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24S-HYDROXYCHOLESTEROL EFFECTS ON LIPID METABOLISM GENES ARE MODELED IN TRAUMATIC BRAIN INJURY

Casandra M. Cartagena, PhD, Mark P. Burns, PhD, and G. William Rebeck, PhD

Department of Neuroscience, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20057

Abstract

Membrane damage during traumatic brain injury (TBI) alters the brain homeostasis of cholesterol and other lipids. Cholesterol 24S-hydroxylase (Cyp46) is a cholesterol metabolic enzyme that is increased after TBI. Here, we systematically examined the effects of the enzymatic product of Cyp46, 24S-hydroxycholesterol, on the cholesterol regulatory genes, SREBP-1 and 2, their post-translational regulation, and their effects on gene transcription. 24S-hydroxycholesterol increased levels of SREBP-1 mRNA and full length protein but did not change levels of cleaved SREBP-1, consistent with the role of 24-hydroxycholesterol as an LXR agonist. In contrast, 24S-hydroxycholesterol decreased levels of LXR independent SREBP-2 mRNA, full-length protein, and SREBP-2 active cleavage product. We examined the downstream effects of changes to these lipid regulatory factors by studying cholesterol and fatty acid synthesis genes. In neuroblastoma cells, 24S-hydroxycholesterol decreased mRNA levels of the cholesterol synthesis genes HMG CoA reductase, squalene synthase, and FPP synthase, but did not alter levels of the mRNA of fatty acid synthesis genes acetyl CoA carboxylase or fatty acid synthase. After TBI, as after 24S-hydroxycholesterol treatment in vitro, SREBP-1 mRNA levels were increased while SREBP-2 mRNA levels were decreased. Also similar to the in vitro results with 24S-hydroxycholesterol, HMG CoA reductase and squalene synthase mRNA levels were significantly decreased. Fatty acid synthase mRNA levels were not altered but acetyl CoA carboxylase mRNA levels were significantly decreased. Thus, changes to transcription of cholesterol synthesis genes after TBI were consistent with increases in Cyp46 activity, but changes to fatty acid synthesis genes must be regulated by other mechanisms.

Keywords

Cyp46; 24S-hydroxycholesterol; lipid metabolism; traumatic brain injury

1. Introduction

Cholesterol 24S-hydroxylase (Cyp46) is a brain enriched enzyme expressed primarily in neurons; it is responsible for converting cholesterol from the plasma membrane into 24S-hydroxycholesterol (Lund et al., 1999; Ramirez et al., 2008). Expression of Cyp46 in the brain

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G. William Rebeck (corresponding author): gwr2@georgetown.edu (T) 202-687-1534, (F) 202-687-0617.

Casandra M. Cartagena: caseyo@umich.edu, (T) 240-277-6939, (F) 202-687-0617

Mark P. Burns: mpb37@georgetown.edu, (T) 202-687-4735, (F) 202-687-0617

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is altered during normal development but is relatively stable in normal adult brain (Lund et al., 1999; Ohyama et al., 2006). Cyp46 is increased after traumatic brain injury (TBI), specifically in microglia (Cartagena et al., 2008). Other models of brain injury including hippocampal kainate injury (He et al., 2006) and acute experimental autoimmune encephalomyelitis (Teunissen et al., 2007) have shown increases in Cyp46 expression at lesion sites. The enzymatic product of Cyp46, 24S-hydroxycholesterol, is increased in the CSF of Alzheimer's disease (AD) patients compared to control subjects (Papassotiropoulos et al., 2002). These findings suggest that while Cyp46 activity in neurons plays a critical role in normal cholesterol homeostasis, increased Cyp46 activity may be a characteristic of the brain response to injury.

Cholesterol synthesized outside the CNS cannot enter the brain across the blood brain barrier (Jurevics and Morell, 1995; Turley et al., 1996). The brain synthesizes cholesterol and cholesterol turnover rates indicate that cholesterol exits the CNS at a rate in the mouse of 1.4 mg/day/kg (Dietschy and Turley, 2004). Cyp46 knockout mouse studies indicate that the conversion of cholesterol to 24S-hydroxycholesterol accounts for 64% of cholesterol efflux out of the brain (Dietschy and Turley, 2004; Xie et al., 2003). The remainder of cholesterol efflux out of the brain may be explained by inclusion of lipoproteins containing cholesterol and apolipoprotein E (apoE) (Pitas et al., 1987) in bulk CSF outward flow through the arachnoidal-lymphatic-venous interfaces (Johanson et al., 2008). 24S-hydroxycholesterol is also an activator of the nuclear transcription factors liver X receptor (LXR) α and β (Janowski et al., 1999; Lehmann et al., 1997), and can induce the upregulation of genes involved in cellular cholesterol efflux (Rebeck, 2004; Tall, 2008). LXR activation increases expression of genes important for cholesterol efflux such as ATP-binding cassette transporter (ABC) A1 in both neurons and glia (Fukumoto et al., 2002) and apoE in astrocytes (Liang et al., 2004). We have shown increases in both apoE and ABCA1 coinciding with increased Cyp46 expression after TBI (Cartagena et al., 2008). When both apoE and ABCA1 are present, cholesterol can be passed from the cell membrane to extracellular lipoproteins (Hirsch-Reinshagen et al., 2005; Huang et al., 2001). These cholesterol efflux mechanisms are important for lowering cholesterol levels when cellular cholesterol is in excess. In addition, the conversion of cholesterol to 24S-hydroxycholesterol makes it more soluble and easier to clear into the extracellular space and across the blood brain barrier into the blood (Bjorkhem, 2006; Olkkonen and Hynynen, 2009; Vaya and Schipper, 2007). Thus, 24S-hydroxycholesterol allows for two separate mechanisms for clearing cholesterol from the cell and from the brain overall.

24S-hydroxycholesterol potentially plays a role in regulating the cholesterol and fatty acid synthesis pathways in the brain both by its LXR activity and also because it is an oxysterol. Oxysterols alter lipid synthesis mechanisms by acting on sterol regulatory element (SRE) binding proteins (SREBPs). SREBPs are expressed as inactive 120 kDa precursors (pSREBP) which are integral to the endoplasmic reticulum (ER) membrane. pSREBPs are translocated from the ER to the Golgi by SREBP cleavage-activating protein (SCAP) where they are cleaved into a 67 kDa active transcription factor, which is not membrane bound. This shorter mature SREBP (mSREBP) alters transcription of genes containing an SRE in the promoter region. These genes are responsible for critical enzymes in both the cholesterol synthesis pathway and the fatty acid synthesis pathway. When intracellular cholesterol levels are in excess, SCAP, which has a cholesterol sensing domain, binds insulin induced gene (Insig) and the Insig-SCAP-pSREBP is retained in the ER (Olkkonen and Hynynen, 2009; Radhakrishnan et al., 2007). Oxysterols synthesized outside the CNS, such as 25-hydroxycholesterol, have been shown to suppress the cleavage of pSREBP to mSREBP (Adams et al., 2004; Du et al., 2004; Thewke et al., 1998), and to bind Insig rather than SCAP (Sun et al., 2007) and induce Insig-SCAP binding (Adams et al., 2004), thus retaining SCAP in the ER and preventing pSREBP translocation to the Golgi for cleavage (Olkkonen and Hynynen, 2009; Radhakrishnan et al., 2007).

There are two genes for SREBPs giving rise to the three isoforms: 1a, 1c, and 2. SREBP-1c differs from SREBP-1a only in a small portion of exon 1 but they are otherwise identical (Brown and Goldstein, 1997). SREBP-1a is constitutively expressed (Raghow et al., 2008). SREBP-2 has been shown to contain an SRE in its promoter region which, when bound, increased transcription of SREBP-2 (Sato et al., 1996). Neither SREBP1a nor SREBP-2 is known to be regulated by LXR. In contrast, SREBP-1c has been shown to be under LXR control (Whitney et al., 2002) and also contains a SRE in its promoter region (Cagen et al., 2005). In both liver and brain, expression levels of SREBP-1c are greater than SREBP-1a (Shimomura et al., 1997). In mice expressing transgenes for mature forms of SREBP-1a, SREBP-1c, and SREBP-2, mSREBP-1c (expressed primarily in liver) has weak transcription activity in comparison to the other isoforms on downstream cholesterol and fatty acid synthesis gene expression, while mSREBP-1a preferentially increases expression of fatty acid synthesis genes and mSREBP-2 preferentially increases expression of cholesterol synthesis genes (Horton and Shimomura, 1999; Horton et al., 1998).

Various oxysterols differ in their biological effects (Gill et al., 2008). For example, 27-hydroxycholesterol is an LXR agonist (Fu et al., 2001), while 25-hydroxycholesterol has only minimal LXR activity (Janowski et al., 1999). In the CYP27 $-/-$ mouse liver and adrenals, SREBP-2 mRNA levels were increased as were mRNA levels of HMG CoA Synthase and HMG CoA Reductase and cholesterol synthesis (Repa et al., 2000). This supports the hypothesis that products of CYP27, including 27-hydroxycholesterol, have inhibitory effects on cholesterol synthesis (Lund et al., 1993). 24S-hydroxycholesterol is the main oxysterol in the brain (Dietschy and Turley, 2004; Karu et al., 2007). In the CYP46 $-/-$ mouse, cholesterol excretion from the brain is reduced, as is cholesterol synthesis, leading to stable total brain cholesterol levels (Xie et al., 2003). These data conflict with the hypothesis that 24S-hydroxycholesterol inhibits the cholesterol synthesis pathway through effects on SREBP cleavage. It is possible that altering one mechanism of cholesterol metabolism leads to compensation in other pathways. For example, CYP27 has been shown to synthesize several oxysterols including 24-hydroxycholesterol (Lund et al., 1993) and transgenic mice overexpressing CYP27 have decreased circulating levels of 24S-hydroxycholesterol (Meir et al., 2002).

Microglia express CYP27 (Gilardi et al., 2009) and express CYP46 after TBI (Cartagena et al., 2008). Astrocytes increase CYP27 expression with LXR agonist treatment (Gilardi et al., 2009) and express CYP46 in AD brain (Bogdanovic et al., 2001; Brown III et al., 2004). In astrocytes, 24S-hydroxycholesterol decreased expression of the rate limiting step in cholesterol synthesis, HMG CoA reductase, and increased LXR regulated apoE expression (Abildayeva et al., 2006). In cortical neurons, 24S-hydroxycholesterol decreased expression of cholesterol synthesis enzymes including HMG CoA synthase and squalene synthase (Wang et al., 2008). In this study, we take a systematic look at 24S-hydroxycholesterol effects on SREBP-1 and 2 levels, post-translational regulation and downstream effects on fatty acid and cholesterol synthesis enzymes. This work also examines the effects of TBI induced increases in 24S-hydroxycholesterol on these two pathways in vivo.

2. Results

Some oxysterols inhibit cleavage of pSREBPs, preventing production of mature transcription factors (Gill et al., 2008; Gimpl et al., 2002; Radhakrishnan et al., 2007). In addition they have effects on gene regulation through activation of LXR proteins (Fu et al., 2001; Janowski et al., 1999). We sought to demonstrate what effects the brain-derived 24S-hydroxycholesterol may have on gene transcription overall. We began by analyzing levels of full-length and cleaved SREBP-1 and 2 in human 293 cells. We treated cells with 5 μ M 24S-hydroxycholesterol for 48 hr. Cells lysates were collected using RIPA buffer to ensure proteins from both cytosolic

and nuclear compartments would be present. Cell lysates were analyzed by western blot. The antibody to SREBP-1 demonstrated bands at 120 kD and 67 kD corresponding to the expected sizes of pSREBP-1 and mSREBP-1, respectively; similar sized bands were seen in membranes probed for SREBP-2 (Figure 1A). 24S-hydroxycholesterol increased pSREBP-1 levels while mSREBP-1 levels were unchanged (Figure 1A, 1st and 2nd panel), consistent with the expected upregulation of pSREBP-1c through LXR activation. In contrast to SREBP-1, levels of both pSREBP-2 and mSREBP-2 were unexpectedly reduced by 24S-hydroxycholesterol treatment (Figure 1A, 3rd and 4th panel). Quantification of pSREBP-1 levels showed an increase of 38% after 24S-hydroxycholesterol treatment, with no significant changes in mSREBP-1 levels (Figure 1B). pSREBP-2 levels showed a decrease of 51% after 24S-hydroxycholesterol treatment while mSREBP-2 levels showed a decrease of 91% (Figure 1C).

We sought to determine whether the observed effects of 24S-hydroxycholesterol were due to changes in mRNA levels of SREBP-1 and 2. We treated 293 cells with 5 μ M 24S-hydroxycholesterol or 1 μ M TO-901317 (an LXR agonist) for 48 hr and isolated RNA. Initial tests by reverse transcriptase PCR demonstrated that levels of SREBP-1 mRNA increased following 24S-hydroxycholesterol and TO-901317 treatments (Figure 2A). By this method, SREBP-2 mRNA levels appeared to have small decreases with 24S-hydroxycholesterol and small increases with TO-901317 treatment (Figure 2A). We used quantitative real-time PCR to test these qualitative findings. Analysis of SREBP-1 mRNA levels showed significant increases of 280% with 24S-hydroxycholesterol and 370% with TO-901317 (Figure 2B). Analysis of relative quantities of SREBP-2 mRNA levels showed a small but significant decrease of 18% with 24S-hydroxycholesterol and no significant change with TO-901317 (Figure 2B).

To determine whether the unexpected effects of 24S-hydroxycholesterol on decreasing SREBP-2 were specific to 24S-hydroxycholesterol, we treated 293 cells with 1 μ M TO-901317, 5 μ M 24S-hydroxycholesterol, 5 μ M 27-hydroxycholesterol, or 5 μ M cholesterol for 48 hr. Levels of both pSREBP-2 and mSREBP-2 proteins were measured by western blot (Figure 3A). pSREBP-2 levels showed a decrease of 50% with 24S-hydroxycholesterol treatment while 27-hydroxycholesterol caused a 36% (but statistically non-significant) decrease in pSREBP-2 levels (Figure 3A–3B). Neither cholesterol nor TO-901317 had a significant effect on pSREBP-2 levels. mSREBP-2 levels showed significant decreases of 84% with 24S-hydroxycholesterol treatment and 80% with 27-hydroxycholesterol treatment (Figure 3C). TO-901317 decreased mSREBP-2 levels by 36%, a significantly smaller decrease than seen with the oxysterols (Figure 3C). Cholesterol did not have a significant effect on either levels of mSREBP-2 levels or pSREBP-2 (Figure 3B and C). Thus, the oxysterols but not cholesterol decreased SREBP-2 levels.

Although 293 cells display many properties of immature neurons (Shaw et al., 2002), we wanted to determine the effects of 24S-hydroxycholesterol on SREBP-1 and 2 levels in a cell line with more established neuronal characteristics. We therefore treated SY5Y neuroblastoma cells with 5 μ M 24S-hydroxycholesterol. In these cells, levels of SREBP-1 and 2 showed similar patterns as those seen in 293 cells (Figure 4). pSREBP-1 levels were increased with 24S-hydroxycholesterol treatment (Figure 4, row 1), while there were no clear changes in mSREBP-1 levels (Figure 4, row 2). pSREBP-2 levels were decreased with 24S-hydroxycholesterol treatment while mSREBP-2 levels were virtually eliminated. We also measured mRNA levels of SREBP-1 and SREBP-2 by real-time PCR. Again, 24S-hydroxycholesterol significantly increased SREBP-1 mRNA levels (by 210%) and significantly decreased SREBP-2 mRNA levels (by 31%) (Figure 5A).

SREBPs regulate the transcription of genes in both the cholesterol and fatty acid synthesis pathways. Previous studies have indicated mSREBP-2 preferentially upregulates genes in the

cholesterol synthesis pathway while mSREBP-1 has greater control over fatty acid synthesis genes (Horton et al., 1998). Because 24-hydroxycholesterol has different effects on SREBP-1 and 2, we wanted to determine its overall effects on the cholesterol synthesis and fatty acid synthesis pathways. Thus, we used real-time PCR to measure relative quantities of mRNA for the cholesterol synthesis rate limiting enzyme gene HMG CoA reductase and the fatty acid synthesis rate limiting gene acetyl CoA carboxylase. In SY5Y cells, 24S-hydroxycholesterol significantly decreased mRNA levels of HMG CoA reductase by 18% (Figure 5B). In contrast, 24S-hydroxycholesterol did not significantly change mRNA levels of acetyl CoA carboxylase (Figure 5C). Because the decrease in HMG CoA Reductase was significant but relatively small we looked at additional genes in this pathway known to be under SREBP control, squalene synthase and FPP synthase. Real-time PCR analysis of these genes revealed that squalene synthase mRNA levels were decreased by 31% and FPP synthase mRNA levels were decreased by 17% after 24S-hydroxycholesterol treatment, showing a small but consistent decrease in multiple enzyme levels across the cholesterol synthesis pathway. Although we saw no significant change in acetyl CoA carboxylase we also wanted to evaluate the effect of 24S-hydroxycholesterol on the other main enzyme in the fatty acid synthesis pathway, fatty acid synthase. We found that 24S-hydroxycholesterol also did not have a significant effect on this enzyme. Thus, in SY5Y cells, 24S-hydroxycholesterol downregulated transcription of several cholesterol synthesis genes but did not affect genes involved in fatty acid synthesis.

Thus, in vitro, 24S-hydroxycholesterol strongly suppresses SREBP-2 levels and the expression of cholesterol synthesis genes but fails to suppress SREBP-1 levels and fatty acid synthesis genes. Since Cyp46 is upregulated following TBI (Cartagena et al., 2008), subsequent changes in 24S-hydroxycholesterol levels may alter levels of both cholesterol and fatty acid synthesis genes. We examined mRNA in cortex of TBI mice and found that SREBP-1 mRNA levels were significantly increased by 86% at the site of injury (Figure 6A) and SREBP-2 mRNA levels were significantly suppressed by 43% at the site of injury (Figure 6B) in comparison to sham control cortex. There were no differences in either SREBP-1 or 2 mRNA between samples from brain contralateral to the TBI compared to sham controls (Figure 6).

As we had previously done in vitro, we determined what overall effects altered SREBP-1 and 2 mRNA levels had on the cholesterol synthesis and fatty acid synthesis pathways in vivo. Following TBI, we found that mRNA levels of HMG CoA reductase were significantly decreased by 38% and squalene synthase mRNA levels were significantly decreased by 33% at the site of injury in comparison to sham cortex (Figure 7A), again consistent with the decreases in SREBP-2 isoforms. However, despite the increase in SREBP-1 mRNA (Figure 6A), we found that acetyl CoA carboxylase mRNA levels were significantly decreased by 26% following TBI, and fatty acid synthase mRNA levels were not changed (Figure 7B).

3. Discussion

In order to maintain cholesterol homeostasis, the brain must regulate both its synthesis and its efflux, processes that are affected by production of oxysterols from cholesterol (Jurevics and Morell, 1995;Lund et al., 2003;Turley et al., 1998;Turley et al., 1996;Xie et al., 2003) Oxysterols activate the nuclear transcription factor LXR, resulting in upregulation of cholesterol efflux molecules (e.g., ABC-A1, ABC-G1, apoE) (Costet et al., 2000;Laffitte et al., 2001;Mak et al., 2002;Repa et al., 2000;Sabol et al., 2005). LXR activation also results in upregulation of pSREBP-1 (Repa et al., 2000), which could increase lipid synthesis and endocytosis through effects on gene transcription. Oxysterols also can inhibit the cleavage of inactive pSREBPs to produce the transcription factors mSREBPs (Adams et al., 2004;Du et al., 2004;Thewke et al., 1998), thus preventing increased transcription of multiple genes in the cholesterol synthesis pathway, including the gene for the rate limiting enzyme, HMG CoA reductase (Horton et al., 2002;Saucier et al., 1989). We found that 24S-hydroxycholesterol had

the expected effect on pSREBP-1, significantly increasing levels of SREBP-1 mRNA and protein (Figure 1, Figure 2, and Figure 5), but that it did not increase mSREBP-1 levels. One plausible explanation is that while 24S-hydroxycholesterol increased pSREBP-1 expression, it also inhibited its cleavage, as observed for 25-hydroxycholesterol (Adams et al., 2004; Thewke et al., 1998). In contrast to increasing SREBP-1, 24S-hydroxycholesterol had an unexpected and dramatic effect of reducing SREBP-2. We observed these effects in both 293 and SY5Y cells (Figure 1, Figure 3, and Figure 4) as well as a mouse microglial cell line (data not shown). We therefore further explored the effects of 24S-hydroxycholesterol on cholesterol homeostatic mechanisms.

24S-hydroxycholesterol reduced both SREBP-2 mRNA and protein levels, decreasing mSREBP-2 protein levels by 80–91%. Another brain oxysterol, 27-hydroxycholesterol, also significantly reduced mSREBP-2 levels, but unaltered cholesterol did not (Figure 3). These data are consistent with the idea that while exogenously applied oxysterols can cross membranes to have intracellular signaling effects, exogenous cholesterol cannot, and thus does not have an effect on intracellular signaling. 24S-hydroxycholesterol also reduced pSREBP-2 levels by at least 50%. The inhibition of pSREBP-2 by oxysterols may be due to a combination of direct inhibition of cleavage by binding Insig (Olkonen and Hynynen, 2009; Radhakrishnan et al., 2007) as well as potential indirect effects from LXR activity (Fu et al., 2001; Janowski et al., 1999). 24S-hydroxycholesterol also decreased transcription of pSREBP-2, although only by 18–31% (Figure 2 and Figure 5). It is important to note that there is an SRE in the promoter region of SREBP-2, resulting in a positive feedback mechanism where more mSREBP-2 promotes its own transcription (Sato et al., 1996). Retention of pSREBP-2 in the ER by 24S-hydroxycholesterol binding Insig would lower mSREBP-2 levels, leading to less mSREBP-2-SRE binding and thus less SREBP-2 transcription.

SREBP's vary in their effects on lipid metabolism: mSREBP-2 has more control over transcription of genes encoding cholesterol synthesis enzymes while mSREBP-1 has more control over transcription of genes encoding fatty acid synthesis enzymes (Horton et al., 2002; Horton and Shimomura, 1999; Horton et al., 1998). Consistent with its effects on decreasing SREBP-2, 24S-hydroxycholesterol suppressed many of the genes in the cholesterol synthesis pathways including HMG CoA Reductase, squalene synthase, FPP synthase in neuroblastomas cells (Figure 5B). Consistent with our finding that mSREBP-1 levels were relatively stable in these cells, 24S-hydroxycholesterol did not have significant effects on mRNA levels of the fatty acid synthesis enzymes, fatty acid synthase or the rate-limiting acetyl CoA reductase. These data suggest that 24S-hydroxycholesterol strongly suppresses cholesterol synthesis but has minimal effect on fatty acid synthesis, despite being a strong LXR agonist. These findings are in agreement with a recent study in cortical neurons showing decreased levels of cholesterol synthesis enzymes HMG CoA synthase and squalene synthase but no significant changes in acetyl CoA carboxylase or fatty acid synthase levels after 24S-hydroxycholesterol treatment (Wang et al., 2008) and a study in STTG glial cells where 24S-hydroxycholesterol decreased mRNA levels of HMG CoA reductase (Abildayeva et al., 2006).

We have previously shown that, although Cyp46 is a neuronally expressed enzyme under normal conditions, its expression is upregulated in microglia after brain injury and after activation (Cartagena et al., 2008). This upregulation was very specific to the injury site while tissue outside the injury site showed little effect on Cyp46 expression (Cartagena et al., 2008). Although the concentrations of 24S-hydroxycholesterol used in these in vitro studies are several fold greater than that seen in CSF, at the site of injury increased Cyp46 levels may lead to sizable increases of 24S-hydroxycholesterol. Increased conversion of lipid debris into 24S-hydroxycholesterol would raise extracellular 24S-hydroxycholesterol levels and would cause regional alterations in both the cholesterol and fatty acid synthesis pathways. Following

brain injury, cholesterol levels in the CSF are increased from the cellular loss and subsequent lipid debris (Fagan et al., 1998; Gasparovic et al., 2001; Kamada et al., 2003). While increases in ABCA1 and apoE after TBI (Cartagena et al., 2008) would facilitate increased cholesterol efflux either for neuronal regeneration in other areas or export from the brain (Cartagena et al., 2008), oxysterol suppression of cholesterol synthesis would also assist in bringing the brain back into cholesterol homeostasis after injury. Previous studies of entorhinal cortex lesions 1 week after injury showed increased apoE levels and decreased HMG CoA reductase activity (Poirier et al., 1993), possibly due to increased 24S-hydroxycholesterol levels. Here, in the CCI model, we show that 7 days after TBI, SREBP-1 mRNA levels were increased and SREBP-2 levels were decreased at the site of injury (Figure 6). While increased expression of Cyp46 after injury is localized to the lesion site (Cartagena et al., 2008; Teunissen et al., 2007), 24S-hydroxycholesterol can enter the CSF (Papassotiropoulos et al., 2002) and thus have more of a gradient effect on the brain as a whole. The idea that high 24S-hydroxycholesterol levels at the injury site diffuse out across the brain is supported by the fact that both SREBP-1 and SREBP-2 mRNA levels in contralateral cortex still slightly elevated compared to sham controls (Figure 6). We found decreases in HMG CoA reductase and squalene synthase mRNA levels after TBI (Figure 7B), indicating that cholesterol synthesis at the site of injury is suppressed.

While 24S-hydroxycholesterol did not appear to have an effect on fatty acid synthesis in vitro, we found that after TBI, transcript levels of the rate limiting enzyme acetyl CoA carboxylase are decreased while levels of fatty acid synthase did not change. Thus we must attribute these changes to factors other than 24S-hydroxycholesterol (Horton et al., 2002). Both palmitic acid and PUFAs are upregulated after TBI (Dhillon et al., 1999; Dhillon et al., 1994; Pilitsis et al., 2003); derivatives of palmitic acid decrease activity and protein levels of acetyl CoA carboxylase (Brun et al., 1997; Natali et al., 2007; Nikawa et al., 1979; Rubink and Winder, 2005), and PUFAs decrease acetyl CoA carboxylase mRNA levels (Brun et al., 1997; Worgall et al., 1998). Thus increased palmitic acid and PUFAs after TBI may counteract any effects of 24S-hydroxycholesterol.

After TBI, increased Cyp46 expression is accompanied by upregulation of cholesterol efflux factors ABCA1 and apoE (Cartagena et al., 2008). Here we show that TBI is also accompanied by suppression of cholesterol synthesis enzymes, which may be accounted for, based on our in vitro experiments, by the increased production of 24S-hydroxycholesterol. We present these findings with the caveat that changes in cholesterol or fatty acid synthesis rates have not been shown directly and it remains to be seen whether the observed changes in enzyme expression alter final cholesterol or fatty acid levels. A recent study showed that increasing Cyp46 expression in the brain by adeno-associated viral therapy significantly reduced A β pathology and improved memory (Hudry et al., 2009). Increases in cholesterol levels have been shown to increase A β deposition (Ehehalt et al., 2003; Levin-Allerhand et al., 2002) and these protective effects of increased Cyp46 expression suggest increased cholesterol clearance. Based on our current findings we suggest that increases in 24S-hydroxycholesterol lead to both increased cholesterol clearance and decreased cholesterol synthesis and that these mechanisms have clear relevance to TBI.

4. Experimental Procedure

Chemicals

Stock solutions of 2 mM TO-901317 (Tocris), 10 mM 24S-hydroxycholesterol (Steraloids), 10 mM 27-hydroxycholesterol (Steraloids) and 10 mM Cholesterol (Sigma) were all made in ethanol and stored at -20°C . These were diluted in media to 5 μM immediately before treating cells. 24S-hydroxycholesterol has been measured in human CSF ranging from 2 nM to 10 nM (Papassotiropoulos et al., 2002; Shafaati et al., 2007), but may increase dramatically at the injury

site following TBI (Cartagena et al., 2008). At least 1 μ M 24S-hydroxycholesterol is necessary to upregulate LXR regulated genes ABC-A1 and ABC-G1 (Fujiyoshi et al., 2007).

Cell Culture

293 cells were cultured in Opti-MEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco). SY5Y cells were cultured in DMEM (ATCC) containing 10% FBS. Cells were plated into 6 well plates. After 24 h cells media was removed and replaced with fresh media containing either 5 μ M 24S-hydroxycholesterol or 0.5 μ l/mL ethanol (vehicle). Cells were treated for 48 h and then collected for either protein or mRNA isolation. Cell culture experiments were performed in triplicate on at least two separate occasions.

Animals

Ten male 4 month old C57BL/6J mice were used in these experiments. All animal protocols were approved by the Georgetown University Institutional Animal Use and Care Committee, and were in compliance with the standards stated in the Committee on Care and the Use of Laboratory Animals Publication [NIH] 85–23 1985.

Mouse Controlled Cortical Impact (CCI) Trauma Model

The CCI-injury device was designed and built at the Georgetown University, and consists of a microprocessor-controlled pneumatic impactor with a 3.5 mm diameter tip (Fox et al., 1998). Moderate injury was induced by the impactor velocity of 6 m/s and deformation depth of 2 mm. Mice were anaesthetized with isoflurane (induction at 4% and maintenance at 2%) evaporated in a gas mixture containing 70% N₂ and 30% O₂ and administered through a nose mask. Depth of anesthesia was assessed by monitoring respiration rate and pedal withdrawal reflexes. The mouse was placed on a heated pad, and a core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and the surgical site was clipped and cleaned with Nolvasan scrubs. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned to the surface of the exposed dura, and reset to impact the cortical surface. After injury, the incision was closed with interrupted 6-0 silk sutures, anesthesia was terminated, and the animal was placed into a heated cage to maintain normal core temperature for 45 min post-injury. All animals were monitored carefully for at least 4 h after surgery and then daily. Five animals underwent CCI-injury. An additional five animals underwent sham injury where a 4-mm craniotomy was made but the dura was not disturbed. At day 7 after surgeries, animals were sacrificed with CO₂ and brains were removed. Isolated cortex was collected from the injury site as well as from the contralateral side. In sham animals, cortex at the site of craniotomy was collected. Tissue was snap frozen in isopentane on dry ice and stored at –80°C until later RNA isolation.

Western blot

Cells were washed in phosphate buffered saline (PBS), lysed in RIPA buffer (Pierce) containing protease inhibitor (Roche). Protein concentrations in lysates were measured by the Bradford method (Biorad). 20 μ g total protein from each sample were loaded onto polyacrylamide gels. Protein was transferred to PVDF membranes and blocked 1 h at room temperature (RT) in 5% milk in PBS. Membranes were probed with anti-SREBP-1 (Abcam, 1:100) or anti-SREBP-2 (BD Biosource, 1:100) overnight at 4°C. Membranes were washed in PBS and incubated with goat anti-mouse HRP tagged antibody at 1:10:000. Membranes were washed in PBS and incubated with DURA (Pierce) and exposed to Kodak MR film. Membranes were reprobbed with anti- β -actin antibody (1:5000) for protein loading controls. Bands were quantified using Quantity 1 software (Biorad). Local backgrounds were subtracted and levels were adjusted for

small variations in loading controls. Statistical analysis was performed using Graphpad Prism 4 and either one way ANOVA with Newman-Keuls multiple comparison post-hoc test or Student's t-test.

mRNA isolation

Total RNA was isolated using the Absolute RNA Miniprep purification kit (Stratagene) according to manufacturer's directions from either cell culture or mouse cortical brain tissue. Briefly, cultured cells in 6-well plates were lysed in 400 μ l/well manufacturer's lysis buffer and mouse hemi-cortex was lysed in 1 mL lysis buffer. Lysate was loaded onto a prefilter column and spun at 14,000 rpm. Filtrate was mixed 1:1 with 70% ethanol, loaded onto a RNA binding column and spun at 14,000 rpm. The column was washed with low salt buffer and treated for 15 min with DNase at 37°C. The column was washed in high salt and then low salt buffers. 60°C elution buffer was added to the column and spun at 14,000 rpm to collect purified RNA. Total RNA concentrations and purity were calculated from spectrophotometer optical density measurements at 260 and 280 nm. RNA was stored at -80°C until further use.

cDNA synthesis

cDNA was synthesized using the AffinityScript QPCR cDNA Synthesis kit (Stratagene). Briefly, 20 μ l reactions contained 3 μ g total RNA, 0.3 μ g oligo(dT) primers, 10 μ l 2 $\times$ mastermix and 1 μ l AffinityScript RT/RNase Block enzyme mixture. The reaction was incubated at 25°C for 5 min, 42°C for 45 min, 95°C for 5 min, 4°C for 5 min, and then stored at -20°C until further use.

Primer design

All primers were designed using Geneious Pro 3.0.6 software (Biomatters). All primers were designed to cross at least two exons. Primers used in rtPCR are listed in Table 1. Primers to human sequences used in real-time PCR are listed in Table 2 while primers to mouse sequences are listed in Table 3. Real-time primers were designed to have zero primer-dimers and to produce product sizes between 120–150 base pairs. Real-time primers were tested using 1:2, 1:4, and 1:8 serial dilutions of cDNA to confirm efficiency of the primer pair. A dissociation curve was performed to confirm the absence of any primer-dimer pair products.

rtPCR

Amplifications of cDNA were performed with a thermocycler using GoTaq Green (Promega) mastermix. Reactions were incubated at 95°C for 2 min, then 30 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, and then 72°C for 5 min. Product was run on 2% agarose gels and bands were digitally imaged and inverted using Quantity 1 (BioRad) software.

Real-time PCR

Relative mRNA quantities were measured by real-time PCR using PowerSYBR green (Applied Biosystems) mastermix and primers listed in Table 2 and Table 3. Amplifications were performed with an ABI 7900 HT sequence detection system and were incubated at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 58°C for 1 min and 72°C for 30 sec. Each sample was tested in triplicate. Δ Ct levels for β -actin were used as an endogenous control to normalize Δ Ct levels for each sample and relative quantities were calculated using the formula $RQ=2^{-\Delta\Delta Ct}$. Analysis of real-time amplification data was done using SDS 2.3 (Applied Biosystems) and relative quantities were calculated using RQ Manager software (Applied Biosystems). Statistical analysis was performed using Graphpad Prism 4 and either one-way ANOVA with Newman-Keuls multiple comparison post-hoc test or Student's t-test.

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Figure 1. 24S-hydroxycholesterol increases SREBP-1 protein levels while uppressing SREBP-2 levels

293 cells were treated for 48 hr with 5 μ M 24S-hydroxycholesterol (24) or control vehicle (C). A) Levels of precursor SREBP-1 (pSREBP-1) and mature SREBP-1 (mSREBP) as well as precursor SREBP-2 (pSREBP-2) and mature SREBP-2 (mSREBP-2) were measured in cell lysate by immunoblot. B) Quantification of pSREBP-1 levels showed an increase of 38% after 24S-hydroxcholesterol treatment (Student's t-test, * $p < 0.05$, $n = 8$) while there were no significant changes in mSREBP-1 levels. C) Quantification of pSREBP-2 levels showed a decrease of 51% after 24S-hydroxycholesterol treatment (Student's t-test, * $p < 0.0001$, $n = 7$)

while mSREBP-2 levels showed a decrease of 91% (Student's t-test, $*p < 0.0001$, $n=7$). Error bars (standard error of the mean).

Figure 2. 24S-hydroxycholesterol and LXR agonist TO-901317 both increase SREBP-1 mRNA levels while 24S-hydroxycholesterol decreases SREBP-2 mRNA levels

293 cells were treated for 48 hr with 5 μ M 24S-hydroxycholesterol (24), 1 μ M TO-901317 (TO) or control vehicle (C). mRNA from these cells was converted to cDNA and amplified using primers against either SREBP-1 or SREBP-2. A) Relative levels of SREBP-1 mRNA showed a clear increase following 24S-hydroxycholesterol and a greater increase following TO-901317 treatment. Relative levels of SREBP-2 mRNA showed small decreases with 24S-hydroxycholesterol treatment and small increases with TO-901317 treatment. B) Real-time PCR quantification of SREBP-1 mRNA levels showed an increase of 280% with 24S-hydroxycholesterol treatment (Newman-Keuls, $*p < 0.001$, $n = 6$) and an increase of 370% with TO-901317 treatment (Newman-Keuls, $*p < 0.001$, $n = 6$). Real-time PCR quantification of SREBP-2 mRNA levels showed a decrease of 18% with 24S-hydroxycholesterol treatment

(Newman-Keuls, $*p < 0.05$, $n=6$) but no significant change with TO-901317 treatment. Control relative quantity=1. Error bars (standard error of the mean).

Figure 3. 24S-hydroxycholesterol but not cholesterol or TO-901317 suppresses SREBP-2 protein levels

293 cells were treated for 48 hr with 5 μ M 24S-hydroxycholesterol (24), 5 μ M 27-hydroxycholesterol (27), 5 μ M cholesterol (Ch), 1 μ M TO-901317 (T) or control vehicle (C). A) Levels of precursor SREBP-2 (pSREBP-2) and mature SREBP-2 (mSREBP-2) were measured in cell lysate by immunoblot. B) Quantification of pSREBP-2 levels showed a decrease of 50% with 24S-hydroxycholesterol treatment (Newman-Keuls, * p <0.001, n =6) while 27-hydroxycholesterol failed to significantly decrease pSREBP-2 levels. Neither cholesterol nor TO-901317 had a significant effect on pSREBP-2 levels. C) mSREBP-2 levels showed a decrease of 84% with 24S-hydroxycholesterol treatment (Newman-Keuls, * p <0.001,

n=6) and a decrease of 80% with 27-hydroxycholesterol treatment (Newman-Keuls, * $p < 0.001$, n=6). TO-901317 also decreased mSREBP-2 levels by 36% (Newman-Keuls, * $p < 0.05$, n=6). Decreases in mSREBP-2 levels by 24S-hydroxycholesterol and 27-hydroxycholesterol were significantly lower than those seen with TO-901317 (Newman-Keuls, $\diamond p < 0.01$, n=6). Cholesterol did not have a significant effect on mSREBP-2 levels. Error bars (standard error of the mean).

Figure 4. 24S-hydroxycholesterol increases SREBP-1 protein levels while suppressing SREBP-2 levels in SY5Y cells

SY5Y cells were treated for 48 hr with 5 μ M 24S-hydroxycholesterol (24S) or control vehicle (C). Results seen in this representative blot were similar to those seen in 293 cells. pSREBP-1 levels were clearly increased while mSREBP-1 levels remained relatively stable with 24S-hydroxycholesterol treatment. Both pSREBP-2 and mSREBP-2 were suppressed by 24S-hydroxycholesterol treatment.

Figure 5. Effects of 24S-hydroxycholesterol on cholesterol and fatty acid synthesis genes in human neuroblastoma SY5Y cells

SY5Y cells were treated for 48 hr with 5 μ M 24S-hydroxycholesterol or vehicle control and mRNA levels were measured by real-time PCR. A) 24S-hydroxycholesterol increased SREBP-1 mRNA levels by 210% (student's t-test, * p <0.001, n =6) and decreased SREBP-2 mRNA levels by 31% (student's t-test, * p <0.001, n =6) in comparison to controls. B) 24S-hydroxycholesterol decreased mRNA levels of the rate limiting cholesterol synthesis enzyme HMG CoA reductase by 18% (student's t-test, * p <0.005, n =6), decreased squalene synthase by 31% (student's t-test, * p <0.005, n =6) and FPP synthase by 17% (student's t-test, * p <0.01, n =6). C) 24S-hydroxycholesterol did not significantly change mRNA levels fatty acid synthesis

enzymes acetyl CoA carboxylase or fatty acid synthase in comparison to controls. Control relative quantity=1. Error bars (standard error of the mean).

Figure 6. Regulation of SREBP-1 and SREBP-2 mRNA levels 7 days after TBI

SREBP-1 and SREBP-2 mRNA levels were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI (tbi icx), contralateral cortex (tbi ccx) and sham cortex ipsilateral to craniotomy (sham icx). A) SREBP-1 mRNA levels at the site of injury were significantly increased 86% in comparison to sham controls (Newman-Keuls, $*p < 0.01$, $n = 5$). B) SREBP-2 mRNA levels at the site of injury were significantly decreased 43% in comparison to sham controls (Newman-Keuls, $*p < 0.05$, $n = 5$).

Figure 7. Regulation of HMG CoA reductase and acetyl CoA carboxylase mRNA levels 7 days after TBI

mRNA levels of acetyl CoA carboxylase, fatty acid synthase, HMG CoA reductase and squalene synthase were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI (tbi icx), and sham cortex ipsilateral to craniotomy (sham icx). A) HMG CoA reductase mRNA levels were significantly decreased 38% (Student's t-test, *p<0.005, n=5) and squalene synthase mRNA level were significantly decreased 33% (student's t-test, *p<0.05) at the site of injury in comparison to sham controls. B) acetyl CoA reductase mRNA levels at the site of injury were significantly decreased 26% (Student's t-test, *p<0.05, n=5) while there was no change in fatty acid synthesis mRNA levels at the site of injury in comparison to sham controls. Error bars (standard error of the mean).

Table 1

Primers used in rtPCR.

Target	Forward Reverse
Human SREBP-1	GACACACCAGCTCCTCGAC GGGACCAAAGTGGCTAGAGA
Human SREBP-2	CTGCCCCCTCCTTCCTCT TGGGCATCTAGTGACAGCAG
Human β -actin	GCAAAGACCTGTACGCCAAC AGTACTTGCCTCAGGAGGA

Table 2

Human sequence primers used in real-time PCR.

Target	Forward Reverse
SREBP-1	CAGCCAGCCTGACCATCT AAGCAGGTCACACAGGAACA
SREBP-2	ATGGGCAGCAGAGTTCCTT CGACAGTAGCAGGTCACAGG
HMG CoA reductase	CTGGGGAATTGTCACTTATGG GAACTGTCGGGCTATTCAGG
squalene synthase	CCTCAAGAGGTTTGGAGCAG TCTTCACTGCCCTTTGAAC
FPP synthase	CTGGAGATGGGGAGTTCTT CCTCCTTCTGCCCGTAATTT
acetyl CoA carboxylase	TGACAGAGGAGGATGGTGTTC GCTGAGTGGGTGATATGTGCT
fatty acid synthase	AGTACACACCCAAGGCCAAG GTGGATGATGCTGATGATGG
β -actin	AAAGACCTGTACGCCAACACA AGTACTTGCGCTCAGGAGGA

Table 3

Murine sequence primers used in real-time PCR.

Target	Forward Reverse
SREBP-1	TCTGGAGCTGCGTGGTTT AGGACTTGCTCCTGCCATC
SREBP-2	AGCCTACCGCAAGGTGTTT GCCAGGTGTCTACCTCTCCA
HMG CoA reductase	AGATAGGAACCGTGGGTGGT GTGCGTTTTCTCCAGGATG
acetyl CoA carboxylase	GCAACTGACAGAGGAAGATGG GGTCATGTGGACGATGGAG
squalene synthase	ACATGCCTGCCGTCAAAG TAGTGGCTTCGGGAGATGAG
fatty acid synthase	TTGACGGCTCACACACCTAC GCTGTGTTCCACATCAAGAAA
β -actin	TGACAGGATGCAGAAGGAGA ACATCTGCTGGAAGGTGGAC