Apolipoprotein E Deficiency Worsens Outcome From Global Cerebral Ischemia in the Mouse

Huaxin Sheng, MD; Daniel T. Laskowitz, MD; G. Burkhard Mackensen, MD; Masaya Kudo, MD; Robert D. Pearlstein, PhD; David S. Warner, MD

Background and Purpose—Apolipoprotein E (apoE) has been found relevant in a variety of central nervous system disorders. This experiment examined the effect of endogenous murine apoE on selective neuronal necrosis resulting from a transient forebrain ischemia insult.

Methods—ApoE deficient (n=16) and wild type (n=17) halothane-anesthetized mice were subjected to severe forebrain ischemia (10 minutes of bilateral carotid occlusion and systemic hypotension). After 3 days’ recovery, brain injury was determined histologically. In other apoE-deficient and wild-type mice, regional cerebral blood flow (CBF) was determined by 14C-iodoantipyrine autoradiography 10 minutes before, 5 minutes after onset of, and 30 minutes after reperfusion from 10 minutes of forebrain ischemia.

Results—The percentage of dead hippocampal CA1 neurons (mean±SD) was greater in the apoE-deficient group (apoE deficient=67±30%; wild type=37±33%; P=0.011). A similar pattern was observed in the caudoputamen (P=0.002) and neocortex (P=0.014). Cerebral blood flow was similar between groups at each measurement interval. Marked hypoperfusion persisted in both groups at 30 minutes after ischemia.

Conclusions—ApoE deficiency worsens ischemic outcome. This is not attributable to effects on CBF. A role of apoE in the cerebral response to global ischemia is consistent with prior reports that murine apoE deficiency increases infarct size resulting from focal cerebral ischemia. (Stroke. 1999;30;1118–1124.)

Key Words: apolipoproteins ■ cerebral blood flow ■ ischemia ■ histology ■ mice

Increasing evidence suggests that apolipoprotein E (apoE), a 34-kD glycosylated protein, plays an important role in the biology of neurological disease. There are 3 common human isoforms of apoE, designated E2, E3, and E4, which differ by single amino acid interchanges.1 In addition to its well-defined role in cholesterol metabolism, apoE is upregulated by astrocytes and oligodendrocytes in the injured central nervous system, suggesting that apoE is a factor in the response to neural injury and repair.2–4 This function would be consistent with clinical data suggesting that apoE influences recovery in an isoform-specific manner after various forms of brain injury, including intracerebral hemorrhage, acute and chronic traumatic brain injury, stroke, and cardiopulmonary bypass.5–10 In general, these studies have all suggested that the APOE4 allele is independently associated with greater neurological dysfunction after central nervous system injury, although 1 recent clinical study has disputed these findings.11

To further explore the role of apoE in ischemic brain injury, we previously examined the effects of apoE deficiency in mice subjected to transient middle cerebral artery occlusion (MCAO). ApoE-deficient mice had larger infarct volumes and worse functional outcome than matched wild-type controls.12 This increased susceptibility of apoE-deficient mice to focal ischemia was not a function of differences in intraischemic cerebral blood flow (CBF) or vascular anatomy.13

Although the technique of transient MCAO is an appropriate model for focal ischemia, it does not accurately reflect the pathophysiology after global cerebral hypoperfusion, such as may occur during hemorrhagic shock, cardiac resuscitation, or cardiopulmonary bypass. We have recently characterized a 2-vessel occlusion recovery model of global ischemia in the mouse14 and now demonstrate that apoE-deficient mice are more sensitive to transient global ischemia than control mice matched for age, gender, and genetic background.

Materials and Methods

This study was approved by the Duke University Animal Care and Use Committee. Homologous recombination in embryonic stem cells (129-derived E14Tg2a ES) was used to produce targeted knockout mutations of the APOE locus.15 The apoE-deficient animals used in this experiment had been back crossed 10 times to the C57Black/6J

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mouse and purchased from The Jackson Laboratory (Bar Harbor, Maine). Wild type animals were age- and sex-matched C57Black/6J mice (The Jackson Laboratory).

Male mice (8 to 10 weeks of age; apoE deficient, n=16; wild type, n=17) were overnight fasted but allowed free access to water. Mice then were anesthetized with 3% halothane. The trachea was intubated with a 20-gauge intravenous catheter (Insite-W, Becton Dickinson). The inspired halothane concentration was reduced to 1.2%, and the lungs were mechanically ventilated at a rate of 130 breaths per minute with a delivered tidal volume of 0.7 mL. Pericranial temperature was monitored from a needle thermistor percutaneously placed adjacent to the skull. Temperature was servo-regulated at 37.0°C by surface heating or cooling during ischemia and for 30 minutes after reperfusion. Via surgical incision, the right femoral artery was cannulated (PE10, Becton Dickinson) to allow measurement of mean arterial blood pressure (MAP) and blood gases. Heparin (6 IU) was given intravenously. Via surgical incision, the common carotid arteries were identified and encircled with suture. The wounds were infiltrated with lidocaine and closed with suture. Mice were allowed to recover in an oxygen-enriched environment (fraction of inspired oxygen, 0.5). After recovery of spontaneous ventilation and the righting reflex, the trachea was extubated, and animals were returned to their cages with free access to food and water.

A recovery interval of 3 days was allowed. The mice were then subjected to a neurological examination designed to detect motor deficits in the rat that was modified for the mouse. Briefly, the mice were placed on a 10×20-cm screen (grid size 0.2×0.2 cm) that could be rotated from 0° (horizontal) to 90° (vertical). The mouse was placed on the horizontal screen, and the screen was then rotated into the vertical plane. The duration of time that the mouse was able to hold on to the vertical screen was recorded, to a maximum of 15 seconds (allowing a total of 3 points). Next, the mouse was placed at the center of a horizontal wooden rod (1.5 cm in diameter), and the time that the mouse was able to remain balanced on the rod was recorded, to a maximum of 30 seconds (allowing a total of 3 points). Finally, a prehensile task was administered. The time that the mouse was able to cling to a horizontal rope was recorded, to a maximum of 5 seconds. From these 3 tests, a total motor score (9 possible points) was computed.

Ischemia was induced by intra-arterial infusion of 0.3 mg of trimethaphan camsylate.14,16 As required, blood (0.5 to 0.6 mL) was withdrawn from the venous catheter to maintain MAP at 30±5 mm Hg. The common carotid arteries were then temporarily occluded and a timer was started. Halothane was discontinued. After 10 minutes of ischemia, the carotid arteries were deoxygenated and any withdrawn blood was reinfused. NaHCO3 (15 mL of 8.4%) was given intravenously, and the vascular catheters were removed. The wounds were infiltrated with lidocaine and closed with suture. Mice were allowed to recover in an oxygen-enriched environment (fraction of inspired oxygen, 0.5). After recovery of spontaneous ventilation and the righting reflex, the trachea was extubated, and animals were returned to their cages with free access to food and water.

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Mice were then anesthetized with halothane, the trachea was intubated, and the lungs were mechanically ventilated. Brains were perfusion fixed in situ with buffered 10% formalin. After overnight stabilization, the brains were removed and stored in 10% formalin. Paraffin-embedded brain sections were serially cut (5 μm thick) and stained with acid fuchsin/celestine blue. With the investigator blinded to the different treatments, injury to the CA1 sector of the hippocampus was evaluated by light microscopy at bregma=−1.70 mm. Viable and nonviable neurons were manually counted, and the percentage of nonviable neurons was calculated (% CA1 dead). At the same anatomical level, damage in the dorsal neocortex was graded using a crude damage index with a scale of 0 to 3 (0 = no damaged neurons; 1 = 1% to 30% neurons damaged; 2 = 30% to 60% neurons damaged; and 3 = >60% of neurons damaged).18 Damage in the dorsolateral caudoputamen was graded using the same scale at bregma=+0.50 mm. Values from the hemisphere with the worst damage were used for the statistical analysis.

For CBF analysis, mice underwent an anesthetic and surgical protocol identical to that described above. In addition, catheters were placed via surgical incision in the left femoral artery and left jugular vein (intraischemic blood flow studies only). One arterial catheter was used for continuous monitoring of MAP. The other arterial catheter was used for collection of timed arterial blood samples. The right jugular vein catheter was used for infusion of isotope. The left jugular catheter was used for withdrawal or infusion of blood as required during ischemia to maintain MAP at 30 mm Hg. Subsets of wild-type and apoE-deficient mice (n=4 to 7 per group) underwent CBF determination 10 minutes before ischemia, at the midpoint of ischemia, or at 30 minutes after onset of reperfusion. Ischemia and reperfusion were produced exactly as described for those animals subjected to histologic outcome analysis.

At the respective CBF measurement intervals, 5 μCi of 4-iodo-n-methyl-[14 C]antipyrine in 50 μL saline (14 C-IAP, specific activity 55.4 μCi/mmol, American Radiolabeled Chemicals, Inc.) was infused into the jugular vein over 60 seconds in a 60-step ramp (0.1 μL/min to 203 μL/min) to produce an increasing 14 C-IAP arterial concentration. For animals in which CBF was measured before or after ischemia, twelve 10-μL arterial blood samples were collected during 14 C-IAP infusion (Unopette capillary pipettes, Becton Dickinson Co) for later determination of arterial 14 C activity. In between sample collections, the arterial catheter was allowed to bleed freely. The blood volume lost was ≈50 μL greater than the volume infused. In preparing for the study, venous (5 cm PE-10 affixed to 5 cm PE-50) and arterial (2 cm PE-10) catheters were standardized for length and volume. For animals in which CBF was measured during ischemia, arterial blood was continuously withdrawn from the right femoral artery at a rate of 200 μL/min into a 90-cm PE-50 catheter. This was required because free flow from the arterial catheter was negligible when MAP equaled 30 mm Hg (see below). At completion of the experiment, this catheter containing radioactive blood was sectioned into 6.6-μL increments (representing 2-second withdrawal intervals sampled at 3-second intervals) for later determination of timed arterial 14 C activity. Simultaneous with the last blood sample and completion of isotope infusion, all animals were decapitated and their brains rapidly frozen in 2-methylbutane (−20°C). Arterial blood samples were placed on filter paper, dried for 24 hours, and then eluted an additional 24 hours in 1 mL normal saline and 10 mL liquid scintillation cocktail (Cytoscient, ICN). Radioactivity was determined via liquid scintillation counting using an external quench correction.

The continuous arterial blood withdrawal method used to define arterial 14 C-IAP concentrations in ischemic mice was examined in an in vitro experiment. The estimated blood volume of the mouse is approximately 2 mL.19 Accordingly, a 2-mL sample of rat blood was obtained and placed in a container with a stir bar. Into this blood we infused 14 C-IAP at the ramp infusion rate used in the in vivo CBF experiments. Simultaneous with this infusion, blood was sampled by continuous aspiration, exactly as was done in the in vivo experiments. Also simultaneous with the infusion, blood was sampled from the container by micropetite at 5-second intervals which simulated the technique developed by Sakurada et al.18 The samples were then analyzed for 14 C-IAP activity using standard scintillation counting techniques. Areas under the respective curves for the 2 sampling techniques were calculated. The area under the curve for the continuous technique (149 988 counts per minute) was 7% less than that of the intermittent (161 388 counts per minute) technique. Thus, the difference between sampling techniques was small, although the continuous sampling technique could theoretically yield flow values less than those provided by the intermittent sampling technique.

Frozen brains were coronally sectioned (20 μm thick) at −1°C. Quadruplicate sections were taken at bregma+1.42, −2.06, −2.39, −3.08, −4.16, and −5.40 mm,21 mounted on glass slides, dried for 5 minutes on a hot plate (36.0°C), and exposed to Kodak SB-5 autoradiographic film for 5 days along with 14 C-methylmecamylate standards (ranging from 0 to 35.0 nCi/mg).

Images from each anatomical level were scanned by a video camera and stored as a 1280×960 matrix of calibrated pixel units (14×15 μm). Digital optical densities from these autoradiographic images, standard radioactivity values derived from 14 C standards, and timed arterial blood 14 C activity were entered into an image analyzer (MCID-M2, version 3.0 revision 1.2, Imaging Research Inc.). Radioactivity values were converted to CBF values according to the
TABLE 1. Physiologic Values for Wild-Type and ApoE-Deficient Mice Subjected to 10 Minutes of Near-Complete Forebrain Ischemia and 3 Days of Recovery

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ApoE Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Body weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic, g</td>
<td>22±1</td>
<td>22±1</td>
</tr>
<tr>
<td>3d posts ischemic, g</td>
<td>21±2</td>
<td>21±2</td>
</tr>
<tr>
<td>10 minutes after ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>75±6</td>
<td>74±6</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.17±0.07</td>
<td>7.14±0.06</td>
</tr>
<tr>
<td>PaCO2, mm Hg</td>
<td>42±6</td>
<td>44±6</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>207±24</td>
<td>195±13</td>
</tr>
<tr>
<td>Pericranial temperature, °C</td>
<td>37.0±0.1</td>
<td>37.0±0.1</td>
</tr>
<tr>
<td>10 minutes after ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>83±10</td>
<td>77±13</td>
</tr>
<tr>
<td>Pericranial temperature, °C</td>
<td>37.0±0.1</td>
<td>37.0±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SD. There were no significant differences between groups. MAP indicates mean arterial pressure; PaCO2, arterial carbon dioxide partial pressure; and PaO2, arterial oxygen partial pressure.

Results

Physiological values for mice subjected to ischemic outcome analysis are presented in Table 1. There were no significant differences between groups. As intended, mice were normocapnic, normothermic, and mildly hyperoxicemic. A moderate metabolic acidosis was present in both groups. Total motor scores were not different between the 2 groups, with most mice showing little or no deficit (wild type, 9±0.25; apoE deficient, 8.5±1.5; P=0.08). Histologic injury is illustrated in Figure 1. The percentage of dead hippocampal CA1 neurons was greater in the apoE-deficient group (apoE deficient, 67±30%; wild type, 37±33%; P=0.011). Crude damage index scores were worsened by apoE deficiency in the caudoputamen (apoE deficient, 3±1; wild type, 1±1; P=0.002) and neocortex (apoE deficient, 1±0; wild type, 0±1; P=0.014).

Physiological values for the CBF study are summarized in Table 2. There were no differences between groups. Regional CBF values are given in Figure 2. No differences were detected between genetic groups 10 minutes before, during, or at 30 minutes after ischemia in the caudoputamen, cortex, hippocampus, and corpus callosum (not shown). Thalamic blood flow was similar between groups before and after ischemia, but ischemic blood flow was greater in the apoE-deficient group (P<0.05). As intended, severe blood flow reduction was restricted to the forebrain with little change in intraischemic or posts ischemic blood flow in caudal structures such as cerebellum and pontine nucleus. Reperfusion at 30 minutes was associated with a persistent state of hypoperfusion in the caudoputamen, cortex, and hippocampus, with CBF values recovering to only 45% to 60% of baseline values. There were no differences between groups for percentage of cross-sectional area where

TABLE 2. Physiologic Values in Wild-Type and ApoE-Deficient Mice Before Ischemia Onset and Cerebral Blood Flow Analysis

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=19)</th>
<th>ApoE Deficient (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>20±2</td>
<td>20±2</td>
</tr>
<tr>
<td>10 minutes before ischemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>73±9</td>
<td>71±6</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.12±0.04</td>
<td>7.14±0.07</td>
</tr>
<tr>
<td>PaCO2, mm Hg</td>
<td>45±5</td>
<td>43±7</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>199±23</td>
<td>206±22</td>
</tr>
</tbody>
</table>

Values are mean±SD. There were no significant differences between groups. MAP indicates mean arterial pressure; PaCO2, arterial carbon dioxide partial pressure; and PaO2, arterial oxygen partial pressure.
flow was <15 mL/100 g/min. Prior to ischemia there were essentially no pixels within this flow bin. During ischemia >98% of pixels in the hippocampus, caudoputamen, and neocortex met this criteria. Data from the reperfusion interval is shown in Figure 3. Although no differences between groups were observed, within the hippocampus ~20% of tissue met the <15 mL/100 g/min criterion after 30 minutes of reperfusion.

**Discussion**

This study demonstrates that mice deficient in apoE have decreased tolerance to transient severe forebrain ischemia, manifested by increased selective neuronal necrosis in the hippocampal CA1, caudoputamen, and neocortex. This effect was not attributable to differences in severity of the ischemic insult or residual hypoperfusion in the apoE-deficient versus wild-type groups.

The worsened histological outcome observed in the apoE-deficient mice is consistent with a prior investigation that demonstrated worsened neurological/histological outcome from focal ischemia in apoE-deficient mice. Total infarct volume resulting from 60 minutes MCAO and 24 hours’ reperfusion was 64% greater in the apoE-deficient group than in the wild-type group. Later work showed that both apoE-deficient and wild-type mice are exposed to similar severities of blood flow reduction during MCAO and that the frequency of vascular anomalies in the circle of Willis is similar between apoE-deficient and wild-type groups. Further, it has been shown that outcome from murine focal cerebral ischemia is modified by apoE genotype. Mice carrying the human APOE3 transgene (on a murine APOE knockout background) had a 38% reduction in total infarct volume resulting from 60 minutes of MCAO and 24 hours of reperfusion compared with mice expressing the human APOE4 transgene. Cumulatively, this evidence provides a strong case for the role of apoE in the pathophysiological response to an ischemic brain insult in the mouse.

We believed that direct measurement of CBF was essential to prove that the severity of the global ischemic insult used in this study was similar between groups. Because apoE plays a central role in lipid transport, a hallmark of apoE deficiency in both the mouse and human is hypercholesterolemia. However, in the apoE-deficient mouse, microscopic preatherosclerotic lesions do not become apparent until 3 to 4 months of age, with more advanced lesions becoming manifest by 9 to 12 months of age. This would not be expected to appreciably alter blood flow in mice at the age of 8 to 10 weeks as used in the current study and is consistent with the absence of differences in blood flow observed in the apoE-deficient and wild-type groups.

The mechanistic basis by which apoE affects ischemic outcome is unknown. While a definitive answer to this question must await further research, there are known effects of apoE deficiency that may be of relevance. Masliah et al reported extensive dendritic vacuolization and disruption of the neuronal cytoskeleton in apoE-deficient mice. Others have observed reduced brain choline acetyltransferase activity in the hippocampus and frontal cortex of apoE-deficient mice. This was correlated with deficits in working but not reference memory and is consistent with the finding that hippocampal tissue taken from apoE-deficient animals exhibits reduced in vitro plasticity in response to stimulation of Schaffer/commissural fibers. Such effects have been found to be reversible if recombinant apoE is infused intraventricularly. ApoE is also known to decrease lymphocyte proliferation and immunoglobulin synthesis of B lymphocytes after mitogenic challenge and suppress glial secretion of inflam-
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matory cytokines, suggesting a potential immunomodulatory mechanism. ApoE also has the ability to protect neurons from oxidative injury. Finally, in the gerbil, apoE is selectively expressed in vulnerable areas of the hippocampus after global ischemia, and apoE accumulates in posts ischemic neurons in the rat. This suggests a potential active role of apoE in response to events initiated during the ischemic insult. The increasing body of evidence from both human and animal studies that indicates a role for apoE in ischemic injury justifies further study of the mechanisms by which such effects occur.

This study also demonstrates potential value of the murine adaptation of the Lund 2-vessel occlusion plus systemic hypotension (2VO) model, which was originally developed for the rat. Despite exciting progress in the development of rat genetic mutants for use in ischemia research, the mouse is likely to play a significant role in such studies in the foreseeable future. Not only are a wide variety of murine mutants available, but also in some circumstances murine genotypes may be more similar to those found in humans as opposed to those found in rats. For example, mice that either overexpress extracellular superoxide dismutase (EC-SOD) or EC-SOD deficient have been found to have worsened/improved outcome, respectively, from focal ischemia when compared with wild-type counterparts. Both murine and human EC-SOD are tetrameric, while that of the rat is dimeric. Further, rats express little EC-SOD, while concentrations in the mouse and human are orders of magnitude greater and similar. This would suggest that the mouse would be a more appropriate species than the rat for study of EC-SOD in ischemic brain.

The 2VO murine model used in this study has recently been shown to be sensitive to expected effects of preischemic hyperglycemia (worsened damage) and mild hypothermia (improved outcome). An alternative murine model uses unilateral occlusion of the carotid arteries (BCAO) in the absence of systemic hypotension. Intervals of occlusion have been varied from 3 to 75 minutes. In the BCAO model, global ischemia can be studied in the absence of any confounds which such effects occur. In conclusion, mice deficient in apoE were subjected to 10 minutes of severe forebrain ischemia and 3 days of recovery. Histologic outcome was compared to that found in wild-type control mice subjected to the same insult. Increased neuronal necrosis was observed in the apoE-deficient hippocampal CA1, caudoputamen, and neocortex. Cerebral blood flow measured before, during, and 30 minutes after ischemia in cohort animals was found to be similar in the apoE-deficient and wild-type groups. This indicates that the severity of the insult was similar and that specific factors associated with apoE deficiency must account for the observed effect on histological outcome. These data are consistent with prior reports that indicate a role for apoE in ischemic brain injury and justifies further investigation into the mechanisms by which apoE enhances tolerance to brain ischemia.

Acknowledgments

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References


It is becoming increasingly clear that the presence of selected isoforms of apolipoprotein E (apoE) influences the response of the brain to injury. In particular, the APOE4 allele has been associated with increased susceptibility to Alzheimer’s disease and with a less-favorable outcome in various types of acute brain pathologies (see Reference 1 for a review). In the accompanying article, Sheng et al demonstrate that mice with a null mutation of the APOE gene develop greater brain damage than wild-type mice after transient global cerebral ischemia. The increased susceptibility to ischemic injury is not related to effects of APOE deletion on cerebral blood flow. The data provide compelling evidence that apoE has a protective effect on cerebral ischemic injury in mice.

Relatively little is known about the neurobiology of apoE. There is evidence that apoE and some of the peptides derived from its proteolytic processing are toxic to neurons in culture.2,3 However, protective effects have also been reported. For example, the apoE3 isoform mitigates β-amyloid–induced neurotoxicity in rat hippocampal neurons.4 The opposing biological actions of apoE are also underscored by clinical studies indicating that the e4 allele increases whereas the e2 allele decreases the risk for Alzheimer’s disease.5,6 The finding of Sheng et al that deletion of the APOE gene worsens the outcome of cerebral ischemia provides additional evidence that selected isoforms of apoE can be protective.

However, only 1 form of apoE has been identified in mice whereas 3 isoforms are known to exist in humans.7 Therefore, it remains unclear how experimental evidence obtained in mouse models relates to the biology of apoE in human diseases. Nonetheless, the elegant study of Sheng et al, in concert with previous studies by the same group, suggest strongly that apoE protects the brain from the consequences of cerebral ischemia. The mechanisms by which apoE modulates brain damage and the role of apoE in the overall process of ischemic brain injury, in rodents as in humans, remain to be defined.

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References
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