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Chronic Administration of Statins Alters Multiple Gene Expression Patterns in Mouse Cerebral Cortex

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d) Abbreviations Aβ, amyloid β-protein; AD, Alzheimer's disease; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LC/MS/MS, liquid chromatography/tandem mass spectometry; OATs, organic anion transporters

e) Neuropharmacology

ABSTRACT

Statins have been reported to lower the risk of developing Alzheimer's disease. However, the mechanism of this potentially important neuroprotective action is not understood. Lowering cholesterol levels does not appear to be the primary mechanism. Statins have pleiotropic effects in addition to lowering cholesterol and statins may act on several different pathways involving distinct gene expression patterns that would be difficult to determine by focusing on a few genes or their products in a single study. In addition, gene expression patterns may be specific to a particular statin. To understand the molecular targets of statins in brain, DNA microarrays were used to identify gene expression patterns in the cerebral cortex of mice chronically treated with lovastatin, pravastatin, and simvastatin. Furthermore, brain statin levels were determined using liquid chromatography-tandem mass spectrometry. These studies revealed 15 genes involved in cell growth, signaling and trafficking that were similarly changed by all three statins. Overall, simvastatin had the greatest influence on expression as demonstrated by its ability to modify the expression of 23 genes, in addition to those changed by all three drugs. Of particular interest, was expression of genes associated with apoptotic pathways that were altered by simvastatin. RT-PCR experiments confirmed the microarray findings. All three drugs were detected in the cerebral cortex and acute experiments revealed that statins are relatively rapidly removed from brain. These results provide new insight into possible mechanisms for the potential efficacy of statins in reducing the risk of Alzheimer's disease and lay the foundation for future studies.

Several different lines of evidence point to a potentially important but not well understood association between Alzheimer's disease (AD) and cholesterol. Experimental studies both *in vitro* and *in vivo* have reported that changes in cholesterol levels alter amyloid precursor protein abundance and amyloid beta-protein (Aβ) levels and conversely that Aβ modifies cholesterol dynamics (reviewed in Wood et al., 2003; Wolozin, 2004). ApoE4, a cholesterol carrier protein, is a major risk factor for AD (Saunders et al., 1993). A most interesting aspect of the association between cholesterol and AD are that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are associated with a reduced risk of developing AD (Wolozin et al., 2000; Jick et al., 2000; Rockwood et al., 2002). Epidemiological data show that patients taking statins had a lower risk of developing AD as compared with individuals not taking statins and effects were cholesterol-independent (Wolozin et al., 2000; Jick et al., 2000; Rockwood et al., 2002). HMG-CoA reductase is the primary regulatory enzyme in cholesterol biosynthesis. A straightforward explanation for the beneficial effects of statins on reducing the risk of AD is a reduction of brain and serum cholesterol levels. However, it is unclear as to whether the apparent efficacy of statins is related to simply lowering cholesterol levels. Data on cholesterol levels in brain of AD patients range from less cholesterol, no differences and more cholesterol when compared with brain tissue of control samples (Wood et al., 2003;Wolozin, 2004). Non-statin drugs that lower cholesterol levels do not lower the risk of developing AD (Jick et al., 2000). Statins appear to reduce the risk of AD however the mechanism underlying statin efficacy does not appear to be simply reduction of cholesterol levels.

There is a growing recognition that statins have pleiotropic effects beyond lowering cholesterol levels. Pleiotropic effects of statins outside of the central nervous system have been described that include for example, activation of protein kinase Akt, upregulation of eNOS

expression, anti-inflammatory actions, and anti-oxidant activity (reviewed in Werner et al., 2002). In an experimental model of stroke, brain tissue of rats treated with either simvastatin or atorvastatin displayed increased synaptogenesis, neurogenesis and angiogenesis that was cholesterol-independent (Chen et al., 2003). In another study however, simvastatin, pravastatin, atorvastatin, rosuvastatin, and mevastatin protected mouse neurons from NMDA-mediated excitotoxicity and cell death and appeared to be cholesterol-dependent because alterations in cholesterol by cyclodextrin modified excitotoxicity (Zacco et al., 2003). The potential neuroprotective effects of statins may include several different pathways involving distinct gene expression patterns that would be difficult to determine by focusing on a few genes or their products in a single study. In addition, gene expression patterns may be specific to a particular statin.Therefore, to understand the molecular targets of statins in brain, DNA microarrays were used to identify gene expression patterns in the cerebral cortex of mice chronically treated with the two hydrophobic statins, lovastatin and simvastatin, and the hydrophilic statin, pravastatin. Lovastatin has been detected in human CSF whereas pravastatin is not thought to cross the blood-brain barrier (Botti et al., 1991) even though patients taking pravastatin were at a lower risk for developing AD (Wolozin et al., 2000; Jick et al., 2000; Rockwood et al., 2002). Statins may accumulate in brain as a result of chronic administration including pravastatin and therefore statin levels were determined in cerebral cortex of mice acutely and chronically administered statins. Statin quantification was accomplished using liquid chromatography-tandem mass spectrometry (LC/MS/MS). We demonstrate that statins have pleiotropic effects on gene expression in brain of mice treated with lovastatin, pravastatin and simvastatin. The three statins altered expression of numerous genes in brain particularly genes associated with cell growth, signaling and trafficking that could be fundamental contributors to neuroprotection. Overlap in

effects of the three statins on gene expression was high. However, simvastatin altered expression of more genes than the other statins that could be due to differences in brain levels or targeting of specific genes. Levels of the three statins were detected in brain suggesting that all three drugs were able to cross the blood-brain barrier and that statin levels declined quickly in brain. Simvastatin was detected at the highest concentration in the brain, followed by lovastatin and pravastatin. Cholesterol levels were reduced between 8% and 13% in the cerebral cortex. Statins have pleiotropic effects on gene expression in brain in addition to acting on cholesterol levels suggesting potential pathways of neuroprotection.

METHODS

Statin administration. Female C57BL/6 mice 2 mo of age were used in this study and were purchased from Charles River, Sulzbach, Germany. Mice were administered lovastatin, pravastatin (100 mg/kg body wt.) or simvastatin (50 mg/kg body wt.) by oral gavage once a day for 21 days. The difference in the drug dose of lovastatin and pravastatin compared with simvastatin is the differences in efficacy of lowering LDL cholesterol levels with simvastatin being more potent (Law et al., 2003). Statin concentrations used were based on earlier studies using mice (Eckert et al., 2001; Kirsch et al., 2003). There were 4 mice per statin group and 4 mice served as controls and received the statin vehicle (0.2% w/v, agarose gel/kg body wt.). Mice were weighed daily to calculate statin and vehicle dosage. Twenty four hours following the last treatment, animals were killed by decapitation. Cerebral cortex was dissected, weighed and either placed at -80^{o} C or homogenized in 5 mM Tris-HCl buffer, pH 7.4 (1 ml buffer per 100 mg brain.). In a separate experiment, mice were acutely treated with one of the three statins and statin levels in cerebral cortex determined at 1, 3, and 6 h following statin administration.

RNA Isolation and Microarray Analysis. RNA was isolated using TRIzol reagent (Invitrogen Life Technologies), chloroform, and 100% ethanol according to manufacturer's instructions. Microarray analysis was performed using the Affymetrix chip Murine Genome U74Av2 (www.Affymetrix.com). Twenty micrograms of RNA was precipitated from stock RNA and used in microarray experiments. Reactions were performed according to the GeneChip Expression Analysis Technical Manual and all reagents were purchased from Invitrogen Life Technologies unless otherwise indicated. Targets were hybridized to MU74UAv2 probe arrays for 16 hours according to the Affymetrix protocol. Washing, staining, and scanning procedures were performed by that Affymetrix Core Unit located at the University of Minnesota. Data was

analyzed using Microarray Suite (MAS) 5.0 and GeneSpring 6 (Silicon Genetics, Redwood City, CA). The following criteria were used to select candidate genes for further analysis: a) gene expression was rated as 'present' by specific Affymetrix criteria; b) hybridization levels were rated as 'changed' by specific Affymetrix criteria; c) there was 1.8 ≥ fold difference in hybridization intensity/average differences; and significantly different as compared with control group.

Reverse transcription polymerase chain reactions (RT-PCR). RT-PCR was used to confirm microarray findings of selected candidate genes satisfying the criteria described above. The gene candidates that were selected for RT-PCR were genes that are well-defined with respect to neuronal function and apoptosis. In addition, because simvastatin had the greatest effect on gene expression as compared with the other statins we elected to do RT-PCR on specific genes whose expression was altered by simvastatin particularly with respect to apoptosis. Reactions were carried out using 5 ug RNA from the same sample stock used in microarray analysis. PCR products were run on a 2% gel, stained with Ethidium Bromide, and visualized using the Stratagene Eagle Eye II system. Densitometry was used to ascertain relative changes in transcriptional levels due to statin treatment.

LC/MS/MS. Statin levels in brain were quantified using LC/MS/MS. Liquid chromatography was carried out on an Agilent 1100 instrument (Palo Alto, CA, USA) using a monolithic reversed-phase column (Chromolith Performance Rod, 100 mm, i.d. 4.6 mm. Merck, Darmstadt, Germany) at a flow rate of 1 mL/min. Separation of the analytes was achieved using a gradient solvent system: Solvent A: water/ acidic acid pH 3.1; Solvent B: 95% acetonitril/ 5%water/acidic acid pH 3.1 0-2 min: linear from 60%A to 20% A 2-5 min: 20% A 5-5.1 min: from 20%A to 60% A5.1-8 min: 60% A. Solvents were degassed by the online degaser of the Agilent 1100

system. Electrospray data was acquired using an AB-Sciex (Concord, ON, Canada) API 4000 triple- quadrupole mass spectrometer in the positive ion mode with a spray voltage of 4.5 kV and a declustering potential (DP) of 40-80, depending on the statin investigated. The orthogonal Turbo-V source's injectors were heated to 450°C to allow connection to the HPLC without mobile phase splitting. The values of the curtain gas (30 psig), nebulizer gas (30 psig) and turbo gas (60 psig) were the same for all statins. The dwell time for the multiple reaction monitoring (MRM) was 200 ms. MS/MS was performed using nitrogen as collision gas (CAD gas setting 6) at a collision cell exhibit potential of 6-11 V. Both, Q1 and Q3 were operated at unit resolution. The instrument parameters were optimized for the transition of the parent ion of each statin to its major fragment. During the preliminary experiments, the following transitions produced the highest signals in the MRM mode: pravastatin (m/z 425-> 269), lovastatin hydroxy acid (m/z 423-> 303), simvastatin hydroxy acid (m/z 437-> 285), lovastatin (m/z 405-> 199) and simvastatin (m/z 419-> 199). Quantification of the statins was based on internal standardization using the peak area ratios of the analyte and the internal standard.

Cholesterol determination. Cholesterol was determined enzymatically using procedures previously reported (Kirsch et al., 2003).

RESULTS

Statins Alter Gene Expression in Cerebral Cortex. Our overall hypothesis was that the action of statins in brain is not fully explainable by inhibition of the HMG-CoA reductase pathway but involves multiple pathways. We used microarray and RT-PCR to identify those gene expression pathways in cerebral cortex of mice treated with either lovastatin, pravastatin, or simvastatin and statin levels were quantified using LC/MS/MS. Simvastatin had the largest effect on the number of genes meeting the criteria for significant change. There were 38 genes whose expression levels were altered by simvastatin; lovastatin 26 genes; and pravastatin 21 genes. For each of the statins, more genes showed an increase in expression than a decrease in gene expression.

Table 1 shows that there were 15 genes whose expression was significantly altered by each of the three statins. These genes could be grouped into the broad categories of Cell Growth, and Signaling and Trafficking. Of particular interest are three genes related to cell growth: *Enc1*, *Cotl1*, and *Arh*. These genes contribute to actin function, a process reported to be disrupted in AD (Butterfield, 2002).

Expression levels of several potentially important genes involved with signaling and trafficking were increased by the three statins (Table 1). Expression levels of *MCT2* were increased by each statin. *MCT2* encodes for a monocarboxylic acid transporter-2 and this transporter family has been recently shown to transport the acid form of lovastatin into glomerular mesangial cells (Nagasawa et al., 2002). Of the 15 genes whose expression was changed by each of the 3 statins, only *Fin15* listed under Cell Growth, showed a decrease in gene expression. *Fin15* belongs to a group of genes that are stimulated by fibroblast growth factors.

Table 2 contains data on gene expression changes induced by either lovastatin, pravastatin, or simvastatin but not all three drugs. Simvastatin altered expression levels of 23

genes, lovastatin 11 genes and pravastatin 6 genes. Overall, genes most affected were in cell growth, signaling and trafficking, and genes associated with apoptosis in the case of simvastatin. Table 2 shows that there was a clustering of genes in the Cell Growth family associated with glucose homeostasis (*Igfbp3*, *Hk1*, and *Gpi1*). Expression of *Igfbp3* was significantly elevated by pravastatin and simvastatin. Lovastatin increased gene expression of *Igfbp3* by 1.7 fold but did not meet the 1.8 or greater cutoff. *HK1* is the gene that encodes hexokinase 1, the key glucose phosphorylating enzyme and expression of *HK1* was significantly increased by simvastatin and pravastatin. Expression levels of *Npy1r* were significantly increased for lovastatin and pravastatin. *Npy1r* encodes the neuropeptide Y receptor Y1. NPY receptor densities and NPY levels were reported to be decreased in AD brains and cerebrospinal fluid (Martel et al., 1990; Nilsson et al., 2001). The neuropeptide gene, *Hcrt*, showed significantly increased expression by lovastatin and pravastatin. *Hcrt* encodes the orexin precursor (hypocretin) that is cleaved, resulting in two peptides (Ferguson and Samson, 2003). The hypocretins play a significant role in energy metabolism and arousal (Ferguson and Samson, 2003). Simvastatin significantly increased expression levels of *Edn1* that encodes the endothelin-1 precursor (Table 2). The mature peptide, endothelin-1 has been reported to contribute to neuroprotection of cells *in vitro* (Walter and Stella, 2003).

Expression levels of specific genes associated with apoptosis (*c-fos*, *c-myc*, *H1.2*, and *Bcl-2*) were altered primarily by simvastatin (Table 2). Lovastatin only significantly increased expression of *c-myc*, and pravastatin did not significantly modify expression levels of *c-fos*, *c-myc*, *H1.2*, or *Bcl-2*. Simvastatin significantly reduced expression of the proto-oncogenic gene, *c-fos*. On the other hand, both lovastatin and simvastatin significantly increased expression of the oncogene, *c-myc*. Myc proteins are involved in basic cellular processes such as proliferation,

growth, apoptosis, and differentiation (Schuhmacher et al., 2001). Simvastatin significantly increased expression levels of *H1.2*, a gene encoding the linker histone H1.2. A potentially important gene whose expression level was increased by simvastatin was the anti-apoptotic gene, *Bcl-2*. This gene has been shown to suppress apoptosis in different cell types and it is thought to play a pivotal role in neuronal cell survival (Akhtar et al., 2004). Lovastatin and pravastatin did increase *Bcl-2* gene expression by 1.75 and 3.8 fold, respectively but within group variability negated statistically significant differences compared with control expression.

A sample of 5 candidate genes (*Enc-1*, *Npy1r*, *c-fos*, *c-myc*, and *Bcl-2*) were selected for confirmation using RT-PCR. Of particular interest, were *c-fos*, *c-myc*, and *Bcl-2* because of the linkage of statins with cell growth and apoptosis. Figure 1, Panels A-E show that expression levels were confirmed.

Statin levels in the cerebral cortex. We determined lovastatin, pravastatin, and simvastatin levels in the cerebral cortex of mice that had received statins for 21 days using LC/MS/MS. Figure 2 shows that relatively low levels of lovastatin and simvastatin were detected in the cerebral cortex following the 24 hour wash out period. Pravastatin was not detected in the cerebral cortex of chronically treated animals 24 hours following treatment. The low levels of lovastatin and simvastatin, and the absence of pravastatin at 24 h were puzzling because of the significant effects of the three statins on gene expression in the cerebral cortex. One possible explanation was that statins were not accumulating in brain but were being transported out of the brain. To examine statin accumulation and removal, we determined statin levels in the cerebral cortex at 1, 3, and 6 hours after an acute statin treatment. At these time points all three statins were successfully detected in the brain (Figure 2). Not surprisingly, brain levels of the three statins reflected their hydrophobicity with simvastatin levels >lovastatin levels> pravastatin

levels. The maximum average concentrations of simvastatin, lovastatin, and pravastatin 600 pmol/g brain, 300 pmol/g brain and 100 pmol/g brain, respectively.

Statins Reduce Cholesterol Levels in Cerebral Cortex. There was a small but significant reduction in cholesterol levels of the cerebral cortex of mice treated with the three statins but only cholesterol levels of pravastatin and simvastatin treated mice were significantly lower when compared with control mice (Figure 3). Percent reduction in cholesterol levels as compared to the cerebral cortex of control mice were: lovastatin 8%, pravastatin 11%, and simvastatin 13%. Cholesterol levels in the cerebral cortex did not differ significantly among the different statin treated mice. Expression levels of the gene, *HMGCR*, encoding HMG-CoA reductase were not significantly changed by the three statins (data not shown).

DISCUSSION

The results of this study show that indeed, statins have pleiotropic effects on gene expression in brain in addition to acting on cholesterol levels. We did observe a small reduction in cerebral cortex cholesterol levels in each statin group as compared with the control group but significant differences were only observed in pravastatin and simvastatin treated mice. In addition, changes in expression levels for genes directly involved in the cholesterol synthesis pathway did not meet the criteria for significant changes in expression levels (data not shown). Statins altered the expression of many genes with potentially protective effects (e.g., *Arhu*, *Enc1*, *Cotl1*, and *Igfbp3*). *Arhu*, *Enc1* and *Cotl1* are genes involved in actin function and whose expression levels were significantly increased by one, two, or all of the statins. *Enc1* is a mammalian Kelch-related gene, highly expressed in brain that encodes an actin-binding protein (Hernandez et al., 1997). *Arhu* is a novel gene that encodes a homolog of the Rho family of GTPases (Tao et al., 2001) and is involved with actin cytoskeleton organization (de Hostos et al., 1993). *Igfbp3* encodes a protein reported to be the primary carrier for insulin-like growth factors (IGF), IGF-1 and IGF-II which are expressed in several cell types including astrocytes and neurons (Lee et al., 1997). There is a growing body of data linking the role of insulin and IGF-1 to AD (Gasparini and Xu, 2003). IGF-1 has been reported to have a neuroprotective effect by regulating Aβ and its clearance from the brain (Carro et al., 2002). It would seem that increased expression of the IGFBP3 protein that transports IGF-1 would be beneficial to brain cell function. Furthermore, IGFBP3 has been linked to humanin, a newly identified protein capable of rescuing neurons from Aβ toxicity (Ikonen et al., 2003). IGFBP3 levels were elevated in AD brains and incubation of cultured human brain pericytes with $A\beta_{1-40}$ increased IGFBP3 mRNA

levels (Rensink et al., 2002). Whether the elevation in IGFBP3 levels of AD brains is indicative of inducing neurodegeneration or inhibiting a neuronal insult is not understood.

Expression levels of specific genes associated with apoptosis (*Bcl-2*, *c-fos*, *H1.2*, and *c-myc*) were altered primarily by simvastatin. With respect to these 4 genes, lovastatin only significantly increased expression of *c-myc*, and pravastatin increased expression but the change was not significant. Simvastatin significantly increased expression of the major anti-apoptotic gene, *Bcl-2*. This gene encodes a protein, apoptosis regulator Bcl-2, that has been shown to suppress apoptosis in different cell types (Adams and Cory, 1998). Increased expression of *Bcl-2* is interesting because the ratio of Bcl-2/Bax has been proposed to be important in the regulation of apoptosis (Adams and Cory, 1998). Overexpression of Bcl-2 in transgenic mice protected neurons from ischemic insult (Martinou et al., 1994) and Bcl-2 can serve as a compensatory mechanism in neurodegenerative diseases to combat reactive oxygen species-induced cell damage (Migheli et al., 1994). Several different lines of evidence indicate that Bcl-2 may play a role in AD. Neurons exhibiting neurofibrillary tangle formation were found to have reduced Bcl-2 levels, however, astrocytes had increased Bcl-2 levels as determined by immunohistochemical staining (Satou et al., 1995). The effect seen in astrocytes may be due to a role in damage repair. Both lovastatin and pravastatin increased Bcl-2 gene expression by 1.75 and 3.8 fold, respectively but within group variability negated statistically significant differences compared with control expression. A recent paper demonstrated that Bcl-w, a member of the Bcl-2 family, was neuroprotective against staurosporine and amyloid β-protein induced apoptosis in a human neuroblastoma cell line (Zhu et al., 2004). Increased protein levels of Bcl-w were observed in brain of AD individuals as compared with age-matched controls (Zhu et al., 2004). Clearly, an upregulation of Bcl-2 and other genes and proteins in this family could provide enhanced

neuronal protection against neurotoxic events and possibly prevent or perhaps delay the neurodegeneration associated with AD. Statins may have neuroprotective effects. However, it has been reported that statins induced apoptosis in neuroblasts, glioma cell lines, and rat primary neurons (García-Román et al., 2001; Murakami et al., 2001; Meske et al., 2003). Protein levels of Bcl-2 were significantly lower in neuroblasts that had been incubated with lovastatin for 24 h (García-Román et al., 2001). The *in vitro* studies used statin concentrations that were several orders of magnitude higher than concentrations used in the present *in vivo* study. Also, it has not been reported as to whether statins administered *in vivo* induce apoptosis in brain. In view of the potential efficacy of statins in reducing the prevalence of AD, much more work is needed in order to understand the pharmacological actions of statins in brain.

We determined statin levels in the cerebral cortex using LC/MS/MS and report the novel findings that pravastatin was detected in the brain at levels above the IC_{50} for inhibition of HMG-CoA reductase activity (Bischoff and Heller, 1998), and that statin levels declined quickly in brain. Both lovastatin and simvastatin also were detected in the cerebral cortex. Detecting pravastatin in brain at the levels observed was surprising because it is thought that little if any pravastatin crosses the blood-brain barrier (Botti et al., 1991). Lovastatin and simvastatin, administered as hydrophobic lactones readily cross the blood-brain barrier. Pravastatin is a hydrophilic drug with a very low octanol/buffer coefficient of -0.47 ($logD_{7.0}$) in comparison to lovastatin, 3.9 and simvastatin 4.8 (Ishigami et al., 2001). Pravastatin, however, is associated with reduced risk of developing AD, alters the transbilayer distribution of cholesterol in synaptic plasma membranes of chronically treated mice, (Kirsch et al., 2003) and as we now show, modifies gene expression in mouse cerebral cortex. One interpretation of our findings with pravastatin is that effects of the drug are occurring outside of brain, and those effects in turn alter

brain gene expression. Arguing against that possibility are several lines of evidence. Pravastatin treated mice showed a small but significant decrease in cerebral cortex cholesterol levels. Cholesterol levels in brain and in serum outside of brain are not in equilibrium. Outside of brain it is well-established that statin-induced inhibition of HMG-CoA reductase reduces mevalonate levels leading to a reduction in the sterol regulatory pool that results in upregulation of HMG-CoA reductase. We found that HMG-CoA reductase brain gene expression was increased in pravastatin treated mice (1.08 fold-increase) but that change did not meet our criteria for significant gene expression. In addition, while pravastatin was not detected in brain after 24 h following statin administration, it was detected after 1, 3, and 6 hours. Therefore, a key question pertains to mechanisms transporting pravastatin into brain. There are two potential mechanisms for pravastatin transport: simple membrane partitioning or active transport. Membrane partitioning of pravastatin acid is unlikely due to its hydrophilicity and an octanol/buffer partition coefficient of -0.47. However, pravastatin can be transported into cells by active transport. Organic anion transporters (OATs) have been identified that transport pravastatin and are expressed in brain (Takeda et al., 2004; Kusuhara et al., 1999). Another transporter that shuttles statin acids and is expressed in the brain is the monocarboxylic acid transporter, MCT2 (Tsuji et al., 1993). Importantly, we found that pravastatin, along with lovastatin and simvastatin, significantly increased *MCT2* gene expression.

Brain levels of the three statins rapidly declined between 1 and 6 h as shown in Figure 2 and could be due to transport out of the brain or metabolism. The OATs described above can also transport acids out of cells and it has been reported that OATs are involved in the excretion of both endogenous and exogenous acids (Kusuhara et al., 1999). An additional transporter is P-glycoprotein (P-gp) that has been identified in brain (Hirrlinger et al., 2002). There is evidence

that statins are substrates for P-gp and that this transporter may play a role in statin efflux from cells (Bogman et al., 2001). An alternative hypothesis is that statins are rapidly metabolized in brain. Statin metabolism has not been studied in brain but data have been reported for serum half-lives in human subjects (Jones et al., 1998). For example, simvastatin lactone and acid have half-lives of between 2.2 to 3.1 h and 3.2 to 4.4 h, respectively. The reduction in simvastatin levels in the cerebral cortex were most notable between 1 and 3 h post-administration (Figure 2) and it is certainly plausible that statin metabolism could occur in brain. Both active transport as well as metabolism may be responsible for the rapid decline in drug statin levels observed between 1 and 6 h following statin administration.

We report the novel findings that the hydrophobic statins, lovastatin and simvastatin and the hydrophilic statin, pravastatin, had pleiotropic effects on gene expression in mouse cerebral cortex and that levels of the three statins were detected in brain. Surprisingly, the genes directly involved in cholesterol synthesis were relatively unaffected by statin treatment when our selection criteria was applied, and cholesterol levels were only slightly lowered. These findings, along with the many potentially protective genes that were changed, suggest that the neuroprotective effects of statins are largely cholesterol-independent. Moreover, these results provide new insight into possible mechanisms for the efficacy of statins in reducing the prevalence of AD and lay the foundation for future studies.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. RT-PCR analysis of candidate gene expression and confirmation of microarray results. The gels displayed here are representative of RT-PCR experiments performed on each sample for each candidate gene normalized by calculating the ratio of specific mRNA to GAPDH mRNA that can be seen in the panel for *Enc-1*. Densitometric analysis provided a measure of changes in relative transcription of statin-treated samples compared to control. Results are expressed as mean densitometric results ± SEM. $N \geq 3$. * $p < 0.05$** $p < 0.01$ as compared with control values. Lov, lovastatin; Pra, pravastatin, Sim, simvastatin.

Figure 2. Statin levels in the cerebral cortex of acutely treated and chronically treated mice. Acute treatment consisted of a single administration and statin levels determined at 1, 3, and 6 h post-administration using LC/MS/MS. Chronic administration was for 21 days and statin levels determined 24 h after the last statin administration. Means ±SEM (n=4 samples per treatment).

Figure 3. Cholesterol levels in cerebral cortex of mice administered statins for 21 days. Cholesterol levels were determined using an enzyme assay. Data are the means ±SEM (n=4 samples/group). *p <0.05 as compared with the control group. Lov, lovastatin; Pra, pravastatin; Sim, simvastatin.

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Table 1. Overlap in Alterations of Gene Expression Induced by Lovastatin, Simvastatin and Pravastatin in Cerebral Cortex of C57BL/6 Mice

Gene	Accession Number	Description	Fold Change & P Values		
Cell Growth			**Lovastatin**	**Pravastatin**	**Simvastatin**
Enc1	U65079	Ectodermal-neural cortex 1	3.3 (0.011)	4.2 (0.004)	6.1 (0.001)
Cotl1	AI837006	Coactosin-like 1 (Dictyostelium)	4.4 (0.003)	5.4 (0.001)	6.4 (0.001)
Arhu	NM_133955	Ras homolog family, member U	3.8 (0.004)	2.0 (0.048)	3.3 (0.007)
Fin15	U42384	Fibroblast growth factor inducible 15	-2.1 (0.019)	-2.2 (0.012)	-2.6 (0.006)
Signaling and Trafficking					
MCT2	AF058054	Monocarboxylic acid transporter-2	2.3 (0.005)	2.5 (0.02)	2.5 (0.024)
Sdc4	D89571	Syndecan 4	3.0 (0.011)	3.2 (0.005)	2.2 (0.009)
Hba-a1	V00714	Alpha-globin.	1.9 (0.007)	1.9 (0.003)	2.2 (0.001)
Hbb-b2	V00722	Beta-1-globin.	2.0 (0.02)	1.9 (0.033)	2.4 (0.003)
LOC55933	AF015811	Putative lysophosphatidic acid acyltransferase	2.3 (0.037)	2.1 (0.05)	2.7 (0.028)
Other					
Polg	U53584	Polymerase (DNA directed), gamma	4.0 (0.005)	3.2 (0.025)	4.0 (0.006)
Zik1	U69133	Zinc finger protein interacting with K protein 1	2.4 (0.004)	2.0 (0.011)	2.2 (0.011)
Avp	M88354	Vasopressin-neurophysin II	2.4 (0.001)	2.2 (0.001)	2.7 (0.016)
Xlr3b	L22977	X-linked lymphocyte-regulated 3b	2.8 (0.002)	2.0 (0.006)	3.1 (0.002)
Vwf	AI843063	Von Willebrand factor homolog	2.6 (0.02)	4.0 (0.003)	2.5 (0.023)
Impact	D87973	Imprinted and ancient	2.9 (0.03)	2.3 (0.003)	2.8 (0.023)

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Table 2. Distinct Alterations in Gene Expression Induced by Lovastatin, Simvastatin, or Pravastatin in Cerebral Cortex of C57BL/6 Mice

Gene	Accession Number	Description	Fold Change & P Values		
			Lovastatin	Pravastatin	Simvastatin
Cell Growth					
Igfbp3	A1842277	Insulin-like growth factor binding protein 3		2.1 (0.003)	2.4 (0.004)
Hk1	J05277	Hexokinase 1		2.1 (0.007)	2.0 (0.02)
Gpi1	AV295044	Glucose phosphate isomerase 1	-1.8 (0.014)		
Lgals8	AA760613	Lectin, β-galactoside binding	2.8 (0.015)		2.6 (0.023)
c-fos	V00727	Mouse c-fos oncogene.			-2.0 (0.015)
c-myc	L00039	c-MYC	2.2 (0.02)		2.5 (0.012)
H1.2	J03482	Mouse histone H1			2.1 (0.005)
Bcl-2	L31532	B-cell leukemia/lymphoma 2			3.4 (0.016)
Col3a1	X52046	Alpha 3 subunit;	-2.0 (0.007)		
Acvr1	L15436	Activin A receptor, type 1		1.9 (0.007)	2.5 (0.004)
Ccl27	AA672499	Chemokine (C-C motif) ligand 27			2.2 (0.018)
Tm4sf8	AI843488	Transmembrane 4 superfamily member 8	-2.4 (0.015)		
Signaling and Trafficking					
Npy1r	Z18280	NPY-1 receptor	2.2 (0.015)	2.5 (0.014)	
Hcrt	AF019566	Hypocretin	2.1 (0.002)	2.0 (0.001)	
Edn1	U35233	Endothelin 1			2.0 (0.002)
Unc13h1	AF115848	Unc13 homolog (C. elegans) 1			2.2 (0.005)
Numb	AV377244	Numb gene homolog (Drosophila)	-2.4 (0.013)		
Cacna1g	AJ012569	Calcium channel, voltage dependent, T type, alpha 1G subunit			1.8 (0.034)
Pld1	AA536939	Phospholipase D1		1.8 (0.033)	
Siat7d	AJ007310	Sialytransferase 7D	2.1 (0.011)		2.3 (0.04)
Catp	AW046747	Cation-transporting ATPase			2.1 (0.008)
Bet1	AF007552	Blocked early in transport 1 homolog (S. cerevisiae)	1.9 (0.035)		
Hbb-b1	J00413	Mouse beta-globin major gene.			2.0 (0.002)
Anp32a	U73478	Acidic nuclear phosphoprotein 32			2.1 (0.01)
Timm10	AW122428	Translocase of inner mitochondrial membrane 10 homolog			1.9 (0.045)
Others					
Zfp354a	L77247	Zinc finger protein 354A			1.8 (0.044)
Nfix	AA002843	Nuclear factor I/X			-2.1 (0.01)
Hs3st1	AF019385	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1			2.6 (0.038)
Pglyrp	AV092014	Peptidoglycan recognition protein			1.9 (0.001)
Col10a1	X67348	Type X collagen gene	2.3 (0.017)		1.9 (0.019)
Myg1	Al842612	Melanocyte proliferating gene 1			1.8 (0.01)

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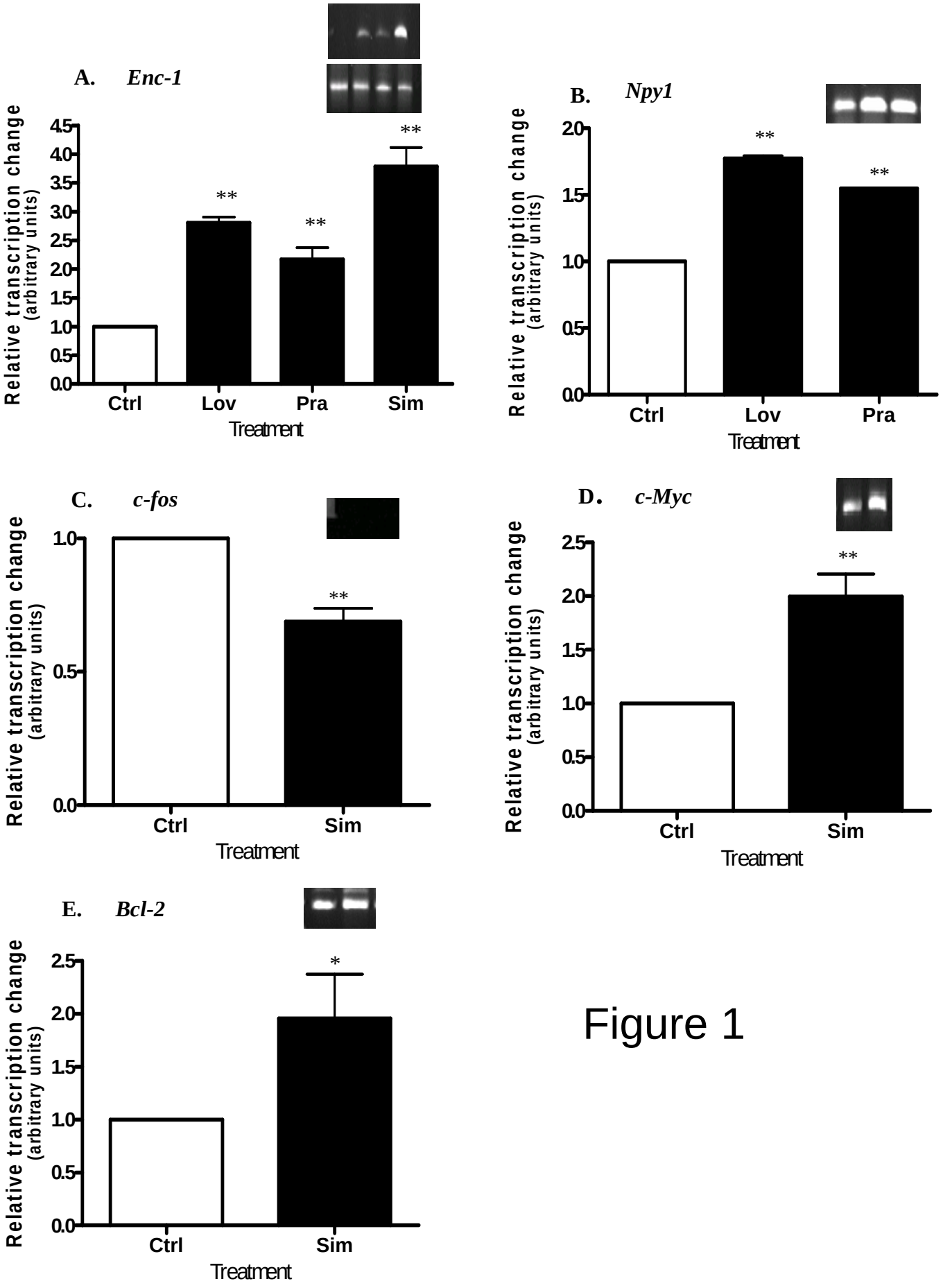

Figure 1

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Figure 2

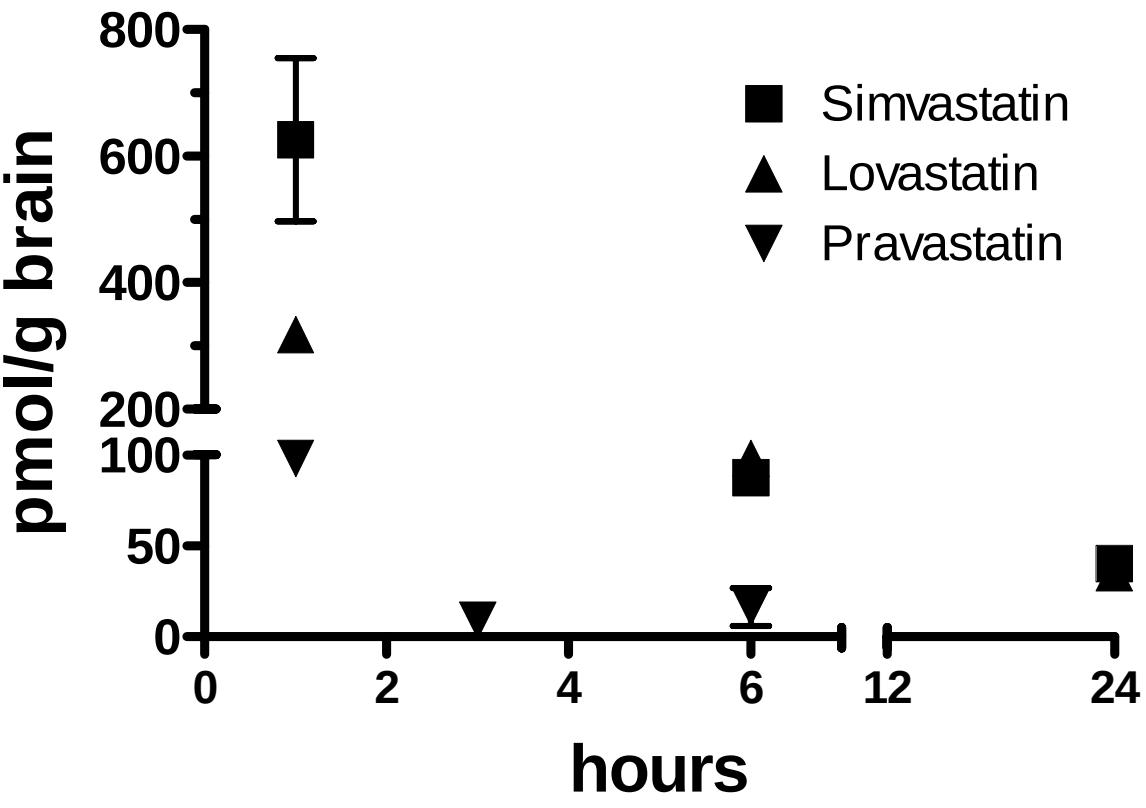

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Figure 3