fold higher in brain tissue than in any other organ, and it has been reported that plasma 24S-hydroxycholesterol concentration may be a peripheral indicator of neurodegeneration in AD, as well as in other neurodegenerative disorders (25, 26). 7-Ketocholesterol and 25-hydroxycholesterol cause apoptosis and significantly decrease viability of microglial cell cultures (5). In non-neural cells, 7-ketocholesterol is known to induce the conversion of pro-interleukin-1β to interleukin-1β, a cytokine that stimulates $cPLA_2$ activity (10, 23).

Large amounts of free radicals and lipid peroxidation products are formed in the hippocampus after kainate-induced excitotoxic brain injury (11). After kainate injury of the hippocampus, for instance, there is a decrease in glutathione (29), and an increase in the lipid peroxidation product, 4-hydroxynonenal (30). It might be expected that high levels of free radicals and lipid peroxidation products could lead to increased formation of COPs in the degenerating hippocampus, and that these, in turn could lead to neuronal injury. The present study was therefore conducted to investigate this possibility.

Materials and Methods

In vivo and in vitro models. *Intracerebroventricular kainate injections*. Wistar rats weighing approximately 250 g were anesthetized with an intraperitoneal injection of 1.2 ml of 7% chloral hydrate, and the cranial vault exposed. Kainate (1.5 μl of a 1 mg/ml solution) was injected into the right lateral ventricle (coordinates: 1.0 mm caudal to bregma, 1.5 mm lateral to the midline, 4.5 mm from the surface of the cortex) using a microliter syringe. The needle was withdrawn 10 minutes later, and the scalp sutured. Experimental control rats were injected with 1.5 μl of saline instead of kainate.

Hippocampal slice cultures. Organotypic hippocampal slice cultures were prepared as previously described (39), with minor modifications (24). In brief, 10-day old Wistar rat pups were anesthetized with intraperitoneal injections of 7% choral hydrate, decapitated, and the brain removed. The hippocampi were dissected out, and sectioned transversely at 400 μm thickness using a tissue chopper. The slices were transferred to 30 mm Millicell CM culture plate inserts with 0.4 μm polytetrafluoroethylene membranes (Millipore, Bedford, Mass), and placed in 6 well culture plates containing culture medium (50% minimum essential medium [Gibco], 25% fetal calf serum [Sigma], 25% Hanks balanced salt solution [Gibco], supplemented with D-glucose [6.5 mg/ml, Sigma], glutamine [2 mM, Gibco], penicillin G [1 unit/ml, Gibco] and streptomycin sulfate [1 μg / ml, Gibco], pH 7.15). The slices were maintained at 37°C, 100% humidity and 95% air and 5% CO_2. The medium was changed to fresh medium after 3 and 7 days in culture.

Hippocampal neuronal cultures. Two to 4-day-old Wistar rat pups were deeply anesthetized by intraperitoneal injections of chloral hydrate, decapitated, and the brains removed. The hippocampi were dissected out and incubated with 2 ml of 0.25% trypsin at 37°C for 10 minutes. The tissues were then washed 3 times with Dulbecco's modified Eagles medium (DMEM). A cell suspension was then prepared by triturating the tissue with a flame polished Pasteur pipette. The cells were plated on poly D-lysine coated culture dishes at a final density of approximately 500 000 cells per cm^2, and maintained in an incubator at 37°C, 100% humidity and 95% air and 5% CO_2. The culture medium was changed to fresh medium after 3 days in culture, and 2×10^{-5} M cytosine arabinoside (Sigma) added to eliminate glial cells. The culture medium was changed to fresh medium after a further 4 days in culture. All procedures involving animals were approved by the Medical Faculty Animal Care and Use Committee.

Specific methods. *Immunocytochemical labeling of cholesterol in hippocampal sections after kainate injections*. Twenty-four rats were used for this portion of the study, of which 16 were injected with kainate and 8 were saline injected controls. The kainate-injected and control rats were sacrificed at 1 day, 3 days, 1 week, and 2 weeks after injection (4 kainate-injected rats and 2 controls at each time point). The rats were deeply anesthetized by intraperitoneal injection of 1.5 ml of 7% chloral hydrate and perfused through the left cardiac

ventricle with a solution of 4% PF in 0.1 M phosphate buffer (pH 7.4). The brains were removed, and a block consisting of the posterior two thirds of the forebrain, including the hippocampi, dissected out. The blocks were sectioned coronally at 100 μm using a vibrating microtome. The sections were divided into 3 sets: the first two were used for immunocytochemistry, while the third was used for histochemical staining for cholesterol.

Sections intended for immunocytochemistry were washed for 3 hours in phosphate buffered saline (PBS) to remove any traces of fixative, and immersed for one hour in a solution of 2% defatted dry (skimmed) milk in PBS to block non-specific binding of antibodies. They were then incubated overnight with an affinity-purified rabbit polyclonal antibody to the AMPA receptor subunit GluR1 (Chemicon, diluted 1 μg/500 μl) or a mouse monoclonal antibody to cholesterol (MAb 2C5-6). The antibody to GluR1 was used as a marker for hippocampal pyramidal neurons. Dense staining for GluR1 is normally present in these neurons, but staining rapidly decreased in CA fields that were undergoing oxidative stress after kainate treatment (29, 30). The antibody to cholesterol has been previously characterized, and shown to be specific for cholesterol (40). The sections were washed in 3 changes of PBS, and incubated for one hour at room temperature in a 1:200 dilution of biotinylated goat anti-rabbit Ig G, or horse anti-mouse Ig G (Vector). Sections were then reacted for one hour at room temperature with an avidin-biotinylated horseradish peroxidase complex, and the reaction visualized by treatment for 5 minutes in 0.05% 3,3,diaminobenzidine tetrahydrochloride solution in Tris buffer containing 0.05% hydrogen peroxide. The color reaction was stopped with several washes of Tris buffer, followed by PBS. Some sections were mounted on glass slides and lightly counterstained with methyl green before coverslipping. The remaining sections were further processed for electron microscopy. Control sections were incubated with PBS instead of primary antibody. They showed absence of staining.

Electron microscopy was carried out by subdissecting some of the immunostained sections into smaller portions that included the lesioned CA fields. These were dehydrated in an ascending series of ethanol and acetone, and embedded in Araldite. Thin sections were obtained from the first 5 μm of the sections, mounted on copper grids coated with Formvar, and stained with lead citrate. They were viewed using a Jeol 1200EX or a Philips EM208 electron microscope.

Filipin histochemical labeling of cholesterol in hippocampal sections, slices, and neuronal cultures after kainate treatment. i) Hippocampal sections. The third set of sections from the kainate or saline injected rats above was stained by a modification of the filipin method for cholesterol as follows: a stock solution of filipin was prepared by dissolving 3.3 mg of filipin III (Sigma) in 100 μl of DMSO. A working solution was prepared by diluting 3 μl of the stock solution in one ml of PBS, and sections were incubated in this solution for 4 hours in the dark. The sections were washed in PBS, coverslipped using a water-soluble mountant, and viewed using a fluorescence microscope (Zeiss Axiophot with a 365 nm UV filter).

ii) Hippocampal slice cultures. Hippocampal slices were treated with 100 μM kainate after 7 days in culture. They were fixed in a solution of 4% PF in 0.1 M phosphate (pH 7.4) buffer the following day, and stained with filipin using the method described above.

iii) Hippocampal neuronal cultures. Hippocampal neurons were treated with kainate (1:10 dilution of a one mg/ml stock solution of kainate in DMEM), or lovastatin (1:10 dilution of a 1mg / ml stock solution of lovastatin in DMEM) followed 16 hours later by incubation with kainate for 3 hours. They were fixed in 4% PF and 0.1 M phosphate (pH 7.4), and stained for filipin. An image of the cultured neurons was then captured using brightfield illumination, followed by a second image using fluorescence illumination to demonstrate filipin-labeled neurons. The number of filipin-positive neurons was expressed as a percentage of the total number of neurons identified at brightfield, and possible statistical differences in this ratio between kainate-treated, and lovastatin plus kainate-treated neurons analyzed, using the Student's t-test.

Gas chromatographic mass spectrometric (GC/MS) analysis of cholesterol and cholesterol oxidation products in hippocampal homogenates after kainate injections. An additional 14 rats were used for this portion of the study, of which 7 were injected with kainate and the remainder were saline-injected controls. They were sacrificed at 1 and 3 days after injection (3 kainate and 3 saline injected rats at 1 day, and 4 kainate and 4 saline injected rats at 3 days after injection). Rats were anesthetized by chloral hydrate injection, decapitated, and the brains removed. The right hippocampus (side of the intracerebroventricular injection) was rapidly dissected out and stored at -70°C until assay.

All reagents for GC/MS analysis were of analytical grade and obtained from Fisher. Standards for cholesterol, COPs and 5α-cholestane were obtained from Sigma.

All the standards were of at least 95% purity. 5α-cholestane was used as an internal standard to correct for minor variations in injection volume, response and retention time. 5α-cholestane has a similar structure to cholesterol and COPs, but is distinct from the analytes by retention time. Stock solutions containing 1.0 mg of cholesterol and COPs were dissolved in methanol. 5α-cholestane was dissolved in hexane. The stock solutions were used to prepare standard solutions of 5 ng of compound/ml by dilution with methanol. Extraction of lipids was carried out using Folch's method (12) with slight modifications.

The hippocampal specimens were homogenized with 0.5 M NaCl, containing 10 μM of butylated hydroxytoluene (BHT) to a final volume 20 times the weight of the tissue sample. Butylated hydroxytoluene was added to prevent oxidation of the samples due to the homogenization procedure. The homogenate (0.5 ml) was then transferred to a 10 ml centrifuge tube, and 4 ml of extracting solvent (chloroform/methanol 2:1, containing 1.0 mg/ml 5α-cholestane and 10 mg/100 ml BHT) added. The mixture was vortexed, shaken on a linear shaker for 15 minutes, and centrifuged at 2500 rpm for 5 minutes. The upper phase was siphoned away and the lower chloroform phase was washed with 1 ml of distilled water. The upper phase of this mixture was then siphoned away, and the lower chloroform phase evaporated to dryness under a stream of nitrogen. N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA, 100 μl) and dry pyridine (20 μl) were added and the mixture was heated for 30 minutes at 60°C to derivatize to trimethylsilyl esters. The resultant mixture (2 μl) was then injected into the GC/MS.

To rule out the possibility of artificial cholesterol oxidation during the extraction procedure, 4 μg/ml of cholesterol was subjected to the same extraction procedure. The concentrations of COPs were found to be under the limit of detection. Controls for the extraction procedure were carried out to validate that the extraction procedures described above were able to extract cholesterol and oxidation products from homogenates of brain tissues. This was carried out by spiking 20 μl of the standard solutions in homogenates of normal hippocampus, and carrying out the extraction procedures as described above for the hippocampal specimens.

A Hewlett-Packard model 5890 gas chromatograph with a 5972 mass selective detector, was used for the analysis. The data system was an HP VE5/233 computer with an HP G1034C MS Chemstation program. The mass spectrometer was operated in the electron impact mode at 70eV with an ion source temperature of 230°C, and mass/charge ratio range for selected ions in the scan mode from 50 to 550 amu. The instrument was autotuned daily with perfluorotributylamine (PFTBA). The GC/MS interface temperature was 280°C. A 25 mm × 0.2 mm i.d. cross-linked 5%-diphenyl 95%-dimethylsiloxane HP-5 column was used for analysis. The helium flow rate was one ml/min at a linear velocity of 35 cm/sec. The operating temperature of the column varied with the analytes. The injection temperature varied form 180 to 265°C, and the injection was splitless. The oven temperature was 300°C, the column temperatures was 100°C for the first minute, followed by a 10°C/min ramp to 300°C and hold for 12 minutes. Run time was 33 minutes with cholesterol trimethysilyl esters (TMS) eluting at 24 minutes, 7β hydroxycholesterol TMS eluting at 25 minutes, and finally, 7-ketocholesterol TMS eluting at 28 minutes. Equilibration time was 0.50 minutes with a 0.75 minute purge off time. The transfer line temperature was 285°C. The solvent delay was 0.3 minutes, and the electron multiplier was set at autotune voltage. The MS was run under the selected-ion monitoring mode. The amount of cholesterol or COPs detected was divided by the amount of internal standard detected and the weight of the starting material to enable comparison across samples. Statistical analysis was carried out using the Student's t-test.

Effects of cholesterol and cholesterol oxidation products on hippocampal slice cultures. At 7 days in culture, hippocampal slices were treated with cholesterol, 25-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α, 6α-epoxide, cholesterol 5β, 6β-epoxide (1:10 dilution from a 10 mM stock solution in 5% ethanol). A 1:10 dilution of 5% ethanol alone was added to some of the cultured slices, as a control. To elucidate the effect of glutathione on toxicity of COPs, separate plates of cultured slices were used, in which 0.33 mM of reduced glutathione was added 30 minutes before addition of COPs. This concentration of glutathione has been shown to be effective in protecting neurons against β-amyloid induced toxicity in vitro (36).

The slices were fixed in 4% PF in 0.1 M phosphate buffer (pH 7.4) one day after treatment. The polytetrafluoroethylene membranes were cut from the culture plate inserts, and processed with the attached slices for immunocytochemistry to GluR1 using the same method described for brain sections. On completion of immunostaining, the slices were detached from the membranes, and mounted on gelatinized slides. Images of the slices were then captured at ×2.5 magnification, using a Zeiss Axiophot microscope and analysed using Image Pro Plus software (Media Cybernatics, Silver

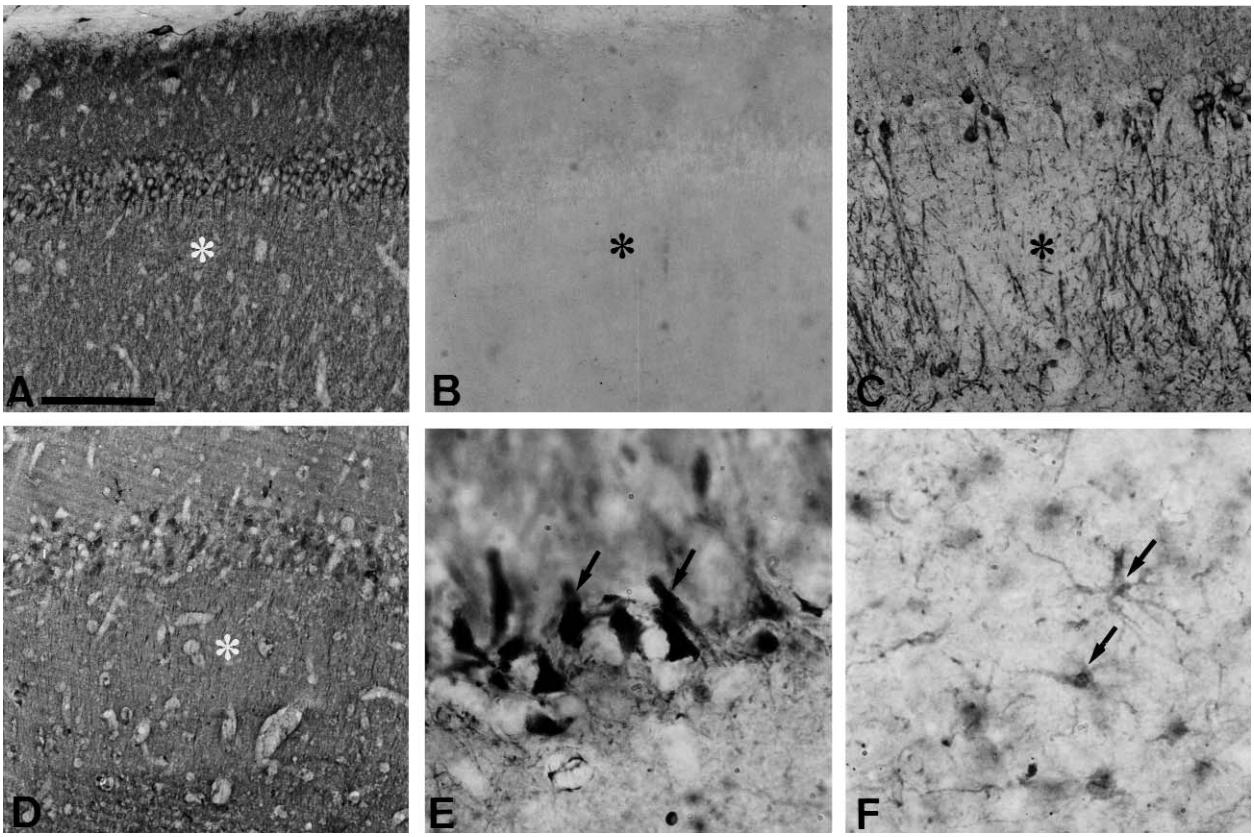


Figure 1. Light micrographs of saline and kainate lesioned hippocampus. **A, B**: sections of field CA1 from the saline treated hippocampus, showing dense staining of the AMPA receptor GluR1 in the cell bodies of pyramidal neurons and the neuropil (**A**, asterisk), but light staining with the antibody to cholesterol (**B**, asterisk). **C, D**: sections of field CA1 from a rat which has been injected with kainate 1 day earlier, showing decrease in staining in the affected CA field (**C**, asterisk), but increase in staining to cholesterol (**D**, asterisk). **E**: higher magnification of field CA3 of a rat which has been injected with kainate 3 days earlier, showing dense cholesterol staining in degenerating pyramidal neurons (arrows). **F**: section through the center of the glial scar of a lesioned CA1 from a rat that has been injected with kainate one week earlier. In contrast to the dense staining of cholesterol in neurons, only light staining to cholesterol is observed in glial cells (arrows). Scale: **A-D** = 150 μm; **E, F** = 40 μm.

Spring, Md). A curved line was traced along the row of hippocampal pyramidal neuronal cell bodies from CA1 to CA4, followed by a second trace, along the pyramidal neurons that showed GluR1 staining. The length of the second trace was expressed as a percentage of the first trace as an indication of "uninjured" pyramidal neurons in the hippocampus. All tracings were done "blind" on coded slides. Six to 12 slices in each treatment category were analyzed. Possible statistical differences between the treatment groups were analyzed, using one-way ANOVA with Bonferroni's multiple comparison test.

Results

Immunocytochemical labeling of cholesterol in hippocampal sections after kainate injections. *Light microscopy. i)* Saline injected rats. The hippocampus of saline injected rats showed dense immunoreactivity for the AMPA receptor subunit GluR1 (Figure 1A) but only very light immunoreactivity for cholesterol (Figure 1B).

ii) One and 3 days after kainate injections. A decrease in GluR1 staining was observed in portions of CA fields at one day post-injection, indicating areas of neuronal injury (Figure 1). The decrease in GluR1 staining was accompanied by an increase in cholesterol immunostaining in the neuropil (Figure 1D) and occasional cell bodies of neurons, in adjacent sections. A further increase in cholesterol staining was observed in the degenerating CA field at 3 days post-kainate injection (Figure 1E). Staining was observed in cell bodies of degenerating neurons and the neuropil in the affected CA fields.

iii) One and two weeks after kainate injections. Areas affected by the kainate injection showed loss of neurons, and a dense glial reaction in Nissl sections. The degenerating neurons at the edge of the glial scar were densely stained for cholesterol, whereas glial cells in the center of the lesions were lightly stained (Figure 1F).

Electron microscopy of cholesterol immunolabeled sections. In contrast to the normal untreated and saline-injected rat hippocampus, which showed very little staining, dense staining for cholesterol was observed in the neuropil, in the one day post-kainate injected hippocampus. Labeling was observed on the cell membranes of processes in the neuropil and the extracellular space, but was absent from endothelial cells of blood vessels (Figure 2A).

The cell bodies of neurons were labeled, in addition to processes in the neuropil, at 3 days post-kainate injection. The nucleus of the neurons was still intact, although large numbers of vacuoles were observed in the cytoplasm (Figure 2B).

At one week post-kainate injection and beyond, labeled degenerating neurons were observed at the edge of the lesioned CA field. The cell and nuclear outlines were indistinct and large numbers of vacuoles were observed in the cytoplasm (Figure 2C).

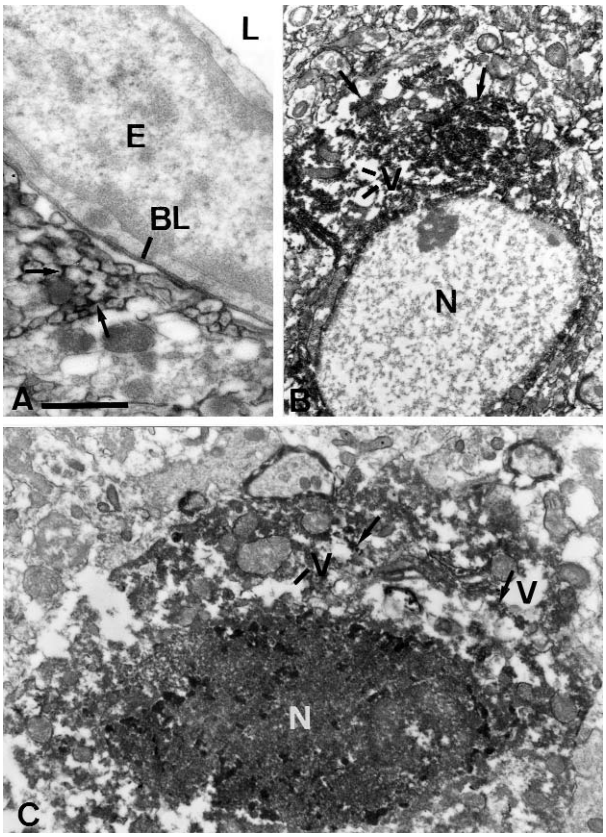


Figure 2. Electron micrographs of kainate lesioned hippocampus stained using an antibody to cholesterol. **A**: section of field CA1, from a rat which has been injected with kainate 1 day earlier, showing dense staining of the intercellular space and cell membranes in the neuropil (arrows), but no staining of the endothelial cells lining a capillary. BL: basal lamina, E: endothelial cells, L: lumen of the capillary. **B**: micrograph of a partially degenerating neuron in a lesioned CA field, in a 3-day post-kainate injected rat. The nucleus (N) is intact, although large numbers of vacuoles (V) are observed in the cytoplasm. Arrows indicate immunoreaction product. **C**: micrograph of a degenerating neuron at the edge of a lesioned CA field, in a one week post-kainate injected rat. The cell and nuclear outlines are indistinct and large numbers of vacuoles are observed in the cytoplasm. Arrows indicate immunoreaction product. Scale: **A** = 1.0 μm; **B** = 1.5 μm; **C** = 2.5 μm.

Filipin histochemical labeling of cholesterol in hippocampal sections, slices, and neuronal cultures after kainate treatment. *Hippocampal sections*. A light, punctate staining was observed in cytoplasmic organelles, in the saline injected rats (Figure 3A). In contrast, a marked increase in staining was observed in the CA field, at one and 3 days post-kainate injection. The cell bodies and dendrites of degenerating neurons were densely stained, but only few labeled glial cells were observed (Figure 3B).

At one and 2 weeks after kainate injection, degenerating neurons at the edge of the glial scar were densely stained for filipin, whereas glial cells in the center of the lesions were unstained or lightly stained.

Hippocampal slice cultures. Untreated slices showed very little filipin staining (Figure 3C). In contrast, an increase in filipin staining was observed after kainate treatment. The increase was observed in degenerating neurons in the CA fields (Figure 3D).

Hippocampal neuronal cultures. Untreated cultured hippocampal neurons showed very little filipin staining (Figure 3E). In contrast, an increase in filipin staining was observed in cultured hippocampal neurons one day after kainate treatment (Figure 3F). Staining was observed in the cell bodies, axons and dendrites of these neurons. Treatment of hippocampal neurons with lovastatin, an inhibitor of cholesterol synthesis, resulted in fewer filipin-labeled cells: the percentage of filipin labeled neurons to the total number of neurons identified at brightfield was 64±8 % for neurons that were treated with kainate only (n = 570), compared to 46±5 % for neurons that were treated with lovastatin plus kainate (n = 791). The difference was significant by t-test ($p<0.05$).

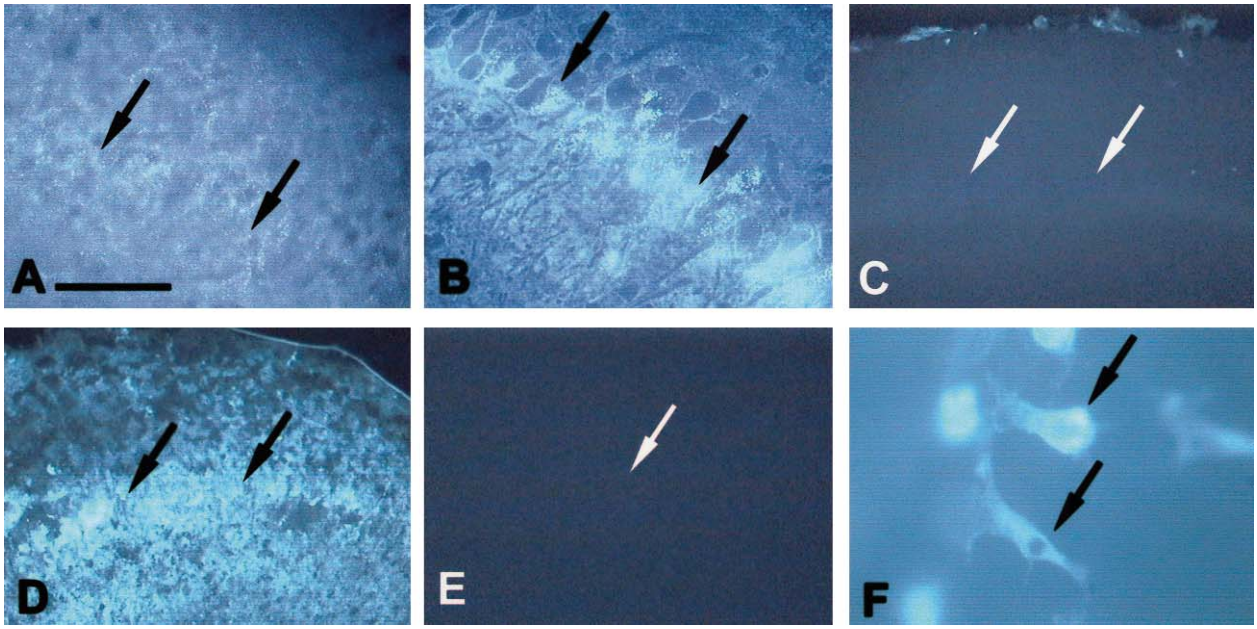


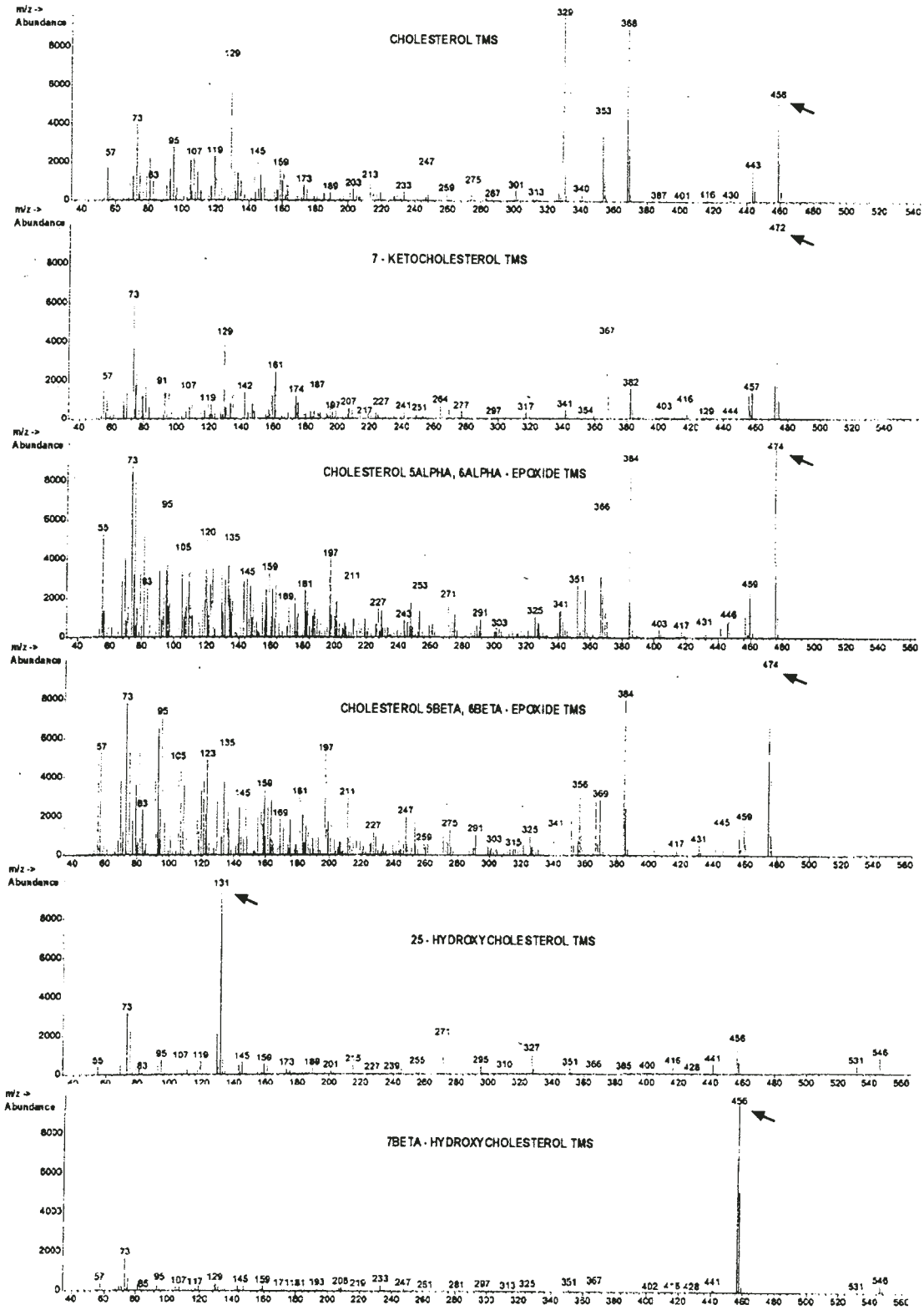
Figure 3. Light micrographs of hippocampal sections, slices and neurons, stained with a histochemical method for cholesterol (filipin stain). **A**: section through field CA3 of a saline injected rat, showing little staining to cholesterol. **B**: section through field CA3 of a rat which has been injected with kainate 1 day earlier, showing intense filipin staining in the affected hippocampal neurons (arrows). **C**: field CA1 of an untreated hippocampal slice, showing very little or no staining in hippocampal neurons (arrows). **D**: field CA1 of a slice that has been treated with kainate, showing dense staining of degenerating pyramidal neurons. **E**: untreated hippocampal neuron (arrow), showing very little or no staining to filipin. **F**: hippocampal neurons that have been treated with kainate 1 day previously, showing increases in staining to filipin (arrows). Scale: **A-D** = 250 μm. **E, F** = 30 μm.

Figure 4. (Opposing page) Detection of cholesterol and COPs in brain tissues spiked with these compounds. Ions at mass charge (m/z) ratios 458 (cholesterol TMS), m/z 131 (25-hydroxycholesterol TMS), m/z 472 (7-ketocholesterol TMS), m/z 456 (7β-hydroxycholesterol TMS), m/z 474(cholesterol 5α, 6α-epoxide TMS and cholesterol 5β, 6β-epoxide TMS) were used to quantify cholesterol and COPs.

Gas chromatographic mass spectrometric (GC/MS) analysis of cholesterol and cholesterol oxidation products in hippocampal homogenates after kainate treatment. *Saline injected rats*. Cholesterol and all COPs could be detected in samples of normal brain that had been spiked with minute quantities of these compounds (Figure 4). Ions at mass charge (m/z) ratios 458 (cholesterol TMS), m/z 131 (25-hydroxycholesterol TMS), m/z 472 (7-ketocholesterol TMS), m/z 456 (7β-hydroxycholesterol TMS), m/z 474(cholesterol 5α,6α-epoxide TMS and cholesterol 5β,6β-epoxide TMS) and ions at m/z 372 (5α-cholestane TMS) were used to quantify cholesterol and COPs as previously described (42). A baseline level of cholesterol was detected in homogenates from the hippocampi of saline injected (Table 1) and one-day post-saline injected rats (Table 1). In addition, a baseline level of the cholesterol oxidation product 7β-hydroxycholesterol and 7-ketocholesterol (mean = 3 and 13 μg/g tissue respectively) was also detected in these extracts (Figure 4). These COPs were detected, despite the addition of BHT to the aqueous homogenization mixture and the organic extracting solvents to protect against oxidative damage, and could represent basal levels of these products that are present in normal brain tissues. Cholesterol added and carried through the extraction procedure did not result in increased formation of COPs.

One day after kainate injection. A trend to an increase (7%), in cholesterol was observed in the kainate injected brains compared to the saline injected brains at one day post-kainate injections, although the difference was not statistically significant. 25-hydroxycholesterol, cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide were found to be under the limit of detection in both the saline and kainate injected brains, even though they were detected in homogenates of normal hippocampi that had been spiked with minute amounts (2 and 4 μg/ml) of these compounds (Figure 5).

Three days after kainate injection. An increase of 70% and 79%, and 32% in cholesterol, 7β-hydroxy-


m/z ->
Abundance
CHOLESTEROL TMS
7 - KETOCHOLESTEROL TMS
CHOLESTEROL 5ALPHA, 6ALPHA - EPOXIDE TMS
CHOLESTEROL 5BETA, 6BETA - EPOXIDE TMS
25 - HYDROXYCHOLESTEROL TMS
7BETA - HYDROXYCHOLESTEROL TMS

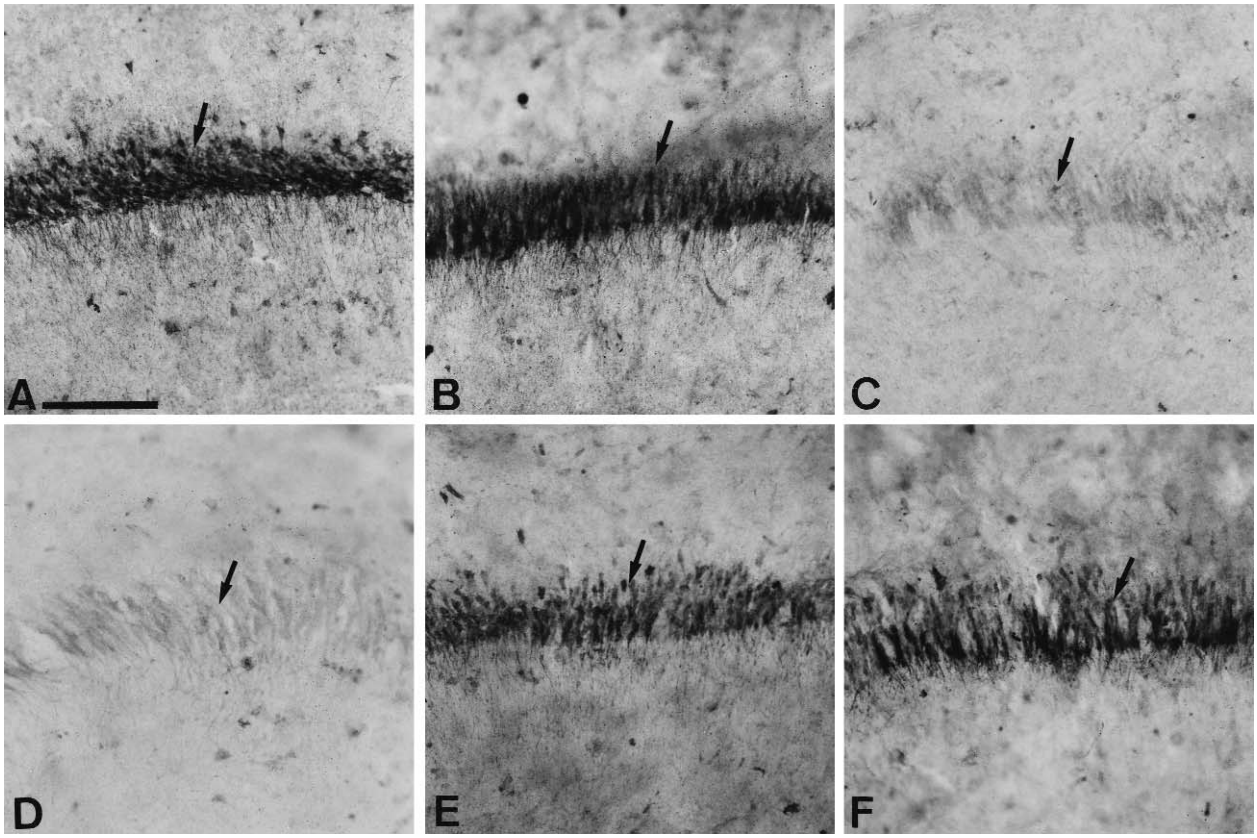


Figure 5. **A-D**: field CA1 of hippocampal slices that have been treated with ethanol, cholesterol or COPs, 7 ketocholesterol and cholesterol 5α, 6α-epoxide, and immunolabelled with antibody to a neuronal marker, GluR1. Dense staining for GluR1 is observed in the cell bodies and dendrites of pyramidal neurons in the slices treated with ethanol (**A**, arrow) or cholesterol (**B**, arrow). This staining is absent in slices treated with COPs 7 ketocholesterol (**C**, arrow) or cholesterol 5α, 6α-epoxide (**D**, arrow). **E, F**: field CA1 of hippocampal slices which have been treated with glutathione, followed by cholesterol oxidation product 7-ketocholesterol (**E**) or cholesterol 5α, 6α-epoxide (**F**), and immnolabelled for GluR1. Dense staining for GluR1 is observed in the cell bodies and dendrites of pyramidal neurons in both cases (arrows in **E** and **F**) indicating a protective effect of reduced glutathione on loss of the neuronal marker. Scale = 250 μm.

cholesterol, and 7-ketocholesterol respectively were observed in the kainate-injected brains compared to the saline injected brains. These differences were found to be statistically significant. 25-hydroxycholesterol, cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide were found to be below the limit of detection in both the saline and kainate injected brains, even though they were detected in homogenates of untreated hippocampi which had been spiked with minute amounts (2 and 4 μg/ml) of these compounds (Table 1).

Effects of cholesterol and cholesterol oxidation products on hippocampal slice cultures. As described previously, dense GluR1 staining was observed in "normal" untreated slices, or slices treated with 5% ethanol alone (Figure 5A; Table 2). Incubation of slices with cholesterol resulted in no significant decrease of GluR1, indicating that cholesterol per se was not harmful to neurons (Figure 5B; Table 2). In contrast, a decrease in GluR1 staining was observed in the CA fields, in rats incubated with COPs (Figure 5C, D; Table 2). The greatest decrease was observed with cholesterol 5β, 6β-epoxide followed by 7-ketocholesterol and cholesterol 5α, 6α-epoxide (Table 2).

Incubation of slices with reduced glutathione prior to addition of COPs was effective in reducing the damage produced by these products. The addition of glutathione prior to addition of 7-ketocholesterol and 5, 6, epoxides significantly prevented the decreases in GluR1 immunoreactivity in the CA fields resulting from treatment with these COPs (Figure 5E, F; Table 2).

	n	Days post kainate injections	Mass Ion	(Kainate-saline/saline) × 100 (%)	P
Cholesterol	3	1	458	7 ± 11	0.59
7-ketocholesterol	3	1	472	0 ± 16	1.00
7β-hydroxycholesterol	3	1	456	-2 ± 8	0.84
Cholesterol 5α, 6α-epoxide	3	1	474	Not detected	-
Choelsterol 5β, 6β-epoxide	3	1	474	Not detected	-
25-hydroxycholesterol	3	1	131	Not detected	-
Cholesterol	4	3	458	70 ± 16	0.005
7-ketocholesterol	4	3	472	32 ± 9	0.022
7β-hydroxycholesterol	4	3	456	79 ± 28	0.030
Cholesterol 5α, 6α-epoxide	4	3	474	Not detected	-
Cholesterol 5β, 6β-epoxide	4	3	474	Not detected	-
25-hydroxycholesterol	4	3	131	Not detected	-

Table 1. Increases in cholesterol and cholesterol oxidation products after kainate treatment. Kainate-saline/saline × 100% indicate the percentage increase in kainate injected hippocampus over saline injected hippocampus. A P value of <0.05 indicates statistical significance.

	Treatment	X ± SEM	P (<0.001)
A	Untreated	97.5 ± 3.8	vs F,H,J
B	5% ethanol	94.5 ± 4.3	vs F,H,J
C	Cholesterol	94.9 ± 3.2	vs F,H,J
D	25-Hydroxycholesterol	90.7 ± 5.1	vs F,H,J
E	Glutathione + 25-hydroxycholesterol	92.3± 4.8	vs F,H,J
F	7-Ketocholesterol	44.1 ± 10.4	vs G,I,K
G	Glutathione +7-ketocholesterol	86.1 ± 12.9	vs H,J
H	Cholesterol 5α, 6α-epoxide	55.4 ± 14.6	vs I,J,K
I	Glutathione + cholesterol 5α, 6α-epoxide	94.1 ± 3.8	vs J
J	Cholesterol 5β, 6β-epoxide	38.0 ± 21.4	vs K
K	Glutathione +cholesterol 5β, 6β-epoxide	85.7 ± 13.4	As above

Table 2. Toxicity of COPs and modulation of toxicity by reduced glutathione. The slices were untreated, treated with 5% ethanol, cholesterol, or COPs 25-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α, 6α-epoxide, and cholesterol 5β, 6β-epoxide. Normal untreated slices, or slices treated with 5% alcohol (a solvent for cholesterol and COPs), or cholesterol showed almost complete staining of the pyramidal cell layer. In contrast, incubation of slices with COPs resulted in significant decreases in GluR1 staining in the CA fields. Incubation of slices with reduced glutathione prior to addition of COPs was effective in reducing the damage produced by COPs. The values represent means ± SEM (n = 10 to 12 in each set) of the percentage of a line drawn along the entire length of the CA field that shows GluR1 positive pyramidal neurons was calculated, and converted to a percentage. Analyzed by one-way ANOVA with Bonferroni's multiple comparison post-hoc test. Significant differences (P<0.001): A vs F,H,J; B vs F,H,J; C vs F, H,J; D vs F,H, J; E vs F,H,J; F vs G,I,K; G vs H,J; H vs I,J,K; I vs J; J vs K.

Discussion

The present study was carried out to elucidate the presence of cholesterol and COPs in the degenerating hippocampus, after kainate induced neuronal injury. Kainate treatment resulted in an increase in cholesterol as shown by 3 different methods (immunostaining, filipin histochemistry and GC/MS) after kainate-induced neuronal injury. The increase in cholesterol is accompanied by accumulation of the COP 7-ketocholesterol 3 days post-kainate treatment. The addition of COPs to hippocampal slices resulted in neuronal injury, and such injury was attenuated by treatment with the antioxidant glutathione, showing that it occurred through oxidative mechanisms. The above results suggesting that COPs may be a factor in continuing and aggravating neurotoxicity, although they are not responsible for the initial stages of kainate-induced neuronal injury.

Little cholesterol was detected by immunostaining in the normal rat hippocampus, even though the brain is known to have a high content of cholesterol. This may be due to the fact that cholesterol is embedded within the lipid bilayer of the membrane (40), and not detected by the anti-cholesterol monoclonal antibody (40) that we employed (MAb 2C5-6).

Kainate injections resulted in a decrease in GluR1 immunoreactivity in select portions of the CA field at one day post-injection. At this time, there was a decrease in immunoreactivity to the antioxidant glutathione, but an increase in immunoreactivity to the lipid peroxidation product 4-hydroxynonenal in the portions of the CA fields that showed decreased GluR1 staining, indicating that these areas were under oxidative stress (29,30). A decrease in GluR1 was therefore taken as a sensitive indicator of areas of neuronal injury.

An increase in cholesterol staining was observed in areas of the hippocampus that showed decreased GluR1 after kainate treatment. The staining was observed in the cell bodies of degenerating neurons. In contrast, glial cells were not labeled or only lightly labeled, indicating that they were not the main cell types that were responsible for the increased cholesterol after kainate lesions. Staining was also absent from endothelial cells in the lesioned CA fields, indicating that increased transport of cholesterol across the blood brain barrier in these regions was unlikely.

The increase in cholesterol as detected by immunostaining with MAb 2C5-6 was corroborated by increased filipin histochemical staining for cholesterol in sections, and in cultured hippocampal slices. As with the immunocytochemical detection of cholesterol, filipin staining showed light staining in the saline injected hippocampus, but dense staining in degenerating neuronal cell bodies in hippocampal sections after kainate treatment.

A similar increase in filipin staining was observed in the degenerating CA fields in hippocampal slice cultures after kainate treatment. The increased filipin staining in slices shows that the increase in cholesterol after kainate treatment is independent of cholesterol that could have been contributed by peripheral organs. The effect of kainate on filipin staining was then studied using dissociated hippocampal neural cultures. Addition of kainate to "pure" neuronal cultures that have been treated with cytosine arabinoside to eliminate glial cells resulted in a marked increase in filipin staining in neurons, showing that the increase in free cholesterol in neurons after kainate treatment did not require glial cells. These observations are consistent with the results of cholesterol immunocytochemistry, and filipin histochemistry, which showed no or light labeling of cholesterol in glial cells.

The addition of lovastatin to neuronal cultures prior to kainate treatment resulted in a significantly smaller proportion of neurons that were labeled with filipin, compared to neurons treated with kainate alone. This indicates that there was increased synthesis of cholesterol in neurons after kainate treatment.

The above observations support the notion that, in addition to cholesterol that could be exposed or released due to the action of membrane degrading enzymes that are activated by kainate treatment (11, 14, 36, 43), much of the increase in cholesterol after kainate treatment was due to increased synthesis in neurons.

The increase in cholesterol in the hippocampus after kainate treatment was further confirmed by gas chromatography/mass spectrometry. An increase in the amount of cholesterol was observed in the hippocampus in the kainate treated rats, compared to saline treated rats. In addition to the increase in cholesterol, an increase in COPs, 7β-hydroxycholesterol and 7-ketocholesterol was also observed in the degenerating hippocampus. This suggests a free radical-mediated attack on the increased cholesterol. Our previous experiments have shown a decrease in glutathione (29) and high levels of lipid peroxidation products (30) in the hippocampus of rats injected with kainate, consistent with a high level of free radical damage in the degenerating hippocampus.

Some of the COPs, including 7-ketocholesterol and cholesterol epoxides were found to cause neuronal injury when applied to hippocampal slice cultures. Since only 7β-hydroxycholesterol and 7-ketocholesterol, but not the other COPs were detected by GC/MS in the present study, the oxidation of C7 position of the cholesterol ring may be more important than that of other carbon positions in generation of COPs after kainate injury. A significant difference in the extent of neuronal injury was observed between slices treated with reduced glutathione and COPs, and those treated with COPs alone. This suggests that COPs could cause neuronal injury by an oxidative mechanism. Glutathione has also been shown to prevent cell damage in non-neural cells, as for instance, in its ability to block 7-ketocholesterol-induced production of reactive oxygen species and apoptosis of human promyelocytic leukemia cells (22).

These results, together with GC/MS results that showed significant increase in 7-ketocholesterol at 3 days post-kainate injury suggest that 7-ketocholesterol

may be a factor in extending oxidative damage to neurons, after the initial stages of kainate-induced neuronal injury.

Further studies are necessary to elucidate possible elevations of cholesterol and COPs in other neurodegenerative diseases, and the precise mechanism of neuronal damage resulting from COPs. In addition to the upregulation of cholesterol synthesis, a possible role for increased phospholipase A_2 activity (36) in the breakdown of neural membrane phospholipids and exposure and release of COPs is worthy of further investigation in kainate-induced neurotoxicity. It is likely that COPs in combination with products of phospholipase A_2 catalyzed reactions (lysophospholipids and oxidized products of arachidonic acid) perturb neuronal membrane integrity and lead to neurodegeneration.

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References

1. Addis PB (1990) Coronary heart disease: An update with emphasis on dietary lipid peroxidation products. Nutr Rev 62:7-10.
2. Addis PB, Warner GJ (1991) The potential health aspects of lipid oxidation products in food. In: Free radicals and food additives aruoma OI, Halliwell B (eds.), pp. 77-119, Taylar and Francis Ltd., London, England.
3. Basu MK, Flamm M, Schachter D, Bertles JF, Maniatis A (1980) Effects of modulating erythrocyte membrane cholesterol on Rho(D) antigen expression. Biochem Biophys Res Commun 95:887-893.
4. Bodovitz S, Klein WL (1996) Cholesterol modulates α-secretase cleavage of amyloid precursor protein. J Biol Chem 271:4436-4440.
5. Chang JY, Chavis JA, Liu LZ, Drew PD (1998) Cholesterol oxides induce programmed cell death in microglial cells. Biochem Biophys Res Commun 249:817-821.
6. Dietschy JM, Turley SD (2001) Cholesterol metabolism in the brain. Curr Opin Lipidol 12:105-112.
7. Dorset DL (1992) Binary phase behavior of angiotoxic oxidized cholesterols with cholesterol. Biochim Biophys Acta 1127:293-297.
8. Eckert GP, Cairns NJ, Maras A, Gattaz WF, Muller WE (2000) Cholesterol modulates the membrane-disordering effects of beta-amyloid peptides in the hippocampus: specific changes in Alzheimer's disease. Dement Geriatr Cogn Disord 11, 181-186.
9. Fan QW, Yu W, Gong JS, Zou K, Sawamura N, Senda T, Yanagisawa K, Michikawa M (2002) Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J Neurochem 80:178-190.
10. Farooqui AA, Ong WY, Horrocks LA, Farooqui T (2000) Brain cytosolic phospholipase A2: localization, role, and involvement in neurological diseases. Neuroscientist 6:169-180.
11. Farooqui AA, Ong WY, Lu XR, Halliwell B, Horrocks LA (2001) Neurochemical consequences of kainate-induced toxicity in brain: involvement of arachidonic acid release and prevention of toxicity by phospholipase A_2 inhibitors. Brain Res Rev 38:61-78.
12. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509.
13. Frears ER, Stephens DJ, Walters CE, Davies H, Austen BM (1999) The role of cholesterol in the biosynthesis of beta-amyloid. Neuroreport 10:1699-1705.
14. Goodrum JF (1991) Cholesterol from degenerating nerve myelin becomes associated with lipoprotein containing apolipoprotein E. J Neurochem 56:2082-2086.
15. Guardiola F, Codony R, Addis PB, Rafecas M, Boatella J (1996) Biological effects of oxysterols: Current status. Food Chem Toxicol 34:193-211.
16. Hartmann H, Eckert A, Muller WE (1994) Apolipoprotein E and cholesterol affect neuronal calcium signalling: the possible relationship to β-amyloid neurotoxicity. Biochem Biophys Res Commun 200:1185-1192.
17. Heron DS, Shinitzky M, Hershkowitz M, Samuel D (1980) Lipid fluidity markedly modulates the binding of serotonin to mouse brain membranes. Proc Natl Acad Sci U S A 77:7463-7467.
18. Higley NA, Taylor SL (1984) The steatotic and cytotoxic effects of cholesterol oxides in cultured cells. Food Chem Toxicol 22:983-992.
19. Imai H, Werthessen NT, Taylor CB, Lee KT (1976) Angiotoxicity and atherosclerosis due to contaminants of USP-grade cholesterol. Arch Pathol Lab Med 100:565-572.
20. Kalmijn S, Launer LJ, Ott A, Witteman JC, Hofman A, Breteler MM (1997) Dietary fat intake and risk of incident dementia in the Rotterdam Study. Ann Neurol 42:776-782.
21. Kutryk MJ, Maddaford TG, Ramjiawan B, Pierce GN (1991) Oxidation of membrane cholesterol alters active and passive transsarcolemmal calcium movement. Circ Res 68:18-26.
22. Lizard G, Gueldry S, Sordet O, Monier S, Athias A, Miguet C, Bessede G, Lemaire S, Solary E, Gambert P (1998) Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. FASEB J 12:1651-1663.
23. Lizard G, Lemaire S, Monier S, Gueldry S, Neel D, Gambert P (1997) Induction of apoptosis and of interleukin-1β secretion by 7β-hydroxycholesterol and 7-ketocholesterol: partial inhibition by Bcl-2 overexpression. FEBS Lett 419:276-280.

24. Lu XR, Ong WY, Halliwell B, Horrocks LA, Farooqui AA (2001) Differential effects of calcium-dependent and calcium-independent phospholipase A2 inhibitors on kainate-induced neuronal injury in rat hippocampal slices. Free Radic Biol Med 30:1263-1273.
25. Lutjohann D, Breuer O, Ahlborg G, Nennesmo I, Siden A, Diczfalusy U, Bjorkhem I (1996) Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proc Natl Acad Sci U S A 93:9799-9804.
26. Lutjohann D, Papassotiropoulos A, Bjorkhem I, Locatelli S, Bagli M, Oechring RD, Schlegel U, Jessen F, Rao ML, von Bergmann K, Heun R (2000) Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. J Lipid Res 41:195-198.
27. Morin RJ, Hu B, Peng SK, Sevanian A (1991) Cholesterol oxides and carcinogenesis. J Clin Lab Anal 5:219-225.
28. Neyses L, Locher R, Stimpel M, Streuli R, Vetter W (1985) Stereospecific modulation of the calcium channel in human erythrocytes by cholesterol and its oxidized derivatives. Biochem J 227:105-112.
29. Ong WY, Hu CY, Hjelle OP, Ottersen OP, Halliwell B (2000). Changes in glutathione in the hippocampus of rats injected with kainate: depletion in neurons and upregulation in glia. Exp Brain Res 132:510-516.
30. Ong WY, Lu XR, Hu CY, Halliwell B (2000) Distribution of hydroxynonenal-modified proteins in the kainate-lesioned rat hippocampus: evidence that hydroxynonenal formation precedes neuronal cell death. Free Radic Biol Med 28:1214-1221.
31. Papahadjopoulos D, Cowden M, Kimelberg H (1973) Role of cholesterol in membranes. Effects on phospholipid-protein interactions, membrane permeability and enzymatic activity. Biochim Biophys Acta 330:8-26.
32. Patterson MC, Vanier MT, Suzuki K, Morris JA, Carstea ED, Neufeld EB, Blanchette-Mackie EJ, Pentchev PG (2001) Niemann-Pick disease type C: a lipid trafficking disorder. In: The metabolic and molecular bases of inherited disease Scriver C, Beaudet A, Sly W, Vale D (eds.), pp. 3611-3634, McGraw Hill, New York.
33. Peng SK, Hu B, Hsiao W, Morin R (1990) Influence of cholesterol oxides on endocytosis of cultured endothelial and smooth muscle cells. Artery 17:84-95.
34. Peng SK, Hu B, Morin RJ (1991) Angiotoxicity and atherogenicity of cholesterol oxides. J Clin Lab Anal 5:144-152.
35. Roher AE, Kuo YM, Kokjohn KM, Emmerling MR, Gracon S (1999) Amyloid and lipids in the pathology of Alzheimer disease. Amyloid 6:136-145.
36. Sandhya TL, Ong WY, Horrocks LA, Farooqui AA (1998) A light and electron microscopic study of cytoplasmic phospholipase A_2 and cyclooxygenase-$_2$ in the hippocampus after kainate lesions. Brain Res 788:223-231.
37. Sevanian A, Peterson AR (1986) The cytotoxic and mutagenic properties of cholesterol oxidation products. Food Chem Toxicol 24:1103-1110.
38. Smith LL, Johnson BH (1989) Biological activities of oxysterols. Free Radic Biol Med 7:285-332.
39. Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173-182.
40. Swartz GM Jr, Gentry MK, Amende LM, Blanchette-Mackie EJ, Alving CR (1988) Antibodies to cholesterol. Proc Natl Acad Sci U S A 85:1902-1906.
41. Tai CY, Chen YC, Chen BH (1999) Analysis, formation and inhibition of cholesterol oxidation products in foods: An overview (Part 1). J Food Drug Anal 7:243-257.
42. Tsuzuki D, Sumino K, Yokoyama M (2000) Analysis of 7-ketocholesterol in low density lipoprotein and fatty acid composition in erythrocyte membranes of patients on maintenance hemodialysis and healthy controls. Clin Chim Acta 295:155-168.
43. Wood WG, Igbavboa U, Rao AM, Schroeder F, Avdulov NA (1995) Cholesterol oxidation reduces Ca^{2+} Mg^{2+}-ATPase activity, interdigitation, and increases fluidity of brain synaptic plasma membranes. Brain Res 683:36-42.