Lovastatin Modulates Increased Cholesterol and Oxysterol Levels and Has a Neuroprotective Effect on Rat Hippocampal Neurons After Kainate Injury

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Abstract
This study was carried out to elucidate the effect of a brain-permeable statin (lovastatin) on cholesterol and oxysterol levels of the hippocampus after neuronal injury induced by the excitotoxin, kainic acid. Increased immunolabeling to cholesterol and the oxysterol biosynthetic enzyme, cholesterol 24-hydroxylase, was observed in the rat hippocampus after kainate lesions. This was accompanied by increased levels of cholesterol, 24-hydroxycholesterol (product of cholesterol 24-hydroxylation enzymatic activity), and 7-ketocholesterol in homogenates of the degenerating hippocampus as detected by gas chromatography/mass spectrometry. Hippocampi from rats or organotypic slices that had been treated with kainate plus lovastatin showed significantly lower levels of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol, compared with those that had been treated with kainate only. Lovastatin also modulated hippocampal neuronal loss after kainate treatment in vivo and in vitro. The level of 24-hydroxycholesterol detected in vivo after kainate treatment (>50 μM) was found to be neurotoxic in hippocampal slice cultures. These results suggest that brain-permeable statins such as lovastatin could have a neuroprotective effect by limiting the levels of oxysterol in brain areas undergoing neurodegeneration.

Key Words: Cholesterol, Cholesterol 24-hydroxylase, Excitotoxic injury, 24-hydroxycholesterol, Oxidative stress, Oxysterol, Statins.

INTRODUCTION
Oxysterol, or cholesterol oxidation products, are products of cholesterol metabolism and play important roles in cholesterol turnover, atherosclerosis, and inflammation (1). Because of their ability to pass through cell membranes and the blood–brain barrier at a faster rate than cholesterol itself, they are also important as transport forms of cholesterol. Oxysterol can be formed by direct oxidation of cholesterol or through the action of oxysterol biosynthetic enzymes (2). 7β-Hydroxycholesterol and 7-ketocholesterol are formed by the direct oxidation of cholesterol (3). In contrast, 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol are produced by enzymatic action. Increased levels of 7β-hydroxycholesterol and 7-ketocholesterol are present in atherosclerotic plaques (4). These 2 oxysterols are also increased in patients with aceruloplasminemia (5).

The enzyme cholesterol 24-hydroxylase synthesizes 24-hydroxycholesterol, and, to a lesser extent, 25-hydroxycholesterol. This enzyme is expressed mainly in the brain and is normally present in neurons (6). The conversion of central nervous system (CNS) cholesterol to 24-hydroxycholesterol, which readily crosses the blood–brain barrier, is a major pathway for brain cholesterol efflux and maintenance of CNS cholesterol homeostasis (7–9). Increased expression of cholesterol 24-hydroxylase has been reported in the brain in Alzheimer disease (10, 11). Oxysterols, including 7-ketocholesterol (12, 13) and 24-hydroxycholesterol (14, 15), are toxic to cells in culture.

Intracerebroventricular injection of kainate, a model of excitotoxic injury, causes acute neuronal death of the affected CA subfields in the hippocampus and proliferation and hypertrophy of glial cells in the glial scar (16). Our previous study has shown that there is significant de novo synthesis of cholesterol in the degenerating hippocampus after neuronal injury (13) and that the cholesterol is directly oxidized to oxysterol at relatively short time intervals (3 days) after kainate injury. The present study was carried out to determine whether there could be changes in oxysterol biosynthetic enzyme expression and oxysterol formation at longer time intervals after kainate injections. We also examined whether treatment by a brain-permeable 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor lovastatin could affect brain cholesterol and oxysterol levels and neuronal survival after kainate injury.

MATERIALS AND METHODS
Animals and Intracerebroventricular Injection
Wistar rats weighing approximately 200 g were anesthetized with an intraperitoneal injection of 1.2 mL of 7% chloral hydrate and the cranial vault exposed. Kainate (1 μL of...
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 microinjection syringe. The needle was withdrawn 10 minutes later and the scalp was sutured. Experimental control rats were injected with 1 μL of saline instead of kainate. All procedures involving animals were in accordance with guidelines of the National Research Council’s guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee.

Western Blots

Western blot was carried out to demonstrate specificity of the cholesterol 24-hydroxylase antibody. Two kainate-injected rats and 2 saline-injected rats were killed 2 weeks after injection. The rats were deeply anesthetized with an intraperitoneal injection of 7% chloral hydrate and killed by decapitation. The right hippocampi from both animals in each treatment group were pooled and homogenized in 10 volumes of ice-cold buffer containing 0.32 M sucrose, 4 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 0.25 mM dithiothreitol. After centrifugation at 1,000 g for 15 minutes, the supernatant was collected and protein concentrations in the preparation were measured using the BioRad protein assay kit. Total proteins (50 μg) were resolved in 10% SDS–polyacrylamide gels under reducing conditions and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Nonspecific binding sites on the PVDF membrane were blocked by incubation with 5% nonfat milk for 1 hour. The PVDF membrane was then incubated overnight with a rabbit polyclonal antibody to cholesterol 24-hydroxylase (diluted to 2 μg/mL in Tris-buffered saline [TBS]). The antibody to cholesterol 24-hydroxylase was raised in the rabbit against amino acids 254–270 of the mouse cholesterol 24-hydroxylase and has been shown to detect a single band at approximately 56 kDa in brain (6). After washing with 0.1% Tween-20 in TBS, the membrane was incubated with horseradish peroxidase-conjugated goat antirabbit immunoglobulin IgG (Amersham) according to the manufacturer’s instructions.

Immunohistochemical Analyses

Immunoperoxidase Labeling

Four kainate-injected rats at each postinjection time intervals were killed at 1 day, 3 days, 1 week, 2 weeks, and 4 weeks after injection. Four saline-injected rats at 2 weeks after injections were used as experimental controls. The rats were deeply anesthetized by intraperitoneal injection of 1.5 mL 7% chloral hydrate and perfused through the left cardiac ventricle with a solution of 4% paraformaldehyde 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 4 sets for cresyl fast violet (Nissl) and immunohistochemical staining as follows: sections were washed for 3 hours in phosphate-buffered saline (PBS) to remove traces of fixative. They were then incubated overnight with a mouse monoclonal antibody to cholesterol (MAB 2C5-6, 1:50 dilution) or a rabbit polyclonal antibody to cholesterol 24-hydroxylase (diluted to 10 μg/mL in PBS). MAB 2C5-6 has previously been characterized and shown to be specific for cholesterol (17). The sections were washed in 3 changes of PBS and incubated for 1 hour at room temperature in a 1:200 dilution of biotinylated horse antimouse IgG or goat antirabbit IgG (Vector, Burlingame, CA). The sections were then reacted for 1 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 (DAB) 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 processed for electron microscopy. Control sections were incubated with cholesterol-absorbed antibody (prepared by incubating 5 μg/mL cholesterol with 1:50 dilution of cholesterol antibody overnight), preimmune rabbit serum, or PBS instead of primary antibodies. They showed absence of staining.

Electron Microscopy

Electron microscopy was carried out by subdissecting some of the immunostained sections into smaller portions that included the lesioned CA1 field. These were osmicated, 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 1010EX electron microscope.

Quantitation of Labeled Cells

The number of cholesterol-positive neurons or cholesterol 24-hydroxylase-positive glial cells in lesioned areas of field CA1 of the right hippocampus of each rat was counted manually using a light microscope. The counts were conducted in a “blind” manner on coded slides at 200× magnification with the help of a grid. A total area of 200 × 300 μm2 from each section and 4 sections from each of the 4 rats in each category were analyzed. The mean number of stained cells/mm2 was then calculated for each group of rats at a specific time interval after kainate injection (n = 4 per group). The “unlesioned” control group consisted of 4 rats at 2 weeks after saline injection.

Double Immunofluorescence Labeling

Four kainate-injected rats and 4 saline-injected rats were killed at 2 week postinjection. The rats were deeply anesthetized by intraperitoneal injection of 1.5 mL 7% chloral hydrate and transcardially perfused with normal saline followed by a fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected out and blocks containing the hippocampus sectioned coronally at 30-μm thickness using a freezing microtome. They were washed for 3 hours in PBS containing 0.1% Triton-X 100 (PBS-Tx) to remove traces of fixative and immersed for
1 hour in a solution of 5% normal goat serum (Vector) in PBS-Tx to block nonspecific binding of antibodies. The sections were then incubated overnight with a rabbit polyclonal antibody to cholesterol 24-hydroxylase (diluted to 10 μg/mL in PBS) and a mouse monoclonal antibody to GFAP (a marker for astrocytes diluted to 1 μg/mL in PBS; Chemicon, Temecula, CA). This was followed by 3 washes of PBS and incubation for 1 hour at room temperature in 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG and Cy3-conjugated goat antimouse IgG (both from Chemicon). The sections were mounted and examined using an Olympus Fluoview FV500 confocal microscopy. Control sections incubated with PBS instead of primary antibodies. They showed absence of staining.

Hippocampal Slice Cultures

Hippocampal slice cultures were prepared as previously described (18) with minor modifications (19). In brief, 10-day-old Wistar rat pups were anesthetized with intraperitoneal injections of 3.5% chloral hydrate, decapitated, and the brains removed. The hippocampi were dissected out and sectioned transversely at 350-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, MA) and placed in 6-well culture plates containing culture medium (50% minimum essential medium [Gibco], 25% horse serum [Sigma, St. Louis, MO], 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, 95% air, and 5% CO2. The medium was changed to fresh medium every 3 days in culture. The effects of kainate and other agents were tested in cultures after 14 days in vitro.

Gas Chromatographic/Mass Spectrometric Analysis

Kainate and Lovastatin Treatment

Initial experiments were performed to elucidate the time course of cholesterol and oxysterol accumulation after kainate injection. Four kainate-injected rats were killed at 3 days, 1 week, 2 weeks, and 4 weeks after injection. The animals were anesthetized by intraperitoneal injections of chloral hydrate and decapitated. The lesioned right hippocampi were quickly removed and snap-frozen in liquid nitrogen and kept in a −80°C freezer until analysis. Four 2-week postsaline-injected rats were used as experimental controls. Subsequent experiments were carried out to investigate possible effects of lovastatin on cholesterol and oxysterol levels after kainate treatment. For in vivo analyses, 11 kainate-injected rats were injected intracerebroventricularly with kainate and intraperitoneally with lovastatin (4 mg/kg) 3 hours after the kainate injection followed by daily injections of the same dose ofLovastatin till the time of death at 1 week (6 rats) or 2 weeks (5 rats) after kainate injection. The rats showed status epilepticus that peaked at 90 to 180 minutes after kainate injection (20), which abated by the time of the first injection ofLovastatin (3 hours after kainate injection). The lovastatin solution was prepared by dissolving pure Lovastatin (gift of Ranbaxy Malaysia Sdn Bhd) in 20% ethanol and diluted to the final concentration with saline (pH 7.4). Fresh solutions were used daily. Another 9 kainate-injected rats were intraperitoneally injected with 20% ethanol in saline for 1 week (4 rats) or 2 weeks (5 rats) and were used as controls. The right hippocampi of these rats were removed for analysis.

In vitro analysis of the effect ofLovastatin was also carried out. Kainate (100 μM final concentration) was applied to slice cultures for 3 hours in serum-free medium. The medium was removed and the slices treated with fresh serum-free medium containing Lovastatin or vehicle for 24 hours. This concentration of kainate has been shown to be toxic to neurons in hippocampal slice cultures (19). Lovastatin was prepared as a 25 mM stock solution in 100% ethanol and stored at 4°C. This was diluted to 2.5 mM solution in sterile water and further diluted in serum-free medium to a final concentration of 1 μM. The vehicle consisted of similarly diluted ethanol. Material from 12 to 16 slices in each treatment group was collected for a single experiment. The mean and standard deviation from 3 separate experiments were then calculated.

All reagents for gas chromatographic/mass spectrometric (GC/MS) analysis were of analytical grade. Standards for cholesterol, 7-ketocholesterol, 25-hydroxycholesterol, cholesteryl 5α,6α-epoxide, cholesterol 5β,6β-epoxide, 5α-cholestanol, and ergosterol were purchased from Sigma and of at least 95% purity. 24S-hydroxycholesterol (nondeuterated) was purchased from Medical Isotopes (Pelham, AL). 5α-cholestanol and ergosterol were used as internal standards. Standard solutions of cholesterol, oxysterols, 5α-cholestanol, and ergosterol were diluted in ethanol.

Lipid Extraction

Extraction of lipids was carried out using the Folch et al method with slight modification (21). Hippocampal specimens were homogenized at 4°C with 1.5 mL PBS (pH 7.4) and 6 mL Folch organic solvent mixture (chloroform/methanol 2:1, containing 0.05% butylated hydroxytoluene [BHT]). The cultured hippocampal slices were homogenized at 4°C with 0.5 mL PBS (pH 7.4) and 2 mL Folch organic solvent mixture. The homogenates were sealed under N2 and centrifuged at 1,000× g for 10 minutes at 4°C. The upper phase was discarded and the lower organic phase carefully transferred to a glass vial and evaporated under a stream of N2.

Lipid Hydrolysis

Two milliliters of 0.5 M KOH (in 50% methanol) was added with 400 ng ergosterol and 10 μg 5α-cholestanol and the vial sealed under N2. The lipid extract was hydrolyzed at 23°C for 2 hours in the dark.

Cholesterol and Oxysterol Extraction

A total of 2.7 mL formic acid (40 mM), 0.2 mL HCl (5 M), and 0.5 mL methanol were added and thoroughly mixed before loading onto a 3 mL, 60-mg Oasis Mixed Anion Exchange (MAX) solid-phase extraction column (Waters, Milford, MA) previously conditioned with 2 mL methanol and 2 mL formic acid (20 mM). The column was
then washed with 2 mL 2% ammonium hydroxide followed by 2 mL methanol/formic acid (40/60). Cholesterol and oxysterol were eluted with 2 mL hexane followed by 2 mL ethyl acetate/hexane (30/70) and collected together into a glass tube containing 50 mg isolate HMN [IST] to remove traces of water. Fifty microliters of the extract was aliquoted into a separate glass vial and evaporated under N₂ for cholesterol analysis and the remaining organic solvent was evaporated under N₂ for oxysterol analysis. Aliquots for cholesterol analysis were derivatized with 25 μL acetonitrile and 25 μL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) for 1 hour at room temperature and injected directly onto GC/MS. Samples for oxysterol analysis were derivatized with 50 μL acetonitrile and 50 μL BSTFA + 1% TMCS for 1 hour at room temperature, evaporated to dryness under N₂, and reconstituted in 30 μL undecane before injection into the GC/MS.

Gas Chromatographic/Mass Spectrometric Measurement

Cholesterol and oxysterol were both analyzed by a Hewlett-Packard 5973 mass selective detector interfaced with a Hewlett-Packard 5890II gas chromatograph (Palo Alto, CA) and equipped with an automatic sampler and a computer workstation. Separations were carried out on a fused silica capillary column (12 m × 0.2 mm inside diameter) coated with crosslinked 5% phenylmethylsiloxane (film thickness 0.33 μm) (Ultra2; Agilent). The carrier gas was helium with a flow rate of 1 mL/minute (average velocity = 55 cm/sec). Selected ion monitoring was performed using the EI mode at 70 eV with the ion source maintained at 230°C and the quadrupole at 150°C. One target ion and 2 qualifier ions selected from each compound mass spectrum were monitored to optimize sensitivity and specificity. Quantitation of modified bases was achieved by relating the peak area of the analyte with its corresponding internal standard.

Cholesterol Analysis

Derivatized samples (1 μL) were injected with a 25:1 split into the GC injection port (280°C). Column temperature was increased from 240°C to 300°C at 25°C/minute after 1 minute at 240°C and then held at 300°C for 4 minutes. Cholesterol was monitored using m/z 329 as target ion and m/z 458, 453 as qualifier ions and 5α-cholestan was monitored as internal standard (target ion = m/z 357, qualifier ions = m/z 372, 232).

Oxysterol Analysis

Derivatized samples (1 μL) were injected with a 5:1 split into the GC injection port (280°C). The column temperature was increased from 175°C to 280°C at 30°C/minute after 1 minute at 175°C and then increased to 291°C at 2°C/minute. Finally, the oven temperature was increased to 306°C at 30°C/minute and held for 1.5 minute. Ergosterol was monitored as internal standard for the quantification of oxysterol. Target ions for 24-hydroxycholesterol and 7-ketocholesterol were m/z 413 and m/z 472, respectively. Quantification of cholesterol and oxysterol was achieved by relating the peak area of the compound with the internal standard peak area and by applying the following formula: ng/mg hippocampus = (A/AIS) × [IS] × (1/k) ÷ hippocampus weight, where A = peak area of product, AIS = peak area of the internal standard, [IS] = concentration of the internal standard (400 ng ergosterol or 10 μg 5α-cholestan), and k = relative molar response factor for each product calculated from the gradient of the calibration curve for each product. Data were expressed as mean ± standard deviation. Calibration curves were constructed from 5 different concentrations in triplicate of cholesterol (5–2,500 μg) as well as oxysterol (5–2,500 ng) and showed good linearity (r² > 0.95).

In Vivo Effect of Lovastatin on Neuronal Survival After Kainate Injury

A further 10 male Wistar rats (each weighing approximately 200 g) were injected with kainate as described. The injected rats were randomly divided into 2 groups (5 rats each). The first group received an intracerebroventricular injection of kainate as described previously followed 3 hours later by an intraperitoneal injection of lovastatin (4 mg/kg dissolved in vehicle consisting of 20% ethanol diluted in saline) followed by daily injections of the same dose of lovastatin until the time of death at 2 weeks after kainate injection. The second group of rats was used as controls. These received intracerebroventricular injection of kainate followed by intraperitoneal injections of vehicle following the same schedule as the lovastatin-treated rats. The rats were killed at 2 week postkainate injection by transcardial perfusion. Blocks containing the hippocampus were sectioned as described. The sections were stained using the Nissl technique or immunostained for microtubule associated protein 2 (MAP2; Sigma; diluted 1:500) to demonstrate viable neurons. The slides were coded and quantitation was carried out in a “blinded” manner. As a result of the patchy nature of the cell loss even within a CA field (i.e. CA1 and CA3) and the enormous total number of cells in the entire hippocampus, direct counting of cells was not carried out. Instead, cell death was estimated by taking digital images of the entire right hippocampus from 6 Nissl- or MAP2-stained sections from each rat and a discontinuous line traced along the row of hippocampal pyramidal neuronal cell bodies from hippocampal fields CA1 to CA3. This was followed by a second trace along the viable pyramidal neurons in the Nissl-stained sections or pyramidal neurons that showed immunolabeling in their cell bodies and dendrites in the MAP2-labeled sections. The ratio of the second to the first trace was calculated to indicate the percentage of uninjured neurons in the CA fields.

In Vitro Effect of Lovastatin on Neuronal Survival After Kainate Injury

Kainate (100 μM) was applied to slice cultures for 3 hours in the serum-free media before treatment of lovastatin (1 μM) or vehicle for 24 hours in fresh serum-free media. Slices (6 slices per group) were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) after treatment. The polytetrafluoroethylene membranes were cut from the culture plate inserts, washed, and immunostained with the attached slices for MAP2. The number of labeled neurons was counted as described.
Cellular injury in the hippocampal slices was also estimated by quantitation of lactate dehydrogenase (LDH) released from the slices into the culture media. The latter were collected after various treatments and analyzed using a LDH cytotoxicity detection kit (Roche, Mannheim, Germany) as follows: neuronal death% = (A - Min)/(Max - Min) × 100, in which A is LDH activity measured in media of test condition, Max is maximum LDH release after 3 hours treatment with Triton X-100 defined as 100% of cell death, and Min is the LDH activity in media of untreated slices. Media from 3 culture dishes in each treatment group was collected for a single experiment. The mean and standard deviation from 3 separate experiments were then calculated.

**In Vitro Effect of Oxysterol on Neuronal Survival After Kainate Injury**

The toxicity of 24-hydroxycholesterol (15 μM and 50 μM of final concentration) on cultured hippocampal slices was determined by MAP2 staining and LDH release as described. Oxysterols were dissolved in 100% ethanol at a concentration of 5 mM and stored at −20°C before use. They were added to slice cultures for 24 hours in serum-free medium. Control slices were treated with vehicle (ethanol). The latter had no effect on cell viability at the concentration used.

**Statistical Analysis**

Experimental data were subjected to statistical analysis using Student t-test or one-way analysis of variance with Bonferroni multiple comparison post-hoc test (SPSS for Windows software). p < 0.05 was considered significant.

**RESULTS**

**Western Blot Analysis**

The antibody to cholesterol 24-hydroxylase detected a single band at approximately 56 kDa in both the saline- and

![Western Blot Analysis](http://jnen.oxfordjournals.org/)

FIGURE 2. (A, B) Light micrographs of sections of hippocampal CA1 field from a saline-injected rat. (A) Cholesterol-immunostained section showing little labeling in neurons (asterisk). (B) Cholesterol 24-hydroxylase-immunostained section showing light labeling of neurons (asterisk). (C, D) Adjacent sections through the affected CA1 field of the right hippocampus from a rat injected with kainate 3 days earlier. (C) Cholesterol-immunostained section showing labeled pyramidal cell bodies (arrows) and diffused labeling of the neuropil (asterisk). (D) Cholesterol 24-hydroxylase-immunostained section showing occasional labeling in glial cells (arrows) but not neurons. (E, F) Adjacent sections through the affected CA1 field of the right hippocampus from a rat that had been injected with kainate 2 weeks earlier. (E) Cholesterol-immunostained section. Labeling is present in the cell bodies and dendrites of pyramidal neurons (arrows). Inset: control section incubated with antigen (cholesterol)-absorbed antibody showing absence of staining in pyramidal neurons. (F) Cholesterol 24-hydroxylase-immunostained section showing labeled glial cells (arrows). (G, H) Adjacent sections through the affected CA1 field of the right hippocampus from a rat that had been injected with kainate 4 weeks earlier. There is little or no staining for cholesterol (G) or cholesterol 24-hydroxylase (H) in the degenerating CA1 field at this time (asterisks). CTRL, saline-injected rat; 3D, 3 days postkainate injection; 2W, 2 weeks postkainate injection; 4W, 4 weeks postkainate injection. Scale bar = 50 μm.
kainate-injected rat hippocampus (Fig. 1). This is consistent with the expected molecular weight of the enzyme and similar to results obtained from the mouse brain (6, 9).

Light Microscopy
Saline-Injected Rats
The saline-injected hippocampus showed light labeling for cholesterol (Fig. 2A; Table 1). The low level of staining may be the result of the fact most of the cholesterol in the brain is associated with cellular membranes and is hidden within the phospholipid bilayer of the membranes (13). Light labeling for cholesterol 24-hydroxylase (Fig. 2B) was also observed. Labeling was observed in the cell bodies and dendrites of pyramidal neurons and punctate profiles in the neuropil.

Kainate-Injected Rats
Three Days After Kainate Injection
Loss of neurons was observed in fields CA1 and CA3 of the hippocampus in Nissl-stained sections (data not shown). The loss was more extensive on the side of the kainate injection than on the contralateral side. An increase in cholesterol immunoreactivity compared with saline-injected rats was observed in the affected CA fields (Fig. 2C). The increased cholesterol staining was observed in the cell bodies and apical dendrites of pyramidal neurons and diffusely throughout the neuropil of the affected areas. In contrast to the increased immunoreactivity to cholesterol, decreased immunoreactivity to cholesterol 24-hydroxylase (Fig. 2D) was observed in neurons of the affected CA field at this time.

One to Two Weeks After Kainate Injection
Loss of neurons and large numbers of glial cells were observed in fields CA1 and CA3 of the hippocampus as judged by Nissl staining. A further increase in cholesterol immunoreactivity compared with 3-day postkainate-injected hippocampus was observed in the affected CA fields (Fig. 2E). The number of cholesterol-positive neurons was significantly greater in the 1-week (919 ± 104 cells/mm²) and 2-week (1,815 ± 225 cells/mm²) postkainate-injected rats compared with saline-injected rats (0 ± 0 cells/mm²) (Table 1). The labeled cells had large-diameter processes that tapered gradually from the cell bodies. They were shown to be astrocytes by

![Image](http://jnen.oxfordjournals.org/)

**FIGURE 3.** (A, B) Electron micrographs of cholesterol 24-hydroxylase-immunolabeled profiles in the hippocampus of a 2-week postkainate-injected rat. (A) Labeled astrocyte (AS) next to an unlabeled microglial cell (M). The nucleus of the astrocyte contains evenly dispersed fine heterochromatin clumps and is distinguished from the marginalized heterochromatin in the microglial cell. F, glial filaments. Arrows indicate reaction product. (B) Astrocytic end foot (AS) on a blood vessel. F, glial filaments; E, endothelial cell; L, lumen of vessel. Arrows indicate reaction product. (C, D) Double, cholesterol 24-hydroxylase (C), red channel, and GFAP (D), green channel) immunofluorescence-labeled sections from field CA1 of a 2-week postkainate-injected rat. The cells that are positive for cholesterol 24-hydroxylase (C, arrows) are also labeled for GFAP (D, arrows), indicating that they are astrocytes. Scale bar = (A, B) 1 μm; (C, D) 100 μm.

### TABLE 1. Number of Cholesterol-Positive Pyramidal Neurons or Cholesterol 24-Hydroxylase-Positive Glial Cells in Field CA1 of the Saline- or Kainate-Injected Rat Hippocampus

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<tr>
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<th>CHO-Neu</th>
<th>CYP46-Au</th>
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<tr>
<td>S</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1D</td>
<td>313 ± 39</td>
<td>157 ± 83</td>
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<tr>
<td>3D</td>
<td>464 ± 63</td>
<td>619 ± 136</td>
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<tr>
<td>1W</td>
<td>919 ± 104</td>
<td>1312 ± 195</td>
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<tr>
<td>2W</td>
<td>1018 ± 145</td>
<td>1815 ± 225</td>
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<tr>
<td>4W</td>
<td>148 ± 81</td>
<td>512 ± 139</td>
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Number of cholesterol-positive pyramidal neurons or cholesterol 24-hydroxylase-positive glial cells in field CA1 of saline- or kainate-injected rat hippocampus. The values are mean ± standard deviation of number of cells/mm² in field CA1. Four sections were counted in each of the 4 rats in each category. Results were analyzed by one-way analysis of variance with Bonferroni multiple comparison post hoc test, p < 0.05 was considered significant. p values < 0.05 are as follows: CHO-Neu: S vs 1D, 3D, 1W, 2W; 1D vs 1W, 2W; 3D vs 1W, 2W; 1W vs 4W; 2W vs 4W. CYP46-Au: S vs 3D, 1D, 1W, 2W; 1D vs 3D, 1W, 2W; 3D vs 1W, 2W; 1W vs 2W; 2W vs 4W; 2W vs 4W. S, saline; 1D, 1 day postkainate injection; 3D, 3 days postkainate injection, 1W, 1 week postkainate injection; 2W, 2 weeks after postkainate injection; 4W, 4 weeks after postkainate injection; CHO, cholesterol staining; Neu, neurons; CYP46, cholesterol 24-hydroxylase staining; As, astrocytes.
TABLE 2. Concentrations of Cholesterol, 24-Hydroxycholesterol, and 7-Ketocholesterol in the Right Hippocampus of the Saline- or Kainate-Injected Rats

<table>
<thead>
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<th>3D</th>
<th>1W</th>
<th>2W</th>
<th>4W</th>
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<tbody>
<tr>
<td>Chol (μg/mg)</td>
<td>23.0 ± 15.8</td>
<td>37.5 ± 16.8</td>
<td>63.0 ± 10.2</td>
<td>98.6 ± 16.7</td>
<td>67.0 ± 4.2</td>
</tr>
<tr>
<td>24-OH-Chol (ng/mg)</td>
<td>7.2 ± 5.4</td>
<td>10.8 ± 3.7</td>
<td>26.2 ± 6.3</td>
<td>30.1 ± 4.9</td>
<td>7.4 ± 2.8</td>
</tr>
<tr>
<td>7-keto-Chol (ng/mg)</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>2.8 ± 0.7</td>
<td>4.2 ± 0.9</td>
<td>2.6 ± 0.1</td>
</tr>
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</table>

Concentrations of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol in the right hippocampi of saline- or kainate-injected rats. The values are expressed by mean ± standard deviation for 4 rats at each time point. Results were analyzed by one-way analysis of variance with Bonferroni multiple comparison post hoc test, p < 0.05 was considered significant. p values < 0.05 are as follows: Chol: S vs 1W, 2W, 4W; 3D vs 2W; 1W vs 2W. 24-OH-Chol: S vs 1W; 2W, 3D vs 1W, 2W, 1W vs 4W; 2W vs 4W. 7-keto-Chol: S vs 2W; 3D vs 2W.

S, saline; 3D, 3 days postkainate injection; 1W, 1 week postkainate injection; 2W, 2 weeks postkainate injection; 4W, 4 weeks postkainate injection. Chol, cholesterol; 24-OH-Chol, 24-hydroxycholesterol; 7-keto-Chol, 7-ketocholesterol.

Four Weeks After Kainate Injection

Little immunoreactivity to cholesterol was observed in the degenerating CA field at this time (Fig. 2G), and the number of cholesterol positive neurons was significantly less than at earlier postinjection time intervals (Table 1). The number of cholesterol 24-hydroxylase-immunoreactive cells was similarly fewer compared with that at 1-week or 2-week postkainate injection (Table 1).

Electron Microscopy

Dense staining for cholesterol 24-hydroxylase was observed in glial cells in the 2-week postkainate-injected rats. The glial cells had large cell bodies with irregular cell outlines. The nucleus contained evenly dispersed fine heterochromatin clumps and absence of dense marginated heterochromatin on the inner aspect of the nuclear envelope. The cytoplasm contained dense bundles of glial filaments. They, thus, had features of astrocytes (Fig. 3A, B).

Double Immunofluorescence Labeling of Cholesterol 24-hydroxylase and GFAP

There was dense staining for cholesterol 24-hydroxylase in glial cells in the 2-week postkainate-injected rats. The glial cells had large cell bodies with irregular cell outlines and were double immunolabeled for GFAP, confirming their identity as astrocytes (Fig. 3C, D).

Gas Chromatographic/Mass Spectrometric Analysis of Cholesterol and Oxyterol in the Kainate-Injected Rat Hippocampus

Kainate injection resulted in significantly greater levels of cholesterol at 1 week (63.0 ± 10.2 μg/mg), 2 weeks (98.6 ± 16.7 μg/mg), and 4 weeks (67.0 ± 4.2 μg/mg) after injection compared with the saline-injected rats (23.0 ± 15.8 μg/mg) (Table 2). Significantly greater levels of 24-hydroxycholesterol were also observed at 1 week (26.2 ± 6.3 mg) and 2 weeks (30.1 ± 4.9 mg) after kainate injection compared with the saline-injected rats (7.2 ± 5.4 mg). The level of 24-hydroxycholesterol returned to baseline level and was not significantly greater than that of the saline-injected rats at 4 weeks after kainate injection. The level of 7-ketocholesterol was also significantly greater at 2 weeks (4.2 ± 0.9 mg) after kainate injection compared with saline injection (1.4 ±

TABLE 3. Effect of Intraperitoneal Injection of Lovastatin on Concentrations of Cholesterol, 24-Hydroxycholesterol, and 7-Ketocholesterol in the Right Hippocampi of 1-Week and 2-Week Postkainate-Injected Rats

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Kainate/Saline</th>
<th>Kainate/Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chol (μg/mg)</td>
<td>1 W</td>
<td>67.6 ± 15.6</td>
<td>41.6 ± 16.0*</td>
</tr>
<tr>
<td></td>
<td>24-OH-Chol (ng/mg)</td>
<td>23.1 ± 10.6</td>
<td>7.2 ± 2.9*</td>
</tr>
<tr>
<td></td>
<td>24-OH-Chol (ng/mg)</td>
<td>27.2 ± 7.8</td>
<td>11.6 ± 3.0*</td>
</tr>
<tr>
<td></td>
<td>7-keto-Chol (ng/mg)</td>
<td>2.9 ± 1.1</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>7-keto-Chol (ng/mg)</td>
<td>3.8 ± 0.8</td>
<td>1.4 ± 0.2*</td>
</tr>
</tbody>
</table>

Effect of intraperitoneal injection of lovastatin on concentrations of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol in the right hippocampi of 1-week and 2-week postkainate-injected rats. The values are expressed by mean ± standard deviation. Results were analyzed by Student test. *p < 0.05 was considered significant.

TABLE 4. Effect of Lovastatin on Concentrations of Cholesterol, 24-Hydroxycholesterol, and 7-Ketocholesterol in Hippocampal Slices

<table>
<thead>
<tr>
<th></th>
<th>CONT</th>
<th>KA</th>
<th>KA/LOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chol (μg/mg)</td>
<td>1.56 ± 0.29</td>
<td>2.17 ± 0.11†</td>
<td>1.68 ± 0.12*</td>
</tr>
<tr>
<td>24-OH-Chol (ng/mg)</td>
<td>0.37 ± 0.05</td>
<td>0.61 ± 0.07†</td>
<td>0.43 ± 0.09*</td>
</tr>
<tr>
<td>7-keto-Chol (ng/mg)</td>
<td>0.42 ± 0.06</td>
<td>1.27 ± 0.46†</td>
<td>0.45 ± 0.09*</td>
</tr>
</tbody>
</table>

Effect of lovastatin on concentrations of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol in hippocampal slices. Data was normalized to the weight of the slices and expressed as mean ± standard deviation of 3 experiments (12–16 slices were used in each treatment group per experiment). Results were analyzed by one-way analysis of variance with Bonferroni multiple comparison post hoc test, p < 0.05 was considered significant.

* Significant difference compared with the KA group.
† Significant difference compared with the CONT group.
CONT, untreated cultures; KA, cultures treated with kainate plus vehicle; KA/LOVA, cultures treated with kainate plus lovastatin; Chol, cholesterol; 24-OH-Chol, 24-hydroxycholesterol; 7-keto-Chol, 7-ketocholesterol.

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Very low or undetectable levels of cholesterol epoxide and 25-hydroxycholesterol were found in the hippocampi of the kainate- or saline-injected rats.

**Effect of Lovastatin on Cholesterol and Oxysterol Concentrations After Kainate Injury**

**In Vivo Analyses**

Systemic administration of lovastatin resulted in significantly lower cholesterol and oxysterol levels in the hippocampus after kainate injection. The level of cholesterol in the 1-week postkainate plus lovastatin-injected rats was 41.6 ± 16.0 μg/mg (Table 3). This was significantly less than that of the kainate plus vehicle-injected rats (67.6 ± 15.6 μg/mg, Table 3), and approximately 65% of the increase in cholesterol level after kainate treatment was blocked by lovastatin.

Lovastatin injection also resulted in significantly lower levels of 24-hydroxycholesterol in the kainate plus lovastatin-injected rats (7.2 ± 2.9 and 11.6 ± 3.0 ng/mg at 1 week and 2 weeks postinjection, respectively) compared with the kainate plus vehicle-injected rats (23.1 ± 10.6 and 27.2 ± 7.8 ng/mg) (Table 3). This effect was even more pronounced than that of cholesterol, and approximately 85% of the increase in 24-hydroxycholesterol after kainate treatment was blocked by lovastatin.

The level of 7-ketocholesterol in the kainate plus lovastatin-injected rats (2.0 ± 0.9 ng/mg) was comparable to that of the kainate plus vehicle-injected rats (2.9 ± 1.1 ng/mg) at 1 week after kainate injection but was significantly lower.

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compared with the kainate plus vehicle-injected rats at 2 weeks after kainate injection (1.4 ± 0.2 ng/mg compared with 3.8 ± 0.8 ng/mg) (Table 3).

In Vitro Analyses

The changes in cholesterol and oxysterol levels after kainate/kainate plus lovastatin treatment in vivo were also observed in vitro, although the absolute levels of cholesterol and oxysterol per weight of “slice tissue” was much lower than the brain. This could be the result of increased extracellular space in the slices. Kainate treatment of hippocampal slice cultures also resulted in significantly greater levels of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol compared with the untreated slices (Table 4). The levels of cholesterol and oxysterol in the kainate plus lovastatin-treated slices were significantly lower compared with the kainate plus vehicle-treated slices (Table 4).

Effect of Lovastatin on Neuronal Survival After Kainate Injury

In Vivo Analyses

Systemic administration of lovastatin partially protected hippocampal neurons after kainate injection as shown by representative Nissl-stained sections (Fig. 4A). Nissl-stained sections showed 48.9 ± 10.6% cell survival in the kainate plus lovastatin-injected rats. This was significantly more than the 17.0 ± 8.7% uninjured neurons in the kainate plus vehicle-injected rats (Fig. 4B). MAP2-immunostained sections similarly showed greater proportion of uninjured neurons (47.3 ± 5.7%) in the kainate plus lovastatin-injected rats compared with the kainate plus vehicle-injected rats (19.2 ± 9.6%, Fig. 4B).

In Vitro Analyses

Lovastatin also partially protected hippocampal neurons from kainate injury in slice cultures. Significantly lower MAP2-positive neuronal cells in fields CA1 and CA3 of the hippocampus was detected after kainate treatment compared with the untreated slices (Fig. 5A, B). Slices that had been treated with the kainate plus lovastatin showed significantly lower neuronal loss compared with those treated with the kainate plus vehicle (Fig. 5A, B). The protective effect of lovastatin was confirmed by detection of lower LDH activity in the culture media of kainate plus lovastatin-treated slices (50.9 ± 10.2% of total LDH release) compared with kainate plus vehicle-treated slices (75.2 ± 8.3% of total LDH release) (Fig. 5C).

Effect of 24-hydroxycholesterol on Neuronal Survival

In Vivo Analyses

Treatment of the lower concentration of 24-hydroxycholesterol (15 μM) had no toxic effect on cultured hippocampal slices as shown by MAP2-stained sections (Fig. 6A). In contrast, the higher concentration of 24-hydroxycholesterol (50 μM) showed significantly fewer numbers of MAP2-positive neuronal cells in both CA1 and CA3 of the cultured hippocampus compared with the untreated slices (Fig. 6A, B).
24-Hydroxycholesterol induced neuronal injury was confirmed by the detection of increased LDH release in the culture media of the 24-hydroxycholesterol (50 μM)-treated slices compared with the vehicle-treated slices (Fig. 6C). Treatment with 7-ketocholesterol (50 μM) also resulted in toxicity (Fig. 6C).

**DISCUSSION**

Increased cholesterol immunoreactivity was detected in the degenerating CA fields of the hippocampus after kainate lesions using a specific antibody to cholesterol. The cholesterol labeling was observed in the cell bodies and dendrites of pyramidal neurons, which are known to degenerate after kainate injury (22). An increased immunolabeling of the oxysterol biosynthetic enzyme cholesterol 24-hydroxylase was also observed in glial cells in the affected areas. Electron microscopy confirmed that the glial cells had dense bundles of glial filaments, whereas double immunofluorescence labeling showed that cholesterol 24-hydroxylase-positive glial cells were double labeled with GFAP, confirming that they were astrocytes.

The increase in cholesterol levels after kainate injury was confirmed by GC/MS analysis. A significant elevation in cholesterol levels was detected at 1 week, 2 weeks, and 4 weeks after kainate injection compared with saline-injected controls. Similar increases in cholesterol were detected in hippocampal slice cultures after kainate treatment. The technique of GC/MS measures the absolute amount of cholesterol in tissue samples regardless of whether it is present within cell membranes or free in the cytosol. Because little cholesterol is thought to enter the brain from the bloodstream (23), the increase in brain cholesterol that is detected by this method therefore indicates de novo cholesterol synthesis. Increases in 24-hydroxycholesterol and 7-ketocholesterol levels were also detected by GC/MS after kainate injury in vivo and in vitro. The in vitro results suggest that the increased cholesterol and oxysterol in brain tissue after kainate injury may be a consequence of increased cholesterol synthesis and cholesterol 24-hydroxylase expression in the degenerating brain tissue and not only or primarily uptake from the bloodstream. This notion is supported by a previous study, which found that most of the 24-hydroxycholesterol in the serum is derived from the brain (8).

The present study adds to our previous study, which showed that there is increased cholesterol and oxysterol levels in the hippocampus at short time intervals (3 days) after kainate injury (13). In this study, we showed that there were further significant increases in cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol levels in the degenerating hippocampus at later time points (i.e. 2 weeks after the initial excitotoxic insult). The increases in cholesterol and oxysterol followed a similar pattern, increasing up to 2 weeks after kainate injection, followed by a decrease to near preinjection levels at 4 weeks postinjection. Other oxysterols such as cholesterol 5a,6a-epoxide, cholesterol 5b,6b-epoxide, and 25-hydroxycholesterol were also analyzed in our samples, but these were not detected.

Neurotoxic effects of cholesterol metabolites have been reported recently (24). 24-hydroxycholesterol and 7-ketocholesterol are toxic to cultured cerebellar granule neurons SH-SY5Y human neuroblastoma cells (14, 25). 24-hydroxycholesterol and 7-ketocholesterol produce neuronal death (apoptosis) by caspase-3 activation, DNA fragmentation, and decreasing mitochondrial membrane potential (12, 15). Our recent studies also showed that 7-ketocholesterol, cholesterol 5a,6a-epoxide, and cholesterol 5b,6b-epoxide were neurotoxic to pyramidal neurons in slice preparations of the rat hippocampus (13). The oxysterols, together with other factors such as iron-induced free radical damage (26), might contribute to increasing neuronal death in the hippocampus with time after kainate injury (22). The toxicity of 24-hydroxycholesterol has been reported to be equal to that of 7-ketocholesterol in SH-SY5Y cells (15).

Slice cultures were treated with low (15 μM) and high (50 μM) concentrations of 24-hydroxycholesterol to determine possible neurotoxicity on hippocampal neurons. These concentrations were chosen based on 24-hydroxycholesterol levels detected in vivo (approximately 7.2 ng/mg tissue in the saline-injected hippocampus and approximately 26.2 ng/mg tissue in the 1-week postkainate-injected hippocampus) and assuming a specific gravity of 1.05 g/cm³ (27). The results showed that the lower concentration of 24-hydroxycholesterol (15 μM or approximately 7.2 ng/mg tissue) was not toxic to hippocampal neurons, whereas the higher concentration (50 μM or approximately 26.2 ng/mg tissue) was neurotoxic. These results suggest that the level of 24-hydroxycholesterol encountered in vivo after kainate injury was sufficient to cause neuronal damage.

The effect of lovastatin on brain oxysterol levels and neuronal survival after kainate injury was also investigated. Rats that had been injected with kainate plus lovastatin showed lower levels of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol at 1 and 2 weeks after injection compared with rats injected with kainate and vehicle. Interestingly, the level of 24-hydroxycholesterol was approximately 7.2 ng/mg tissue (nontoxic range) after lovastatin treatment. This could be related to an observed protective effect ofLovastatin on hippocampal neurons after kainate treatment. Nissl-stained or MAP2- immunostained sections showed significantly lower neuronal death in the hippocampus in kainate plus lovastatin-injected rats compared with kainate plus vehicle-injected rats. Lovastatin was also found to show neuroprotective effect on kainate-treated hippocampal slices.

Although high concentrations (10–25 μM) of lovastatin can suppress cell proliferation and induce apoptosis in various cell lines (28–30), low concentrations (4 μM) of lovastatin did not affect cell viability of cultured hippocampal neurons (31, 32). The concentration of lovastatin (1 μM) used in the present study is therefore unlikely to cause cellular injury. The findings of neuroprotective effect of lovastatin in slice cultures support the notion that inhibition of excess cholesterol synthesis could have a neuroprotective effect apart from its effects on the microvasculature (see subsequently). Together, these findings suggest that increased brain cholesterol synthesis and oxysterol formation play a role in propagation of neuronal death after kainate injury.

The findings of this article may have implications in neuropathology. The use of statins has been reported to reduce the risk of AD (33, 34) and also improve the outcome after
stroke (35, 36). Statins have also been shown to protect cortical neurons from excitotoxicity in cell culture (37). There are several ways in which statins could exert their beneficial effects. One possibility is that statins might upregulate brain endothelial nitric oxide synthase, thus increasing blood flow, inhibiting platelet aggregation, and improving neurologic outcome (38, 39). A second possibility is that statins might have antiinflammatory actions, including reducing interleukin-1 and tumor necrosis factor production, thus inhibiting the consequences of neuronal damage (40). A third possibility is that statins may enhance brain plasticity by increasing vascular endothelial growth factor, angiogenesis, endogenous cell proliferation, and neurogenesis, thus improving functional outcome after neuronal injury (41). A fourth possibility is that neuroprotective effects of statins may be related to preservation of copper/zinc superoxide dismutase activity (42). A fifth possibility, in view of the present study, is that the neuroprotective effect of statins may be related to decreased cholesterol and oxysterol synthesis in the brain areas undergoing neurodegeneration/neuroinflammation. It is to be noted, however, that severe depletion of cholesterol in the brain (>23% by use of experimental compounds compared with lovastatin, <10%) could result in seizures (43). Further studies are necessary to elucidate changes in brain oxysterol and possible protective effect of statins on other forms of neuronal injury.

ACKNOWLEDGMENTS

The authors thank Ranbaxy Malaysia Sdn Bhd for generous supply of lovastatin and Professor D. W. Russell for generous gift of cholesteryl 24-hydroxylase antibody and helpful comments on the manuscript.

REFERENCES


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