

Dietary cholesterol and estrogen administration elevate brain apolipoprotein E in mice by different mechanisms[#]

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Apolipoprotein (apo) E plays an important role in the whole body cholesterol homeostasis. Recent studies suggest that it may also be involved in the local cholesterol transport in the brain, and influence the pathogenesis of Alzheimer's disease (AD) by interacting with the β -amyloid protein and brain lipoprotein receptors. Since apoE expression is highest in the brain, next only to the liver and associated with the pathogenesis of AD, we hypothesized that dietary and hormonal intervention, known to regulate hepatic apoE expression may also regulate brain apoE and thereby influence local cholesterol transport. To test this hypothesis, groups of male C57BL mice were fed either regular rodent chow or high fat (HF) and high cholesterol enriched diet for 3 weeks. In a separate study, groups of male mice were administered pharmacological doses of 17- β estradiol for 5 consecutive days and sacrificed on the 6th day. As expected, HF diet elevated liver apoE mRNA and apoE synthesis. Similar to liver, brain apoE mRNA and synthesis also increased, following HF feeding. Estradiol administration increased liver apoE synthesis without affecting apoE mRNA. Interestingly, estradiol administration also increased the brain apoE synthesis, but without altering the brain apoE mRNA. These studies suggested that dietary cholesterol and estrogen administration elevated the brain apoE by different mechanisms.

Keywords: Apolipoprotein E, Brain, Cholesterol, Alzheimer's disease, Estrogen, Mouse

Apolipoprotein (apo) E, a 34 kDa protein is a major component of circulating lipoproteins¹ that plays an important role in maintaining the whole body cholesterol homeostasis². It is abundantly expressed in the liver, brain, and steroidogenic tissue³, but unlike the major apoproteins A1 and B, is not expressed in the gut⁴. The high level expression of apoE in the brain, its presence in the β -amyloid-containing neuritic plaques^{5,6} and association of an isoform $\epsilon 4$ of apoE in the pathogenesis of Alzheimer's disease (AD)⁷ suggest apoE's role in the pathogenesis of AD. The high avidity binding of apoE to the β -amyloid protein⁸ has further established a strong link between brain apoE and AD. ApoE may either promote

aggregation of diffuse amyloid deposition or its presence in the neuritic plaque may have resulted from its role in the uptake of either native or modified lipoproteins via apoE receptors. Indeed, lipoprotein receptors LRP⁹, LDL receptor^{10,11}, and SR-BI¹²⁻¹⁴ are expressed in the brain. Given the implications of apoE in the pathogenesis of AD, and its regulation by nutritional¹⁵ and hormonal¹⁶ stimuli, the modulation of apoE in the brain by these stimuli may influence progression and pathogenesis of AD.

ApoE-containing large lipoproteins secreted by astrocytes are taken up by the neurons, possibly involving an apoE receptor and resulting in the stimulation of increased number of synapses¹⁷. Cholesterol has been implicated in the decreased release of secreted amyloid precursor protein (APP) in cultured cells¹⁸. Furthermore, animal studies have demonstrated influence of cholesterol on APP processing and requirements of apoE in this process¹⁹. A correlation of fibrillar A β (1-42 aa) with circulating total cholesterol and LDL cholesterol further suggests²⁰ a link between cholesterol and A β deposition in the brain. Thus, lowering cholesterol may lower A β ^{21,22}. In a preliminary study, dietary fat

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Abbreviations: AD, Alzheimer's disease; ApoE, apolipoprotein E, APP, amyloid precursor protein; 17-BE, 17- β estradiol, HF, high fat.

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has shown increase brain in apoE mRNA in the Zucker lean rat²³, but no changes in the levels of brain apoE have been noted in cholesterol-fed rabbits²⁴. A separate study in rabbits²⁵ has shown increased density of cortical apoE immunoreactivity in the brain neurons after dietary cholesterol feeding. These conflicting results warrant a careful study to examine brain regulation of apoE by dietary lipids.

Estrogen is suggested to play a protective role against AD pathogenesis through a variety of mechanisms including upregulation of glutamate transporter²⁶, activation of protein kinase C²⁷, protein kinase B²⁸, antioxidant effect²⁹, A β -modulation³⁰, and modulation of apoptotic pathway³¹. Reduced cerebrospinal fluid estradiol levels are associated with increased β -amyloid levels in female patients with AD³². Since estrogen also regulates hepatic apoE expression through a novel mechanism¹⁶, it is hypothesized that estrogen may influence brain apoE and thereby impact AD pathogenesis. The present study looks into the mechanistic insights of cholesterol and estrogen-mediated regulation of the brain apoE. The data herein suggest that dietary cholesterol and pharmacological doses of estrogen both regulate brain apoE, albeit by different mechanisms.

Materials and Methods

Animals and treatment

Mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Female C57BL mice were fed either rodent chow containing 5% corn oil or a high fat diet containing 0.5% cholesterol and 20% hydrogenated coconut oil as previously described^{33,34}. At the end of the 3 weeks feeding and a brief fasting for 4 h, mice were sacrificed using sodium pentobarbital and blood was collected in EDTA-coated tubes and centrifuged for 10 min at 10,000 rpm in a table top centrifuge to obtain plasma. Plasma was analyzed for lipoprotein profile and levels of apo E. Liver and brain were removed for the preparation of RNA. Part of the liver and brain was used to measure apoE synthesis. Male Sprague Dawley rats were fed high fat and high cholesterol diet in a similar manner for 3 weeks. Plasma isolated after an overnight (12 h) fast was used to measure lipid levels and profile.

In a separate experiment, male C57BL mice were administered 17 β -estradiol as described previously³⁵. Estradiol treatment was performed for 6 days, and mice sacrificed on the 7th day under sodium pentobarbital. Mice had free access to rodent chow

diet or a high fat diet³³ and tap water. Plasma was used for the analysis of lipid and lipoprotein, and tissues were excised for the preparation of RNA and protein synthesis. Plasma apoE and hepatic cholesterol measurements were done as described¹⁶.

RNA Analysis

Total RNA from liver and brain tissues were isolated following one step RNA isolation method⁴. The quality of RNA was examined by electrophoresing a 10 μ g sample of RNA in 1.2% agarose gel containing formaldehyde and formamide. The ratio of 28S and 18S RNA was measured to determine the quality of RNA. ApoE mRNA was quantitated by Northern blotting³⁶ as well as by RNase protection assay³⁷. Measurements of LDL receptor mRNA were done using RNase protection assay³⁸. The recombinant probes for apoE and the reagents for RNA analysis were obtained from Clonogen Biotechnology, India (www.clonogenbiotech.com).

Protein synthesis

ApoE synthesis was measured in the freshly isolated tissues of liver and brain as described previously³⁹. In brief, tissues were sliced into 1-3 mg pieces, incubated in the previously oxygenated KRB buffer in the presence of ³⁵S-methionine. Protein concentration was determined for normalization purposes. After 1 h, tissues were homogenized and S100 prepared. Protein synthesis was allowed to occur for 1 h at 30°C, followed by termination of synthesis by transferring the tubes to the ice bath. ApoE protein was immuno-precipitated by anti-mouse apoE antibody (Clonogen Biotech, India, www.clonogenbiotech.com), and subsequent processing of samples was done as described³⁹. The immunoprecipitates were separated in a denaturing gel electrophoresis and the apoE protein bands were visualized by autoradiography for quantification.

Statistical analysis

All values were expressed as mean \pm standard error of mean (SEM). Data were analyzed for statistical significance compared to vehicle-treated control group using the analysis of variance (ANOVA). A *p* value of <0.05 was considered as significant.

Results

High fat feeding modulates ApoE in the brain

The effects of diet-derived fat and cholesterol on brain apoE regulation in rats and mice were examined

after feeding a high fat high cholesterol diet for 3 weeks. As expected, plasma cholesterol increased significantly both in rats as well as in mice^{33,34}. The total cholesterol increased from 106 ± 9 mg/dl to 151 ± 9 mg/dl ($p < 0.025$) in rats and from $158 \pm$ mg/dl to 249 ± 15 mg/dl in mice ($p < 0.025$) (Fig. 1). Both LDL and HDL cholesterol also showed increase in rats and mice. To examine, if diet-derived lipid influenced hepatic cholesterol levels, liver cholesterol was also measured. As shown in Fig. 1, hepatic cholesterol increased 2.5-fold (control 3.18 ± 0.2 , HF 7.81 ± 0.8 mg/g liver) in the rats and 4-fold (control 2.69 ± 0.2 , HF 11.04 ± 0.7 mg/g liver) in the mice. Since cholesterol is known to down-regulate LDL receptor gene expression, the measurements of hepatic LDL receptor mRNA by a sensitive ribonuclease protection assay were performed. As shown in Fig. 1, hepatic LDL receptor mRNA decreased both in rats (control 3.7 ± 0.4 pg/ μ g RNA, HF 2.2 ± 0.3 pg/ μ g RNA, $p < 0.05$) and in mice (control 3.5 pg/ μ g RNA, HF 2.3 ± 0.2 pg/ μ g RNA, $p < 0.05$).

The levels of plasma apoE increased about 50% both in rats (control 14.4 ± 1.6 mg/dl, HF 22.5 ± 2.5 mg/dl, $p < 0.025$) and in mice (control 7.6 ± 2.4 mg/dl, HF 13.2 ± 2.1 mg/dl) (Fig. 2), suggesting that high fat and cholesterol feeding elevated plasma levels of apoE. To examine, if the changes in the plasma levels of apoE occurred via transcriptional or post-

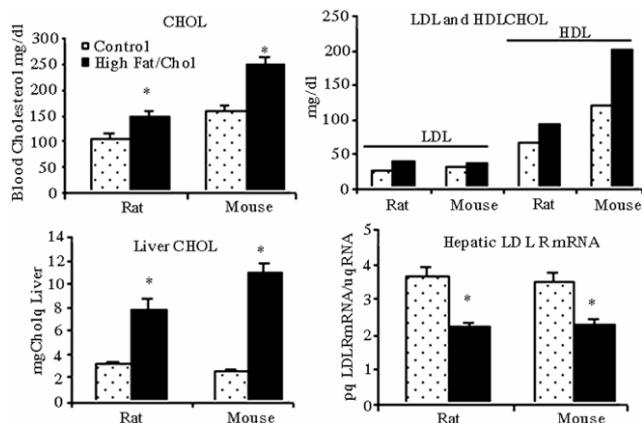


Fig. 1—High fat and high cholesterol feeding elevates plasma levels of cholesterol, hepatic cholesterol and down-regulates hepatic LDL receptor [Mice and rats were fed high fat and cholesterol diets for 3 weeks, and plasma lipids were determined. LDL and HDL were measured by performing FPLC and combining the values of their corresponding fractions. LDL receptor mRNA was quantitated by RNase protection assay in total RNA isolated from liver tissues as described^{37,38}. * $p < 0.025$ compared to control group]

transcriptional mechanism, hepatic apoE mRNA was quantitated by ribonuclease protection assay. First, an assay for apoE mRNA quantitation was established as shown in Fig. 3. In this assay, protected apoE mRNA fragment intensity increased with increasing RNA concentration (Fig. 3). Using this optimized protection assay, brain apoE mRNA measurements were done. As shown in Fig. 4, HF feeding increased mouse brain apoE mRNA. Liver apoE mRNA also increased significantly (data not shown). To find out, if the increased apoE levels represented increased rate of apoE synthesis, *in vitro* translation of apoE on

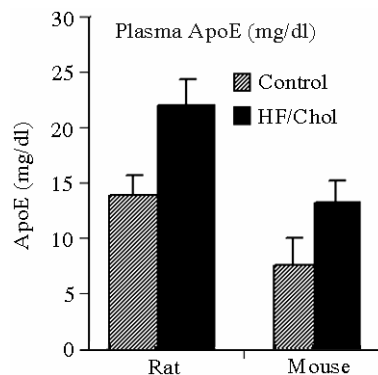


Fig. 2—High fat and high cholesterol feeding elevates plasma levels of apoE in rats and mice [Groups of mice ($n = 4$) and rats ($n = 4$) were fed high fat and cholesterol diet for 3 weeks, followed by isolation of plasma by collecting blood in EDTA containing tubes and centrifuging for 10 min. ApoE measurements were done by ELISA¹⁶. High fat diet caused elevation of plasma levels of apoE both in rats and in mice. * $p < 0.025$ compared to control group]

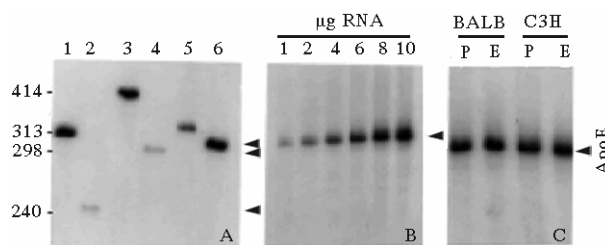


Fig. 3—RNase protection assay for the quantitation of apoE mRNA [Riboprobes were synthesized *in vitro* using an *in vitro* RNA synthesis kit (Ambion) with radiolabeled UTP [³²P] and linearized recombinant plasmids as described^{37,38}. The synthesized riboprobes were purified using RNase-free Sephadex G-25 column. Panel A: The quality and size of riboprobes were checked by running a sequencing gel. Panel B: ApoE riboprobes were hybridized with increasing amounts of total hepatic RNA as indicated and separated in a sequencing gel. Panel C: Five microgram of total pooled hepatic RNA from 4 mice in each group were hybridized with riboprobes, processed as described, and separated in a sequencing gel. P, placebo, E, estradiol treated].

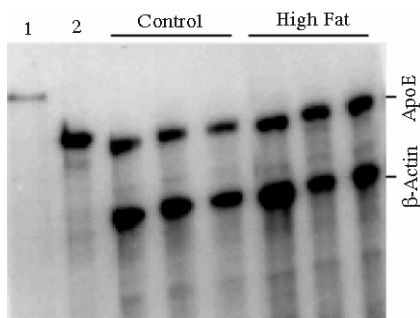


Fig. 4—High fat feeding elevates apoE mRNA in the brain [Mice fed chow or high fat diet for 3 weeks as described under the 'Materials and Methods' section were sacrificed and liver excised for RNA preparation. Ten microgram RNA was taken for RNase protection assay. Three RNA samples from each group were analyzed. Lanes 1 and 2 show apoE and β -actin riboprobes synthesized *in vitro*, and remaining lanes indicate protected fragments after hybridization with the total RNA. When the intensities of the apoE protected fragments were scanned, the high fat-fed group showed significantly higher intensities compared to the control group ($p < 0.025$)]

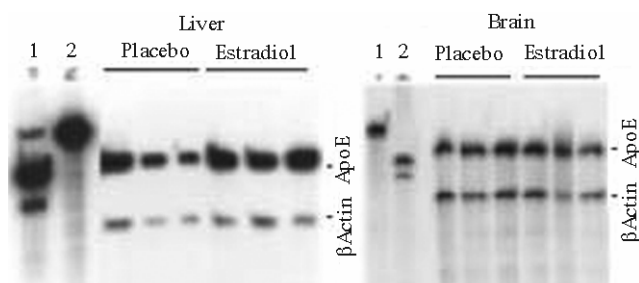


Fig. 6—Groups of mice were administered estradiol as described in the 'Materials and Methods' section. At the end of treatment period, mice were fasted, blood withdrawn and tissues collected for total RNA preparation. Northern blotting analysis was done using 15 μ g RNA (not shown), and detection of respective mRNA was done using mouse apoE riboprobe as described¹⁶.

isolated liver and brain tissues was performed *ex vivo*. As shown in Fig. 5, HF feeding increased apoE synthesis in the liver as well as in the brain. These results suggested that increased plasma levels of apoE occurred partly via up-regulation of the hepatic apoE gene expression, since liver is the main organ expressing apoE.

Estradiol administration regulates brain apoE expression

To investigate, if brain apoE was regulated by estradiol administration, groups of mice were administered estradiol for 6 days, and on the 7th day, mice were sacrificed for plasma analysis as well as hepatic and brain RNA analysis for apoE regulation. Lipid changes in the estradiol administered rats and mice have been described before^{16,35}. In this study,

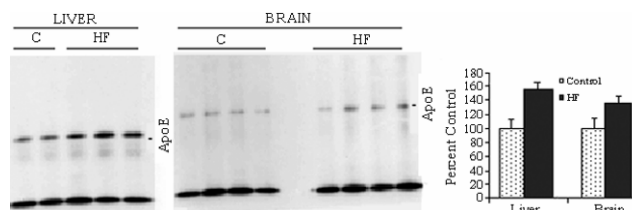


Fig. 5—High fat feeding increases apoE synthesis in the liver and brain [Slices of tissues (2-3 mg) from low and high fat fed mice were subjected to *in vitro* synthesis in the presence of ³⁵S-methionine using wheat germ translation system (Ambion) as described in the 'Materials and Methods' section. Thereafter, the contents were put on ice and immunoprecipitated using mouse anti apoE antibody, and run in a polyacrylamide gel. Following the electrophoresis, the gel was dried under heated vacuum and exposed to X-ray film. The arrow indicates newly synthesized apoE protein. Left panel shows apoE synthesis in the liver and the right panel shows apoE synthesis in the brain. C indicates control group and HF indicates high fat-fed group]

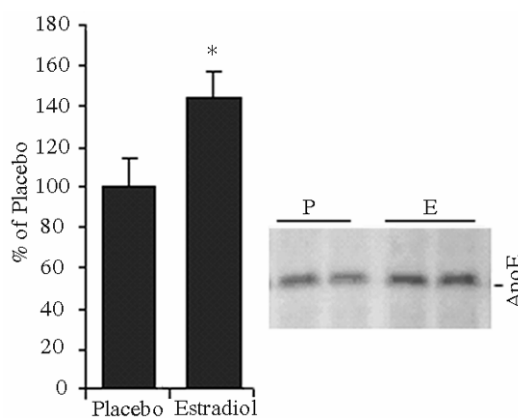


Fig. 7—*Ex vivo* translation of brain apoE in estradiol treated mice [Brain tissues were sliced into small pieces as described in the legend to Fig. 5. Protein synthesis was allowed to occur for 1 h at 30°C, followed by termination of the synthesis by transferring the tubes to the ice bath. *In vitro* translated apoE was immunoprecipitated and separated in a polyacrylamide gel electrophoresis. The results shown are average of two separate experiments. In the inset, the results of a representative protein gel after immunoprecipitation and autoradiography is shown. P indicates placebo, E, estradiol-treated group]

estrogen mediated regulation of brain apoE was investigated. As shown in Fig. 6, hepatic and brain apoE mRNA did not change, following estradiol administration. However, when *ex vivo* translation on brain tissues was performed using an *in vitro* translation system, apoE protein synthesis increased both in the liver and in the brain (Fig. 7).

Discussion

ApoE plays an important role in brain cholesterol homeostasis and transport, and is implicated in the

pathophysiology of AD^{6-8,22}. Cholesterol influences apoE gene regulation¹⁵ as well as cleavage of γ -secretase¹⁸, which is involved in the pathophysiology of AD through cleavage of β -amyloid peptide. Since apoE may play a role in AD pathogenicity, we have in the present study, examined, if dietary cholesterol and estrogen, both known to modulate liver apoE expression also influence expression of brain apoE. Rodent model, earlier shown to upregulate hepatic apoE expression by dietary cholesterol¹⁵ has been used in the present study.

The results presented here corroborate earlier findings that dietary cholesterol increases the expression of hepatic apoE gene regulation, and estrogen administration increases plasma levels of apoE by post-transcriptional mechanism¹⁶. In the present study, cholesterol feeding raised brain apoE mRNA similar to liver, suggesting that apoE gene is regulated by cholesterol feeding, similarly in the liver and the brain. As expected, the increased brain apoE mRNA levels result in increased apoE synthesis. This is an important finding, suggesting the role of dietary cholesterol on brain apoE regulation. Thus, diets rich in cholesterol are likely to influence pathogenicity of AD via modulation of apoE gene. This is the first study demonstrating a distinct association between dietary cholesterol and brain apoE. Role of disrupted cholesterol metabolism in a transgenic mouse model of AD has been studied, which shows that the diet-induced chronic changes in plasma cholesterol also increase apoE content in the liver and the brain⁴². These findings have been further corroborated by the increased secretion of apoE by glial cells following cholesterol loading, and decreased apoE, following treatments with statins⁴². These data corroborate the findings in the present study that dietary cholesterol up-regulates brain apoE by transcriptional mechanism. Thus, dietary cholesterol influences brain apoE metabolism which may impact pathogenesis of AD.

Studies suggest that estrogens play a protective role against AD pathogenesis through a variety of mechanisms. Among other mechanisms, estrogen-induced apoE regulation could play an important role in the process of AD development. Since estrogens regulate hepatic apoE expression through a novel mechanism¹⁶, it was hypothesized that similar mechanism might operate in the brain as well. Similar to earlier studies, estrogen did not influence hepatic

and brain apoE mRNA, but increased *ex vivo* apoE synthesis in hepatic and brain tissues. These data suggest that estrogen modulates apoE regulation in liver and brain by similar mechanism. Although detailed studies on the rates of translation on isolated monosomes and polysomes have not been carried out in this study, but an earlier study investigating the effects of estrogen on apoE regulation has shown a shift of apoE mRNA to polysomal fractions¹⁶, which correlates with increased rates of apoE synthesis. It is plausible that similar mechanism may have resulted in increased apoE synthesis in the brain.

In summary, present study provides experimental data for the mechanistic insights of cholesterol and estrogen-mediated regulation of the brain apoE. Both the dietary cholesterol and estrogen increase apoE synthesis in the brain, but they differ in terms of their loci of regulation.

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