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Changes in Brain Cholesterol Metabolome After Excitotoxicity

Wei-Yi Ong · Ji-Hyun Kim · Xin He · Peng Chen · Akhlaq A. Farooqui · Andrew M. Jenner

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Abstract Excitotoxicity due to excess stimulation of glutamate receptors in neurons is accompanied by increased Ca^{2+} influx, stimulation of Ca^{2+}-dependent enzymes, ATP depletion, increase in lipid peroxidation products, and loss of glutathione. These changes resemble neurochemical alterations in acute neuronal injury (stroke, spinal cord injury, and traumatic brain injury) and chronic neurodegenerative diseases such as Alzheimer's disease. Intracerebroventricular injection of the potent glutamate analog kainate in rats results in increased cholesterol concentration in the hippocampus at short to medium time intervals, i.e., 3 days–1 week post-injection, as detected by gas chromatography–mass spectrometry in the lesioned hippocampus. This is accompanied by an early increase in levels of cholesterol biosynthetic precursors and increases in both enzymatically derived oxysterols such as 24-hydroxycholesterol and cholesterol oxidation products (COPs) generated by reactive oxygen species, including cholesterol epoxides and 7-ketocholesterol. In contrast to COPs, no change in concentration of the neurosteroid pregnenolone was found after KA injury. Cholesterol and COPs significantly increase exocytosis in cultured PC12 cells and neurons, and both oxysterols and COPs are able to induce cytotoxic and apoptotic injuries in different cell types, including neurons. Together, the findings suggest that increased cholesterol and COPs after KA excitotoxicity could themselves lead to disturbed neuronal ion homeostasis, increased neurotransmitter release, and propagation of excitotoxicity.

Keywords Cholesterol · Oxysterols · Cholesterol oxidation products · Brain injury · Kainate · Excitotoxicity

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Glutamate Receptors, Memory Formation, and Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter in the vertebrate central nervous system (CNS). Nerve impulses trigger release of glutamate from the synaptic vesicles in the presynaptic neuron and in the postsynaptic neuron, and glutamate receptors, such as the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, bind glutamate and are activated. Phosphorylation or dephosphorylation of

AMPA and NMDA receptors have been proposed to play important roles in modulation of synaptic strength and synaptic plasticity. In the CA1 region, increase in synaptic strength and induction of long-term potentiation (LTP) occurs through Ca^{2+}-calmodulin kinase II (CaMKII)-dependent phosphorylation of postsynaptic AMPA receptors. Entry of Ca^{2+} via NMDA receptors during strong synaptic stimulation, provides the stimulus to trigger phosphorylation of AMPA receptors. In addition, LTP induction requires activation of a protein kinase C-dependent tyrosine kinase signal cascade and a concomitant up-regulation of NMDA receptors [1, 2]. AMPA and NMDA receptors are not static components of synapses, but are continuously delivered or removed from postsynaptic membranes [3]. AMPA receptors incorporated into synapses during LTP are from lateral diffusion of spine surface receptors containing GluR1, an AMPA receptor subunit. Relatively little is known about the contribution of kainate or kainic acid (KA) receptors in learning and memory formation. LY382884, a selective antagonist of KA receptor with no effect on AMPA or NMDA receptors, blocks the induction of induction of LTP at mossy fiber synapses in the CA3 region of the hippocampus, an effect attributed to the presence of presynaptic GLU (K5)-subunit-containing KA receptors at mossy fiber terminals [4, 5].

Excitotoxicity is a pathological process by which nerve cells are damaged and killed by glutamate and its analogs released from injured neurons. This occurs when receptors for glutamate such as the NMDA receptor and AMPA receptor are over-activated. Glutamate and its analogs, including KA, bind to these receptors and produce excitotoxicity by allowing high amounts of Ca^{2+} to enter the cell. KA excitotoxicity increases intracellular calcium and activates calcium-dependent enzymes, including phospholipases A_2, nitric oxide synthase, calpains, sphingomyelinases, and cytochrome P450 oxidases generating glycophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators. These changes resemble neurochemical alterations in acute neuronal injury, e.g., stroke, spinal cord injury, and traumatic brain injury, and chronic neurodegenerative disease such as Alzheimer's disease [6, 7]. In ischemic and traumatic brain and spinal cord injuries, neurons degenerate rapidly (in minutes to hours) because of massive glutamate release, sudden lack of oxygen, a quick drop in ATP, and alteration in ion homeostasis. In contrast, massive release of glutamate does not occur and oxygen, nutrients, and ATP are available to neurons in neurodegenerative diseases so ion homeostasis is maintained to a limited extent and neuronal death may take a longer time [8]. Neurodegenerative diseases are also accompanied by increased production, misfolding, and pathological aggregation of proteins such as beta-amyloid, tau-protein, huntingtin, and alpha-synuclein. These proteins intensify the interplay between excitotoxicity, oxidative stress, and neuroinflammation [8].

KA injections produce significantly increased cytosolic phospholipase A_2 ($cPLA_2$) mRNA levels in the hippocampus at 3 and 7 days after injection [9], which is accompanied by increased $cPLA_2$ immunolabeling in neurons at 1 and 3 days after KA injection and increased immunoreactivity in astrocytes after 1, 2, 4, and 11 weeks [10]. Increased $cPLA_2$ activity in neurons may contribute to neurotoxicity, whereas elevation in astrocytes is associated with gliosis. The increased expression of PLA_2 is accompanied by increased levels of both $cPLA_2$ and secretory PLA_2 ($sPLA_2$) activities in homogenates from the hippocampus [11]. Elevated levels of arachidonic acid released by PLA_2 and its metabolites (eicosanoids) produce a variety of detrimental effects on neural membrane structures, changes in activities of membrane enzymes and neurotransmitter uptake systems, and generate reactive oxygen species (ROS) [12, 13]. PLA_2 activity is also stimulated by ceramide, ceramide-1-phosphate and 24-hydroxycholesterol which are lipid mediators of sphingolipid and cholesterol metabolism, respectively [14–16] (Fig. 1). Lipidomic analysis of the hippocampus after KA injection shows significant reductions in glycerophospholipid species with polyunsaturated fatty acids in the hippocampus after KA lesions, consistent with increased PLA_2 activity [17]. Elevation in $cPLA_2$ and $sPLA_2$ activity following KA-induced toxicity can be prevented by PLA_2 inhibitors [13].

Injections of KA also produce significant increase in ceramide in the hippocampus at 1 and 3 days post-injection. Tandem mass spectrometric profiling of lipids extracted from the hippocampus shows significant increases in ceramide species with 16:0, 18:0, 20:0, 22:0, and 24:1 fatty acids after KA injection [17]. The increase in ceramide levels is associated with increased expression and activity of the first enzyme in the sphingolipid biosynthetic pathway, serine palmitoyltransferase (SPT) [18]. Immunohistochemical analysis indicate baseline expression of SPT in neurons and increase in astrocyte immunoreactivity post-KA treatment [18]. Inhibition of SPT by L-cycloserine or myriocin produces a significant neuroprotective effect in hippocampal slice cultures after KA treatment [18]. In addition, neurons lacking acidic sphingomyelinase, an enzyme that generates ceramide from sphingomyelin, exhibit decreased vulnerability to excitotoxicity [19]. Increased ceramide may facilitate the opening of the mitochondrial permeability transition pore which disrupts the mitochondrial transmembrane potential, causing release of cytochrome c and activation of caspase-3 and PLA_2 and neuronal death [6] (Fig. 1). The purpose of this commentary is to review the maintenance and regulation of CNS cholesterol

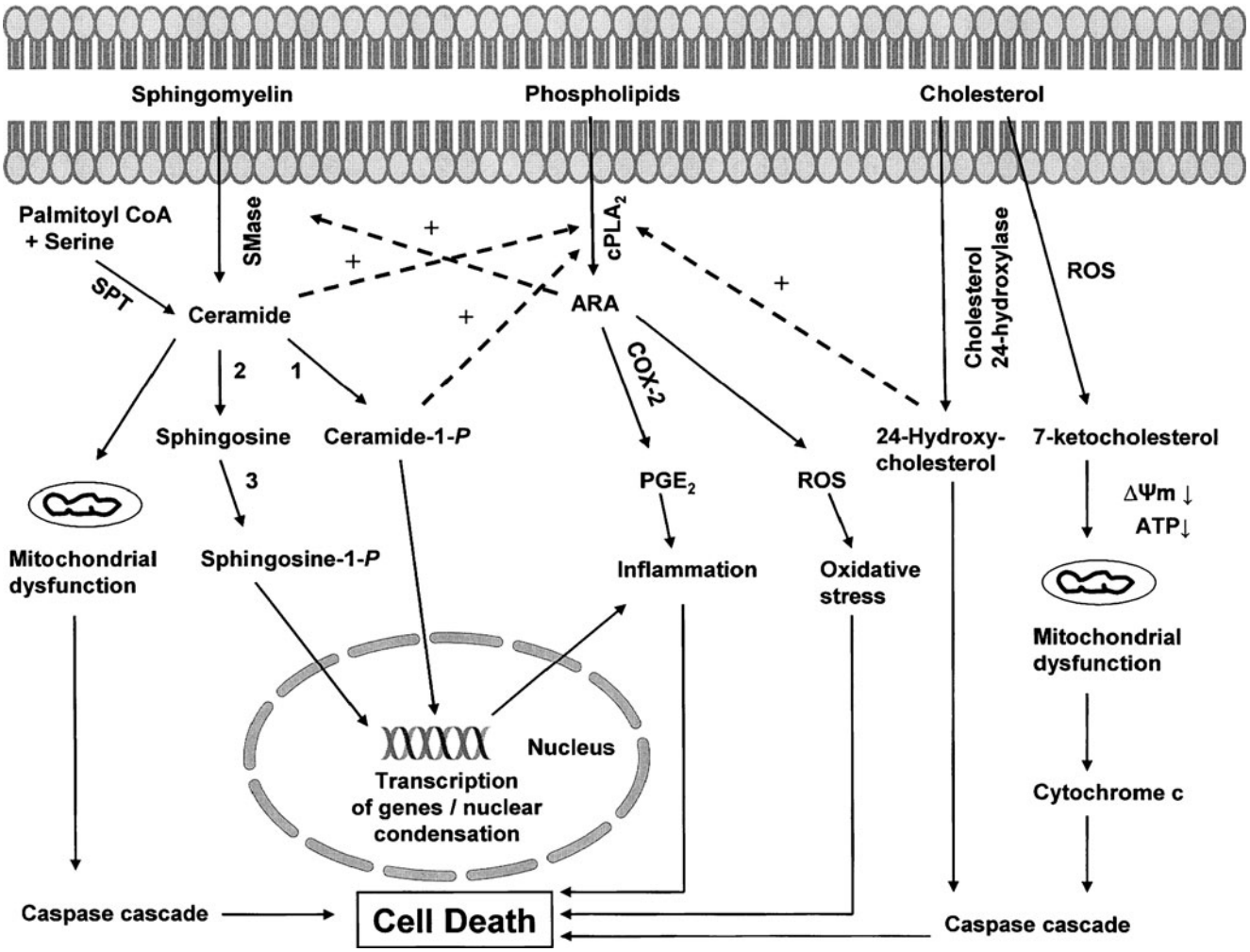

Fig. 1 Hypothetical diagram showing interplay among glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators and their involvement in neuronal cell death after KA excitotoxicity. *PLA_2* cytosolic phospholipase A_2, *SMase* sphingomyelinase, *SPT* serine palmitoyltransferase, *ARA* arachidonic acid, *COX-2* cyclooxygenase-2, *ROS* reactive oxygen species,*ceramide-1-P* ceramide-1-phosophate, *sphingosine-1-P* sphingosine-1-phosphate. Ceramide-1-kinase (*1*); ceramidase (*2*); sphingosine-1-kinase (*3*)

synthesis/metabolism and to discuss the influence of KA excitotoxicity on cholesterol metabolic profiles.

Brain Cholesterol Metabolism

Cholesterol Biosynthesis

Even though the CNS accounts for only 2% of the body weight, it contains 23% of the sterol, predominantly cholesterol present in the whole body pool. Cholesterol is a vital component of cell membranes, determining their fluidity and biophysical properties, and is implicated in the assembly and maintenance of lipid rafts. It is particularly important for the formation of synapses, modulating the activities of membrane-bound enzymes, receptors and ion channels, and neural signaling [20, 21], and is a key component of the myelin sheath [22]. The blood–brain barrier effectively blocks uptake of cholesterol from the circulation, and currently, there is no evidence for net transfer of cholesterol from the blood to the CNS [23]. Brain cholesterol is predominantly derived from de novo synthesis [24] and requires multiple regulatory mechanisms involving synthesis, transport, and metabolism to maintain physiological levels. Cholesterol biosynthesis in brain involves an extensive and energy-consuming pathway comprising multiple intermediates and mediating enzymes (Fig. 2). The regulation of biosynthesis of cholesterol is believed to involve insulin-induced genes and sterol regulatory element binding proteins, in particular SREBP-2 [25], and controlled through feedback regulation by sterols, including cholesterol itself. HMG-CoA synthase, a key enzyme in cholesterol biosynthesis, is a target of cholesterol regulation [26, 27]. Other enzymes further

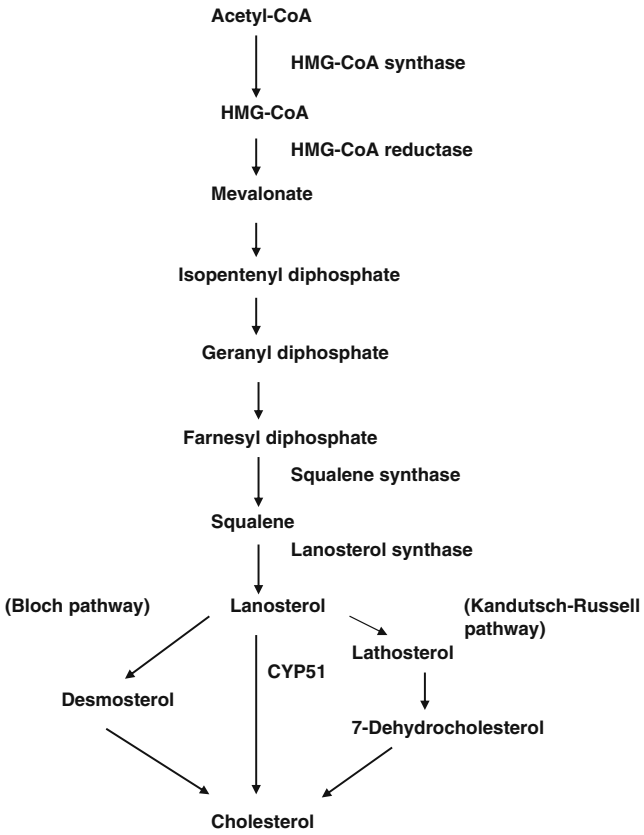

Fig. 2 Overview of cholesterol biosynthesis

downstream in the cholesterol synthetic pathways include squalene synthase, lanosterol synthase, and cytochrome P450 lanosterol 14 alpha-demethylase (CYP51) which produce squalene, lanosterol, and cholesterol, respectively [28]. Two alternative pathways exist in the later stages of cholesterol synthesis. The Bloch pathway occurs via zymosterol and desmosterol, while the Kandutsch–Russell pathway is through lathosterol and 7-dehydrocholesterol [29] (Fig. 3). Most growth and differentiation of the CNS occurs in the first few weeks or years after birth, and cholesterol required for such growth apparently comes exclusively from de novo synthesis. The highest rate of synthesis occurs during the first postnatal weeks in humans and rodents [30, 31]. The Bloch pathway contributes more to cholesterol biosynthesis in young animals and the alternative pathway in aged animals [32]. The majority of brain cholesterol is present in myelin sheath, and while there is a clear role of oligodendrocyte cholesterol in myelin formation, the roles of other glial cells and neurons in cholesterol metabolism in the adult CNS are less well understood [22]. The prevailing view is that in the adult nervous system, astrocytes are responsible for cholesterol synthesis and synaptic plasticity is modulated by cholesterol homeostasis, which is controlled by cell export proteins and lipoproteins such as apolipoprotein E (apoE). The latter plays an important role in the translocation of cholesterol between astrocytes and neurons [33]. Compared to glial cells, neurons show different profiles of cholesterol biosynthetic enzymes, post-squalene sterol precursors, and cholesterol metabolites, and produce cholesterol less efficiently, possibly due to low levels of lanosterol-converting enzymes [22, 34]. Nevertheless, there have been suggestions that besides glial cells, adult neurons are capable of cholesterol synthesis [35, 36].

Sterol regulatory element binding proteins (SREBPs) activate the expression of genes involved in the synthesis and uptake of fatty acids, triglycerides, glycerophospholipids, and cholesterol [37]. SREBP is synthesized from a membrane-bound, 125-kDa precursor that is transported from the endoplasmic reticulum to the Golgi apparatus [38, 39]. It is then cleaved to release a transcriptionally active mature form (56–70 kDa) which enters the nucleus to bind to sterol-responsive element containing promoters on target genes, including those encoding cholesterol biosynthetic enzymes such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, HMG-CoA synthase, squalene synthase, and farnesyl diphosphate synthase [37, 40]. Both SREBP-1 and SREBP-2 are localized to neurons in the cortex and hippocampus in the normal brain [41, 42]. In addition, the first and last enzymes in the cholesterol biosynthetic pathway, HMG-CoA reductase and 7-dehydrocholesterol reductase, respectively, are expressed in neurons throughout the adult mouse brain and are particularly prominent in cortical, hippocampal, and cholinergic neurons [43]. The co-expression of SREBP-1 and -2 and the first and last enzymes of the cholesterol biosynthetic pathway in neurons suggest that these cells require endogenous cholesterol for their normal function. Moreover, brain-derived neurotrophic factor elicits transcription of enzymes in the cholesterol biosynthetic pathway and cholesterol biosynthesis in cultured cortical and hippocampal neurons, but not glial cells [36]. Apoptotic neuronal death induced by the free radical-generating xanthine/xanthine oxidase system is associated with activation of the cholesterol biosynthesis pathway, suggesting that neuronal cholesterol biosynthesis may be affected by oxidative stress [44].

Cholesterol Metabolism

Cholesterol is metabolized into side chain oxysterols 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol by oxygenases, enabling them to cross the blood–brain barrier. Cholesterol 24-hydroxylase (CYP46) is a highly conserved cytochrome P450 that is responsible for the majority of cholesterol turnover in the vertebrate CNS. The enzyme is expressed almost exclusively in neurons in the normal brain, including hippocam-

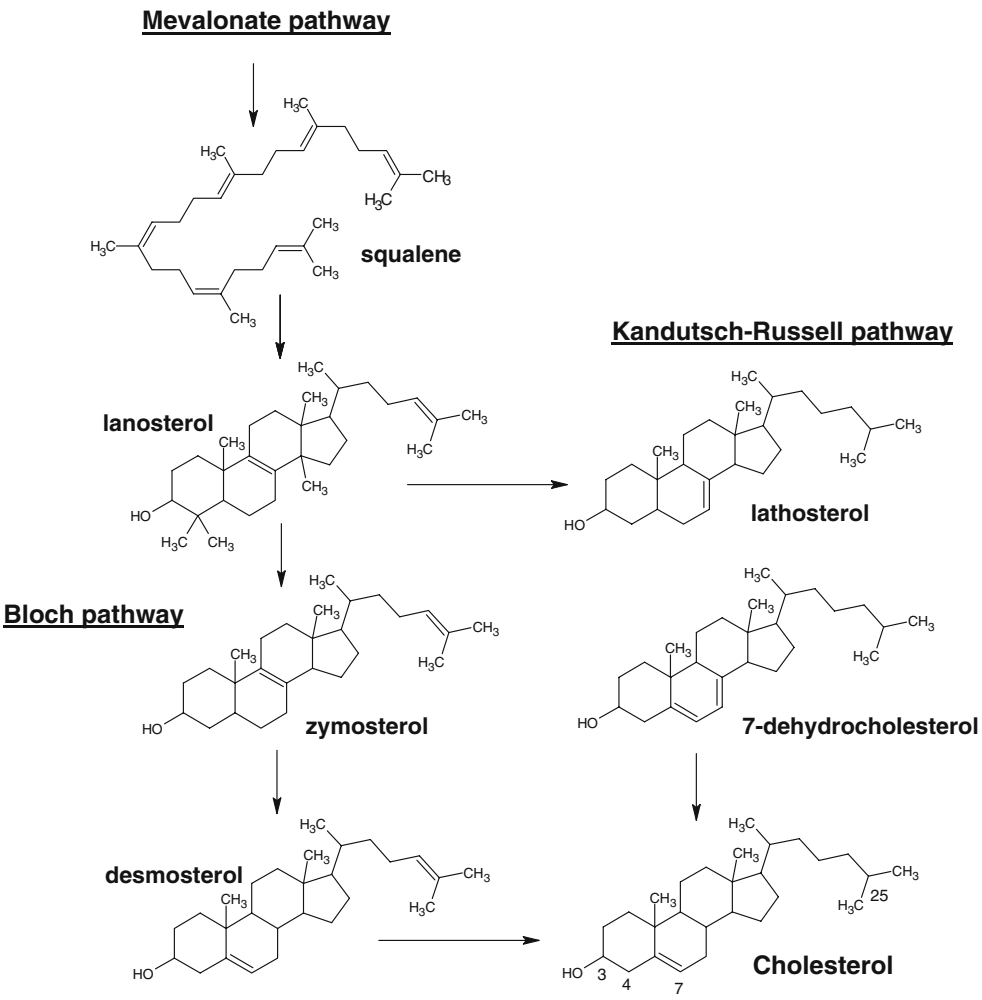

Fig. 3 Late stages in cholesterol biosynthesis

pal and cortical neurons that are important for learning and memory formation [45, 46]. 24-Hydroxycholesterol is the major brain cholesterol metabolite which is eliminated in the bloodstream and is responsible for the removal of excess cholesterol from the brain (Fig. 4). In humans, the concentration of 24-hydroxycholesterol is 30- to 1,500-fold higher in the brain than in any other organ except the adrenals, indicating that the majority of 24-hydroxycholesterol present in the circulation originates from the brain [47, 48]. The ratio between 24-hydroxycholesterol and cholesterol in plasma is approximately five times higher during the first decade of life than the sixth [48]. 27-Sterol hydroxylase (CYP27A1) is expressed in multiple organs and catalyzes the formation of 27-hydroxycholesterol. 27-Hydroxycholesterol is metabolized in brain by a combination of three different enzymes in a novel metabolic route forming 7 alpha-hydroxy-3-oxo-4-cholestenoic acid and subsequently eliminated [49, 50]. In contrast to efflux of 24-hydroxycholesterol from the brain to the circulation, there appears to be a net flux of 27-hydroxychrolesterol from the serum to the brain, which has relatively lower CYP27A1 levels and negligible CNS endogenous 27-hydroxycholesterol production [49] (Fig. 4). 27-Hydroxycholesterol and 25-hydroxycholesterol, but not 24-hydroxycholesterol, also undergo 7 alpha-hydroxylation with subsequent oxidation to 7 alpha-hydroxy-3-oxo-4 steroids in Schwann cells, astrocytes, and neurons [51].

The ATP binding cassette (ABC) transporters ABCA1 and ABCG1 play important roles in cholesterol export from the brain, largely through neuronal expression of ABCA1 in the normal rat brain [52]. ABCA1 is induced by treatment with retinoic acid and several oxysterols including 22(R)-hydroxycholesterol and 24-hydroxycholesterol and is dramatically up-regulated in neurons and glial cells in lesioned areas of the hippocampus after AMPA treatment [52]. ABCG1 and ABCG4 have overlapping functions in astrocytes, promoting efflux of cholesterol, desmosterol, and possibly other sterol biosynthetic intermediates to high-density lipoprotein (HDL) [53]. Mice lacking ABCA1 in the CNS have reduced plasma high-density lipoprotein (HDL) cholesterol levels, decreased brain cholesterol content, and enhanced brain uptake of esterified cholesterol from plasma HDL [53]. Nuclear receptor liver X-receptors (LXR)

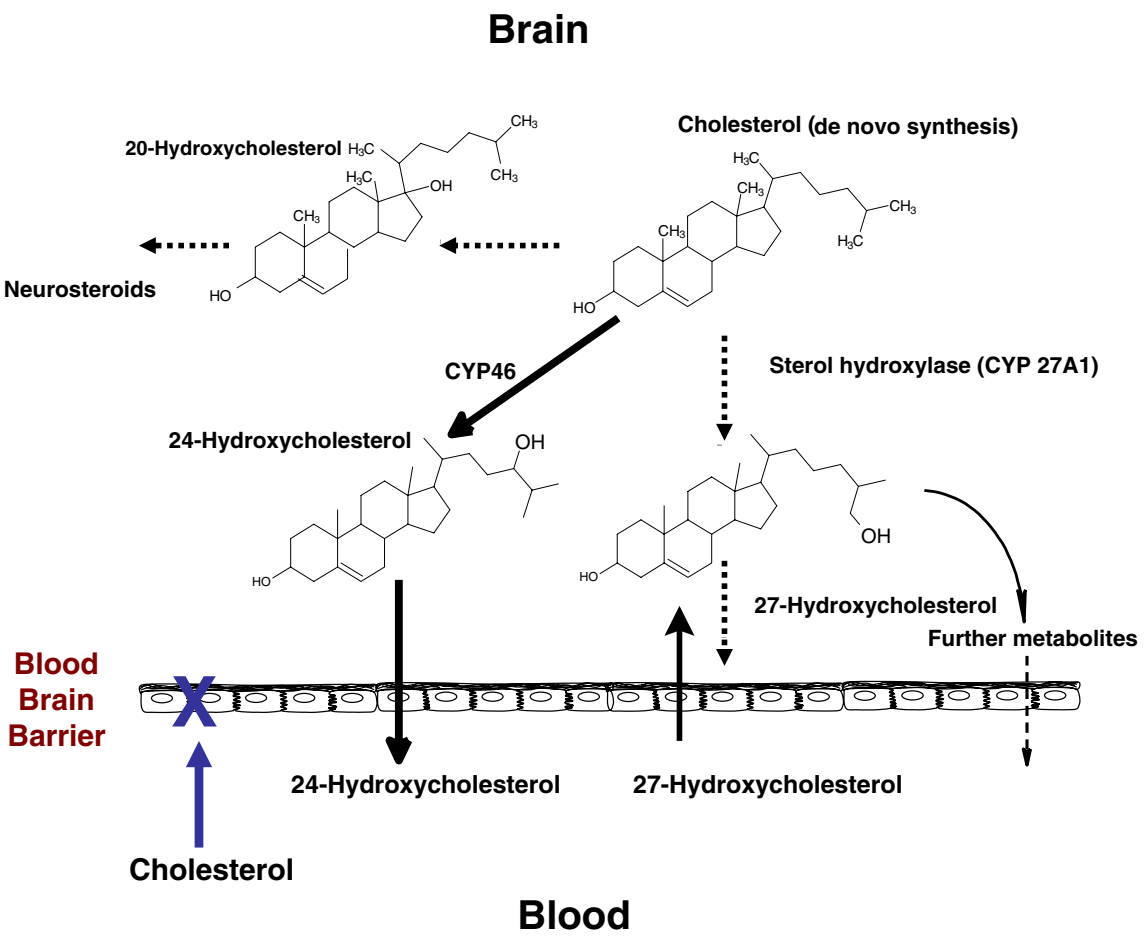

Fig. 4 Brain cholesterol metabolism and enzymatic generation of oxysterols. *Arrows* indicate relative flux of sterols across the blood–brain barrier between brain and the circulation

function as cellular "sterol sensors" and play a critical role in the regulation of ABCA proteins. The LXR agonist (T0901317) increases protein levels of the cholesterol transporters ABCA1 and ABCG1 [54]. Glial cells influence ABCG1, A1, 2, 7, and low-density lipoprotein receptor-related protein 1 transcription, suggesting a role of these cells in modulating cerebral lipid supply/elimination [55].

Oxysterol-mediated LXR activation also induces apoE biosynthesis in astrocytes, further enhancing cholesterol redistribution and removal [56]. ApoE plays a major role in lipid metabolism and is synthesized in various organs, particularly the CNS. It is present in high concentrations in CNS interstitial fluid where it appears to participate in cholesterol redistribution from cells with excess cholesterol to those with less cholesterol. Humans possess three major isoforms, apoE2, apoE3, and apoE4, which result from the expression of the E2, E3, and E4 alleles of the APOE gene. ApoE4 in particular is strongly associated with familial and sporadic late-onset Alzheimer's disease [57, 58]. ApoE also appears to be involved in the repair response to tissue injury; for example, markedly increased amounts of apoE are found at sites of peripheral nerve injury and regeneration [59]. Treatment of mice with the LXR agonist T0901317 results in the up-regulation of apoE mRNA and protein in both hippocampus and cerebral cortex [60]. Of the various isoforms of apoE, ApoE3-HDL induces marked cholesterol release from neurons, while apoE4-HDL induces little [61]. Moreover, pulse-chase experiments show enhanced degradation and reduced half-life of newly synthesized apoE4 compared with apoE3. These data suggest that different apoE isoforms have different abilities to stimulate cholesterol release from neurons, probably due to their different rates of degradation [62]. However, recent data from studies of human ApoE knock-in mice indicate that ApoE isoform has relatively little influence on total brain cholesterol and enzymatically generated oxysterol levels [63]. Astrocytes are also the primary producers of brain lecithin cholesterol acyltransferase, an enzyme that esterifies free cholesterol on nascent apoE-containing lipoproteins secreted from glia [64].

Cholesterol trafficking in the brain is regulated by the Niemann Pick C (NPC) proteins. Both cholesterol and 25-hydroxycholesterol bind to NPC1, which is competitively inhibited by 24-, 25-, and 27-hydroxycholesterol [65]. NPC2 binds a range of cholesterol-related molecules (cholesterol precursors, plant sterols, some oxysterols, cholesterol sulfate, cholesterol acetate, and 5-alpha-cholestan-3-one) [66]. Immuno-electron microscopic analyses of primate and rodent brains show that NPC1 is localized in astrocytic processes at the sides of synapses, while NPC2 is observed mainly in small-diameter dendrites or dendritic spines [67–69]. Loss-of-function mutations in the NPC1 gene lead to a failure of Ca^{2+}-mediated fusion of endosomes with lysosomes, resulting in the accumulation of cholesterol and glycolipids and progressive neurodegeneration [70]. The metabolic defects and excess lipid storage in neurons from humans with NPC disease result in extensive growth of new, ectopic dendrites as well as formation of neurofibrillary tangles [71]. In addition, NPC1 knockout mice show mitochondrial cholesterol accumulation, mitochondrial glutathione depletion, and release of pro-apoptotic proteins [72].

Changes in Brain Cholesterol Metabolism After KA-Induced Excitotoxicity

Cholesterol Levels and Biosynthesis

Intracerebroventricular injection of the potent glutamate analog, KA, in rats results in increased cholesterol concentration in the hippocampus at short to medium time intervals, i.e., 3 days–1 week post-injection, as detected by gas chromatography–mass spectrometry (GC-MS) [73, 74] (Fig. 5). The increase was confirmed by increased cholesterol immunoreactivity in hippocampal neurons in brain sections and increased cholesterol staining of neurons by filipin in brain sections, hippocampal slice cultures, and neuronal cultures after KA treatment [73]. The increase in filipin staining of slice cultures implies that the increase in cholesterol in neurons after KA is not the result of exogenous transfer from the bloodstream. Likewise, increased filipin staining in neuronal cultures after KA suggests that the increase in cholesterol could be due to increased biosynthesis and/or defective cholesterol export in these cells [74]. KA excitotoxicity induces significant changes in the expression of SREBP-2 in the rat hippocampus [42]. Western immunoblot analysis shows a dense band at 70 kDa corresponding to the transcriptionally active form of SREBP-2 in homogenates from normal rat hippocampus, cortex, and striatum. SREBP-2 immunolabel is observed in the nucleus, somatic cytoplasm, and dendrites in the hippocampus. After KA lesions, there is loss of SREBP-2 immunoreactivity in areas of gliosis, but increased SREBP-2 staining in a cluster of neurons at the edge of the lesions. This may result in decreased cholesterol biosynthesis in the glial scar, but increased cholesterol biosynthesis in neurons that are affected by ongoing excitotoxic injury [42].

Quantitative RT-PCR analysis of whole hippocampal homogenates shows significant decrease in mRNA expression of SREBP-2 and the rate-limiting enzyme for cholesterol biosynthesis HMG-CoA reductase at all time points after KA injection, as well as a decrease in lanosterol synthase and CYP51 at 1 and 2 weeks post-KA injection, respectively [74]. These findings indicate decreased mRNA expression of cholesterol biosynthetic genes after KA injury. GC-MS analysis, however, shows a significant increase in cholesterol biosynthetic precursors in both the Bloch and Kandutsch–Russell pathways, lanosterol, desmosterol, and 7-dehydrocholesterol, at 1 day after KA treatment compared to untreated controls. Subsequently, levels of precursors are significantly reduced at 1 and 2 weeks post-KA treatment compared to controls (Fig. 5). These time-dependent changes suggest that the brain mounts an acute response to excitotoxic injury by inducing an increase in cholesterol synthesis despite the absence of increases in mRNA expression of synthetic enzymes [74]. Since increased level of precursors occurs at 1 day after KA injection, at a time before substantial glial reaction, this suggests increased cholesterol biosynthesis in neurons affected by excitotoxic injury. On the other hand, no net increase in cholesterol precursors is observed at 1 and 2 weeks after KA injection, i.e., a time of significant glial reaction in the degenerating hippocampus [10], indicating that a primarily glial source of the increased cholesterol is unlikely [74]. It is postulated that ongoing neuronal injury due to release of neurotransmitters from dying cells and excitotoxicity could continue to contribute to prolonged neuronal cholesterol biosynthesis after KA injection, leading to significantly elevated cholesterol in the hippocampus at short to medium time intervals after KA injection [73, 74].

Cholesterol Metabolism

Besides cholesterol biosynthesis, another factor which could result in cholesterol accumulation in the hippocampus is possibly changes in expression of cholesterol transporters. Quantitative RT-PCR analysis of the KA lesioned hippocampus shows significant up-regulation of mRNA of ABCA1 at 1 and 2 weeks after KA lesion. Immunohistochemical analysis shows loss of ABCA1 immunoreactivity in neurons, but increased labeling in astrocytes of hippocampal areas affected by KA lesions [74]. It is unknown whether these changes in biodistribu-

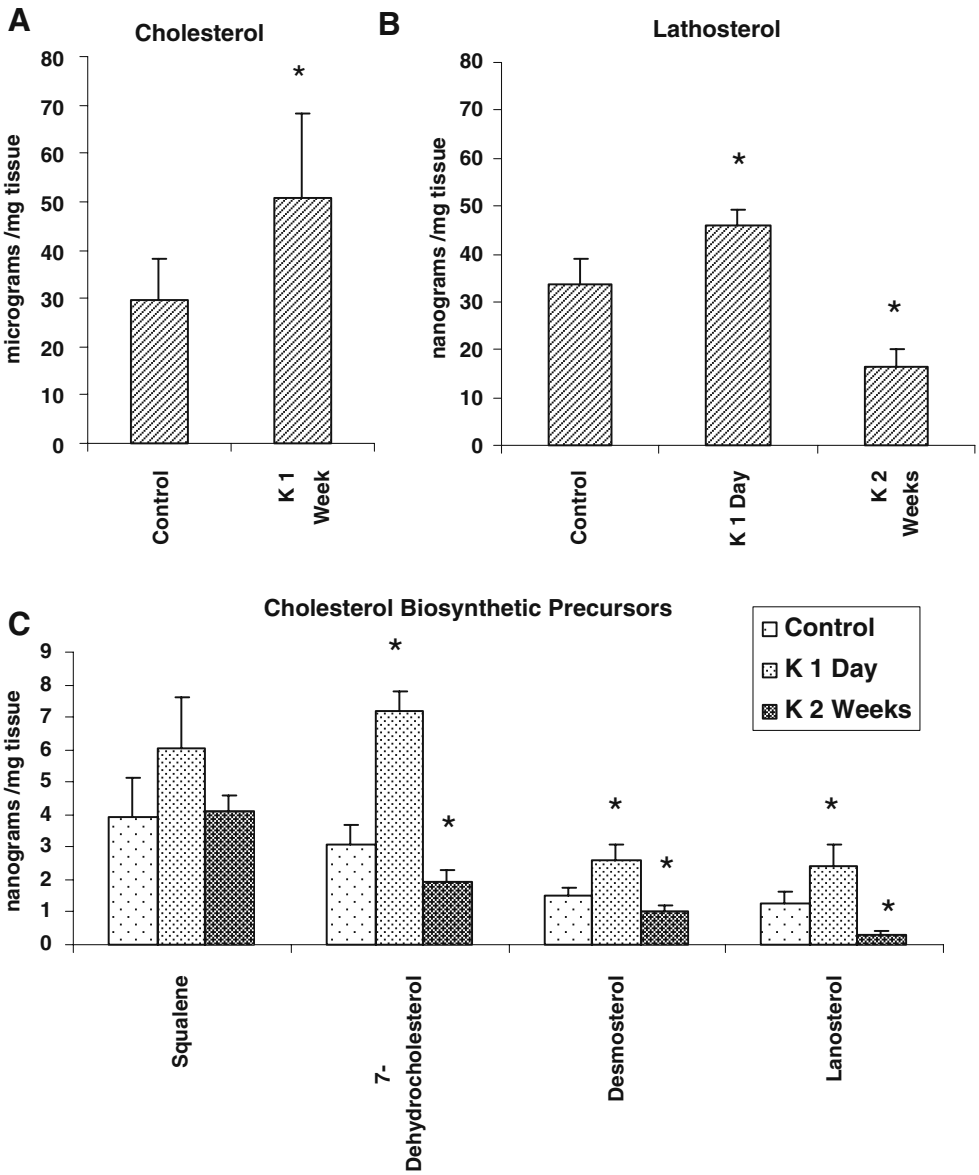

Fig. 5 Changes in cholesterol and cholesterol biosynthetic precursors in the rat hippocampus at 1 day (**a**) and 1 day and 2 weeks (**b**) after KA (*K*) injection. *Significant difference compared to untreated controls by Student's *t* test or one-way ANOVA with Bonferroni's multiple comparison post hoc test ($P<0.05$)

tion of ABCA1 could contribute to cholesterol accumulation in the hippocampus after KA lesions. A further factor which could increase in brain cholesterol is that some cholesterol may be converted to cholesterol esters, which may not be good substrates for cholesterol transporters, so that these accumulate after brain injury [75]. Metabolomic analysis of hippocampal homogenates using magnetic resonance spectroscopy shows significant increase in cholesterol esters in the hippocampus at 1 and 2 weeks after KA lesions. The increase in cholesterol esters was accompanied by significant increase in acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) mRNA expression in the lesioned hippocampus (Kim JH and Ong WY, unpublished). It is possible that increased levels of arachidonic acid in the degenerating hippocampus could contribute to the formation of cholesterol esters, and further studies examining KA-induced cholesterol accumulation are necessary to elucidate the exact mechanisms responsible for cholesterol accumulation.

GC-MS analysis of the hippocampus demonstrates that cholesterol accumulation after KA injection was accompanied over 1–2 weeks by an increase in several oxysterols, including enzymatically mediated products, particularly 24-hydroxycholesterol as well as COPs formed by auto-oxidation of cholesterol via direct attack by ROS, including 7-ketocholesterol and cholesterol epoxides [74] (Figs. 6 and 7). COPs can be measured in trace amounts in human tissue [76, 77] and are elevated in diseases that involve oxidative stress including atherosclerosis, liver cirrhosis, and multiple sclerosis [76, 78, 79]. Since there is considerable evidence for oxidative damage during neurodegeneration and cholesterol constitutes a

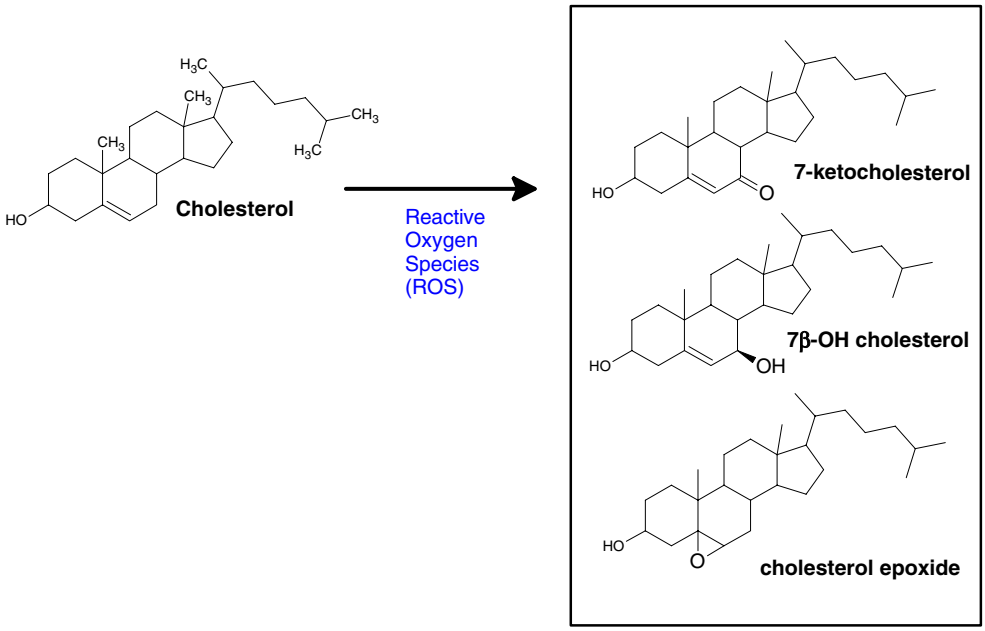

Fig. 6 Formation of cholesterol oxidation products (*COPs*) via oxidative attack by ROS. 7-Alpha hydroxycholesterol can also be formed by ROS as well as 7-alpha cholesterol hydroxylase (*CYP7A1*)

major lipid target in brain (~2%, *w*/*w*), these oxysterols have the potential of being important biomarkers of oxidative damage in this organ. COPs formation adds further evidence of lipid peroxidation after KA lesions, similar to that provided by other ROS lipid damage biomarkers, including 4-hydroxynonenal and F_2-isoprostanes [80]. The increase in 24-hydroxycholesterol after KA is likely due to increased expression of CYP46 in astrocytes of the affected CA fields [81]. CYP46 immunoreactivity is also induced in glial cells after fluid percussion cortical injury in rats [82]. Proteomic data indicate that 24-hydroxycholesterol down-regulates cholesterol biosynthetic enzymes possibly via SREBP-2 and may be a mechanism to regulate intracellular cholesterol levels [55]. This may explain the decrease in mRNA expression of cholesterol biosynthetic enzymes after KA lesions. Moreover, 24-hydroxycholesterol induces apoE transcription and protein synthesis in a dose- and time-dependent manner in cells of astrocytic origin [83] (Table 1). COPs generated by ROS are also increased after KA lesions. Increase in 7-ketocholesterol is found at 1 and 2 weeks, increase in cholesterol 5,6, alpha epoxides at 1 week, and cholesterol 5,6, beta epoxides at 1 day and 1 and 2 weeks after KA injections [74]. Both enzymati-

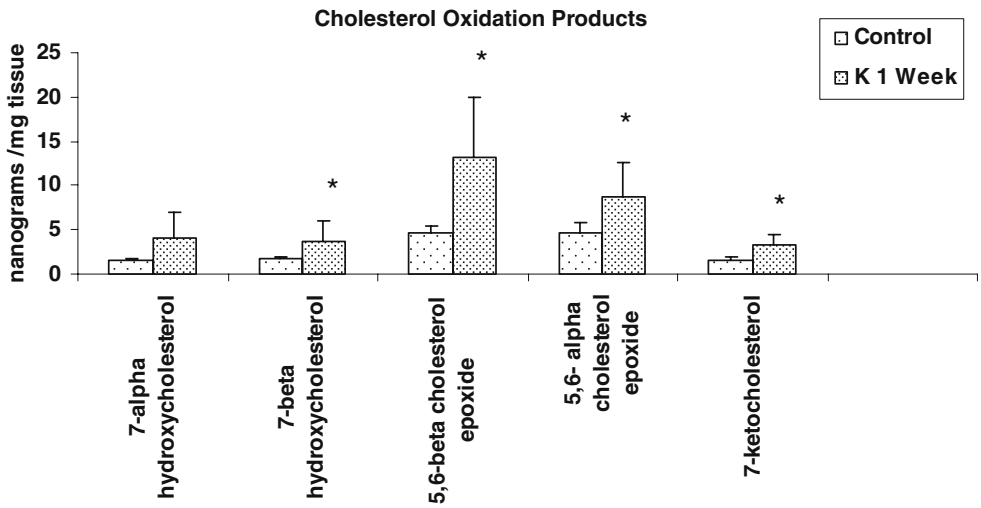

Fig. 7 Changes in cholesterol oxidation products in the rat hippocampus at 1 week after KA (*K*) injection. *Significant difference compared to untreated controls by Student's *t* test ($P<0.05$)

Table 1 Effect of 24S-hydroxycholesterol on enzymes associated cholesterol synthesis and oxidative stress

Enzymes	Effect	Reference
$cPLA_2$	Up-regulated	[14]
COX-2	Up-regulated	[14]
3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Down-regulated	[27]
Diphosphomevalonate decarboxylase	Down-regulated	[27]
Isopentenyl-diphosphate delta isomerase	Down-regulated	[27]
Farnesyl-diphosphate synthase	Down-regulated	[27]
Squalene synthase	Down-regulated	[27]
Methylsterol monooxygenase	Down-regulated	[27]
Apolipoprotein E	Down-regulated	[27]
Protein kinase C	Down-regulated	[112]
eNOS	Down-regulated	[113]

cally formed oxysterols and COPs are able to induce cytotoxic and apoptotic injuries in different cell types including neurons [84]. The marked increases in cholesterol and COPs including 7-ketocholesterol and cholesterol epoxides after KA treatment may therefore be a factor in aggravating oxidative damage to neurons after KA-induced excitotoxicity. Treatment of rats or hippocampal slice cultures with a brain permeable statin, lovastatin, results in modulation of increases in cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol and partial protection against KA-induced neuronal injury [81]. This suggests that brain-permeable statins could have neuroprotective effects by limiting cholesterol or COP levels in brain areas undergoing neurodegeneration.

The importance of cholesterol and arachidonic acid oxidation products in excitotoxicity is supported by studies on apolipoprotein D (apoD). The latter is a member of the lipocalin family of transport proteins with a molecular structure consisting of an eight-stranded beta barrel that forms a binding pocket for small hydrophobic cargo, including arachidonic acid and cholesterol [85]. Increased apoD immunoreactivity is observed in pyramidal neurons of the affected CA fields after KA injection in rats, and hippocampal apoD protein levels peaked 3 days after KA injection. Besides pyramidal neurons, apoD immunoreactivity is present in a small number of reactive glial cells in the affected CA fields [86]. Increased apoD immunoreactivity is also observed in pyramidal neurons and glial cells after experimental stroke [87]. Addition of physiological levels of ApoD (10 μg/ml) to hippocampal slice cultures attenuates increase in COPs and F_2 isoprostanes (decomposition product of arachidonic acid peroxidation) and partially prevents loss of microtubule-associated protein immunostaining and lactate dehydrogenase release from hippocampal neurons after KA treatment [88]. In view of its molecular structure, it is postulated that apoD may promote its neuroprotective effects by binding to cholesterol and arachidonic acid, thus preventing their oxidation to neurotoxic products such as 7-ketocholesterol and 4-hydroxynonenal. These results are consistent with recent studies which show a neuroprotective effect of apoD. Overexpression of the homolog of apoD, lazarillo, in *Drosophila* results in increased resistance to hyperoxia and extension of life span under nomoxia [89], while apoD knockout flies are less resistant to oxidative stress and starvation and males have shorter life span [90]. *Drosophila* expressing the human form of apoD are also long-lived and protected against stress conditions associated with aging and neurodegeneration [91]. Moreover, human apoD overexpression increases survival in flies and prevents brain lipid peroxide formation after oxidant treatment [92].

Neurosteroids are steroid hormones synthesized in the brain from cholesterol in a multistep enzyme-catalyzed process. The first step in neurosteroid biosynthesis is the conversion of cholesterol to pregnenolone by the enzyme cytochrome P450 side chain cleavage (P450scc) which is localized in neurons [93, 94]. The latter is then metabolized to other hormones such as dehydroepiandrosterone (DHEA) and estradiol which are believed to have neuroprotective functions. KA lesions result in an overall decrease in hippocampal P450scc protein in Western blots and reduced immunohistochemical labeling of the enzyme in neurons, but induction of labeling in a small number of astrocytes. Analysis of the neurosteroids pregnenolone and DHEA using a GC-MS technique with heavy isotope dilution shows a non-significant trend to a decrease in the level of pregnenolone at 2 weeks post-lesion [94]. The results indicate the absence of increased formation of neurosteroids despite higher levels of cholesterol after KA lesions due to overall down-regulation of the P450scc enzyme expression in the damaged hippocampus. Increased P450scc expression in astrocytes and a small number of microglia is also reported in the hippocampus after pilocarpine-induced status epilepticus [95].

Possible Contribution of Cholesterol Metabolites to Excitotoxicity

The distribution of cholesterol in neural membranes is asymmetric across the plane of the membrane, with an outer to inner ratio of 1:3 [96, 97]. Cholesterol condenses the lipid bilayer by positioning between the hydrocarbon chains below the large head groups of the sphingolipids and not only controls exocytosis by modulating vesicle fusion and motion during synaptic transmission but also regulates the activities of membrane-bound enzymes, receptors, and ion channels [21]. Ordered domains (lipid rafts) on the cell membrane are stabilized by the intercalated interactions between the small cholesterol molecules and the hydrocarbon chains of sphingolipids [98]. By recruiting specific proteins and excluding others, lipid rafts modulate cellular functions such as signal transduction [99], ion channel activities [100], and particularly vesicular exocytosis that is facilitated by assembly of secretory proteins such as soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAREs) into lipid rafts. Chelation of cholesterol by cyclodextrin disrupts lipid rafts and inhibits exocytosis, whereas addition of cholesterol perturbs the semi-stable hemifusion structure and directly induces vesicle fusion [21]. Due to its inverted cone shape that favors formation of a negative membrane curvature, cholesterol facilitates dilation of the fusion pore and the formation of hemifusion structure between the vesicular and the plasma membrane prior to vesicular fusion [101]. SNARE proteins are essential for intracellular membrane fusion and, cholesterol modulates fusion kinetics by inducing conformational changes in transmembrane domains of SNARE proteins [101]. Cyclodextrin, a membrane cholesterol-depleting agent, strongly reduces neuronal synaptic transmission and synaptic plasticity associated with activation of AMPA, KA, and NMDA receptors [102]. Besides cholesterol, oxysterols also strongly promote membrane fusion and exocytosis. An increase in exocytosis is observed after external infusion of 24-hydroxycholesterol, 7-ketocholesterol, or cholesterol 5,6 beta epoxide as demonstrated by total internal reflection microscopy, membrane capacitance measurements, and direct measurement of catecholamine release from PC12 cells by amperometry (Ma MT and Ong WY, unpublished). It is postulated that increased cholesterol and oxysterol after KA injury could lead to increased exocytosis from axon terminals of surviving neurons, leading to enhanced glutamatergic stimulation and excitotoxicity (Ma MT and Ong WY, unpublished).

Exposure of PC12 cells to 7-ketocholesterol results in nuclear damage, decrease in the mitochondrial transmembrane potential, cytosolic accumulation of cytochrome c, activation of caspase-3, increase in the formation of reactive oxygen species, and depletion of glutathione [103, 104]. Treatment with antioxidants or nitric oxide scavengers including *N*-acetylcysteine, trolox, and carboxy-PTIO or caspase inhibitors reduces the cytotoxic effect of 7-ketocholesterol [103, 104]. COPs may cause cytotoxic injury by disturbing the function of calcium channels. Cytotoxic effects, including phosphatidylserine externalization, loss of mitochondrial potential, increased permeability to propidium iodide, and occurrence of cells with swollen, fragmented, and/or condensed nuclei, are identified with 7-beta-hydroxycholesterol, cholesterol-5beta, 6beta-epoxide, and 7-ketocholesterol, and these effects are partially blocked by the calcium channel blockers verapamil and nifedipine [105, 106]. 7-Ketocholesterol also triggers sustained increase of cytosolic-free Ca^{2+}, which in turn elicits the mitochondrial pathway of apoptosis by activating calcium-dependent phosphatase, calcineurin, and subsequent dephosphorylation of the Bcl-2-associated death promoter protein [107]. Antioxidants are able to modulate the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome c that occur during 7-ketocholesterol-induced apoptosis [108].

Oxysterol cytotoxicity may also be due to their effects on cell signaling. Oxysterols or COPs with oxygen substitution on the side chain or sterol nucleus are markedly different from cholesterol in physiochemical characteristics, molecular configurations, and binding affinities to other lipids and proteins due partly to their wider range of potential molecular orientations. Moreover, some oxysterols or COPs are poor substrates for pathways that detoxify cells of excess cholesterol, which might account for some of their toxicity [109]. There could also be more indirect interactions between cholesterol or oxysterols and other lipids and proteins on the cell membrane. Studies in hypercholesterolemic rabbits and cultured cells show that 7-betahydroxycholesterol markedly inhibits the desaturation and elongation of fatty acids interfering with the production of long-chain polyunsaturated fatty acid from their precursors [110]. On the other hand, treatment of neurons with alpha-tocopherol or an inhibitor of sphingomyelin synthesis prevents the accumulation of ceramides and cholesterol after oxidative stress induced by amyloid beta peptide [111].

Conclusion

Intracerebroventricular injection of the potent glutamate analog KA in rats results in increased cholesterol concentration in the hippocampus at short to medium time intervals, i.e., 3 days–1 week post-injection, as detected by gas chromatography–mass spectrometry. This is accompanied by an early increase in levels of cholesterol biosynthetic precursors and increases in both enzymatically derived oxysterols such as 24-hydroxycholesterol and

COPs generated by ROS, including cholesterol epoxides and 7-ketocholesterol in the lesioned hippocampus. In contrast to COPs, no change in concentration of the neurosteroid pregnenolone was found after KA injury. Cholesterol and cholesterol oxidation products significantly induce exocytosis in cultured PC12 cells and neurons, and both oxysterols and COPs are able to induce cytotoxic and apoptotic injuries in different cell types, including neurons. The lipocalin apoD significantly reduced cholesterol and oxysterol levels in the KA lesioned hippocampus, consistent with its neuroprotective effect. Together, the findings suggest that increases in cholesterol and oxysterols after KA excitotoxicity could themselves lead to disturbance to neuronal ion homeostasis, increased neurotransmitter release, and propagation of excitotoxicity.

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