Pharmacology Biochemistry and Behavior, Vol. 56, No. 4, pp. 669–673, 1997

0091-3057/97 $17.00 + .00

PII S0091-3057(96)00412-1

Reduced Levels of Antioxidants in Brains of Apolipoprotein E-Deficient Mice Following Closed Head Injury

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Received 1 August 1996; Revised 2 October 1996; Accepted 2 October 1996

LOMNITSKI, M., R. KOHEN, Y. CHEN, E. SHOHAMI, V. TREMBOVLER, T. VOGEL AND D. M. MICHAELSON. *Reduced levels of antioxidants in brains of apolipoprotein E-deficient mice following closed head injury.* PHARMACOL BIOCHEM BEHAV **56**(4) 669–673, 1997.—Recent animal model studies using apolipoprotein E (apoE)-deficient (knockout) mice revealed that these mice have memory deficits and neurochemical derangements and that they recover from closed head injury less adequately than control mice. In the present study, we examined the possibility that the diminished recovery of apoE-deficient mice from head injury is related to a reduction in their ability to counteract oxidative damage. Measurements of reducing agents by cyclic voltammetry revealed that cortical homogenates of apoE-deficient and control mice contain similar levels of these compounds, whose oxidation potentials for the two groups of mice are at 400 ± 40 mV and 900 ± 50 mV. The responses of the apoE-deficient and control groups to closed head injury were both biphasic and were composed of initial reductions followed by subsequent increases in the levels of reducing antioxidant equivalents. However, the two groups differed markedly in the magnitude of their response. This difference was most pronounced with the 400-mV reducing compounds, such that at 4 h after injury their levels in injured control mice increased over twofold relative to the noninjured control mice, whereas the corresponding anodic current of the apoE-deficient mice recovered only to its original level and did not increase further even by 24 h after injury. In vitro studies using recombinant apoE allele E3 and β very low density lipoprotein revealed that this lipoprotein can delay Cu^{2+}-induced lipid peroxidation. This suggests that the inability of the apoE-deficient mice to respond to brain injury by a surge in brain reducing compounds may be related, at least in part, to direct antioxidant activity of apoE. © 1997 Elsevier Science Inc.

Alzheimer's disease Apolipoprotein Head trauma Apolipoprotein E-deficient mice
Reducing currents Antioxidants

GENETIC studies of familial Alzheimer's disease (AD) suggest that the etiology of this disease may be associated with several factors which include the amyloid precursor gene and allele E4 of apolipoprotein E (apoE4) [see (6,22)] as well as the two recently described presenilin 1 and presinilin 2 genes (15,24). Of these genes, only the apoE4 allele has been linked thus far to sporadic AD (7,22). Furthermore, the size of senile plaques, a major histopathological hallmark of AD, and their β amyloid content are greater in subjects with apoE4 (22). These compelling findings imply that the biology of apoE is extremely relevant to the expression of AD.

Head injury is an important environmental factor that has been implicated in the neuropathology of AD (10). Furthermore, it has been suggested that subjects with the apoE4 allele are much more vulnerable to head trauma than subjects who lack this apoE allele (21). These findings and the observation that the deleterious effects of apoE4 are apparent late in life suggest that apoE plays a key role in neuronal maintenance and repair.

ApoE-deficient homozygous mice whose apoE gene was knocked out (20) are a useful system for studying the role of apoE in neuronal function and repair. Indeed, Masliah and

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colleagues (17) have demonstrated age-dependent decreases in synaptic density in distinct brain areas of apoE-deficient mice. We have shown that apoE-deficient mice have memory impairments that are associated with specific derangements in basal forebrain cholinergic neurons (11) and that their brain neurons have cytoskeletal abnormalities (i.e., hyperphosphorylation of the cytoskeletal protein tau) reminiscent of those that occur in AD (9). Furthermore, we have recently shown that apoE-deficient mice recover less well neurologically from closed head injury than do control mice and that, unlike the controls, they are cognitively impaired for at least a month following the head trauma (4,16). The cellular and biochemical mechanisms that render the apoE-deficient mice more susceptible to head trauma are not known.

Oxidative stress, namely an imbalance between the levels of reactive oxygen species and antioxidants, plays a major role in neuronal damage elicited by head injury and has been suggested to play an important role in neurodegenerative processes underlying AD (2,3,12). Antioxidants may be classified into two major groups: antioxidant enzymes and low molecular weight antioxidants. The reductive power of the latter can be evaluated by cyclic voltammetry, which enables the determination of their type (i.e., oxidation potentials) and their concentration (i.e., anodic currents). In the present study, we employed cyclic voltammetry for examining the possibility that the neurochemical and cognitive deficits of apoE-deficient mice and their diminished ability to recover from head trauma are associated with alterations in their brain antioxidants, either prior to or after the head injury.

EXPERIMENTAL

Closed Head Injury

Four-month-old apoE-deficient male mice (~23 g) and a matched group of control mice from the same original parent litter (20) were used in this study. Closed head injury to seven mice in each group was produced under anesthesia as modified by Chen et al. (4) after Shapira et al. (23). A weight-drop device was employed in which a calibrated weight (333 g) was allowed to fall from a height of 3 cm onto the exposed skull, over the left cervical hemisphere, 1–2 mm lateral to the midline of the midcoronal plane. Following recovery from anesthesia, the mice were returned to their home cages with free access to food and water. Parallel groups of apoE-deficient and control mice were anesthesized; their skulls were exposed but no trauma was induced. These mice served as sham controls.

Measurements of Brain Reducing Capacity

Brains of closed head injured and sham-treated mice were removed by decapitation prior to and at 5 min, 4 h, and 24 h after head trauma ($n = 7$ for each group of mice at each time point). The left and right cortical hemispheres and the cerebellum, which is located distal to the site of injury, were then rapidly excised and stored at −70°C. The reducing capacity of the excised brain areas was determined by cyclic voltammetry (1), using a BAS model CV-IB cyclic voltammetry apparatus (West Lafayette, IN, USA) modified for a 250-μl cell. The brain samples were thawed and sonicated (1 g wet weight per 10 ml phosphate-buffered saline). Measurements for the resulting homogenates were then performed between 0 and 2 V, using a three-electrode system as previously described (14). Results obtained from brain areas of the closed head injured mice were compared with those of the corresponding noninjured mice by the Mann–Whitney nonparametric test. All reagents used were of analytical grade (Sigma Chemical Co., MO, USA).

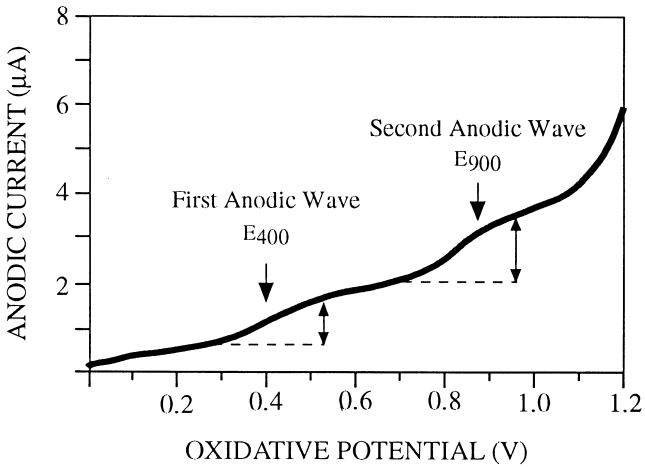

FIG. 1. A representative voltammogram of a cortical homogenate of control mice. Two anodic waves were recorded at oxidative potentials 400 mV and 900 mV (denoted by E_{400} and E_{900}). The corresponding anodic currents are marked by double-headed arrows. The cyclic voltammetry measurements were performed as described in "Experimental."

In Vitro *Measurements of the Effects of apoE on Lipid Peroxidation*

Cu^{2+}-induced peroxidation of rabbit β very low density lipoprotein (β-VLDL) (8) was monitored spectrophotometrically at 234 nm (19). In brief, $CuCl_2$ (5 μM) was added to β-VLDL (6.6 μg/ml) suspended in phosphate-buffered saline and in the presence or absence of recombinant apoE allele E3 (apoE3) (25), after which the resulting effects of apoE on the rate and extent of lipid peroxidation were monitored at 234 nm.

RESULTS

The possibility that the diminished recovery of apoE-deficient mice from closed head injury is related to a diminution in their ability to counteract oxidative damage was examined by cyclic voltammetry. This was pursued by measurements using homogenates of distinct brain areas of injured and noninjured apoE-deficient and control mice. A representative voltammogram thus obtained from a cortical homogenate of a control mouse prior to closed head injury is presented in Fig. 1. Cortical homogenates contained reductive substances that yielded two waves of anodic currents whose reductive potentials were, respectively, 400 ± 50 mV and 900 ± 50 mV (Fig. 1). Similar measurements of the levels of anodic currents in the cerebelli of the two groups of mice also revealed two anodic waves, except that the oxidation potential of the first anodic wave of this brain area was 570 ± 50 mV and not 400 ± 50 mV. However, as with the cortex, there were no significant differences in cerebellar reducing agents between the apoE-deficient and control mice as measured by their anodic currents (Fig. 2).

The effects of closed head injury on the levels of the 400-mV antioxidant anodic currents of cortical homogenates of the contused hemispheres of the two groups of mice are depicted in Fig. 3. The responses of the two groups of mice

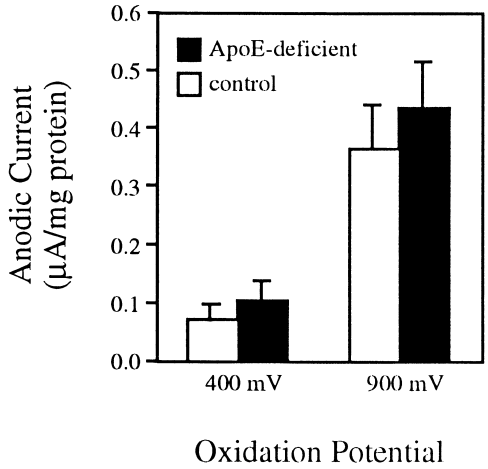

FIG. 2. Comparison of the levels of reducing agents in cortical homogenates of apoE-deficient and control mice. Results presented were obtained by cyclic voltammetry and correspond to the anodic currents (mean + SD) of the 400-mV and 900-mV reducing currents of seven mice in each group. The experiments were performed as described in "Experimental."

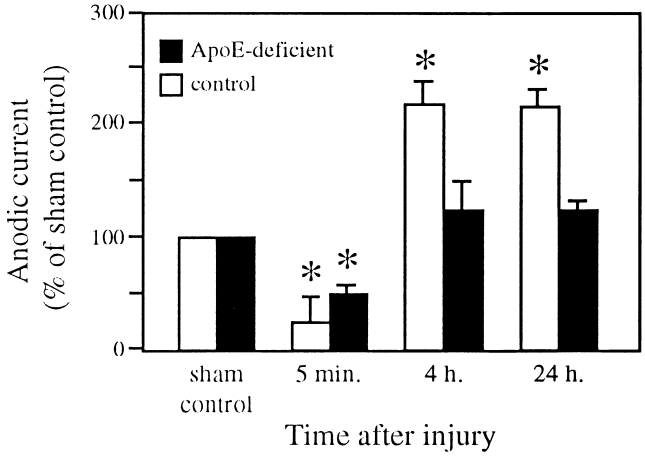

FIG. 3. Effects of closed head injury on cortical levels of reducing compounds with an oxidation potential of 400 mV in apoE-deficient and control mice. Twenty-eight mice in each group were subjected to closed head injury; seven mice of each group were then sacrificed at the indicated time points and their levels of 400-mV antioxidant reducing compounds (first anodic wave) were measured by cyclic voltammetry. Results presented for each group (mean + SD) are in percent relative to results for the corresponding untreated group. The 400-mV reducing currents for the noninjured control and apoE-deficient mice were, respectively, 0.072 ± 0.01 and 0.1 ± 0.02 μA/mg protein. *Significant difference ($p < 0.01$) from the corresponding sham control.

were both biphasic and were composed of initial reductions followed by subsequent increases. There were, however, quantitative differences between the responses of the two groups. The initial decrease in anodic current of control mice measured at 5 min after injury was about 75%, whereas the corresponding decrease for the apoE-deficient mice was less pronounced. Furthermore, by 4 h following injury, the 400-mV anodic currents of the control mice increased significantly ($p < 0.01$) to more than 200% of those of the untreated mice, whereas the corresponding increase in the apoE-deficient mice was only back to the level of the untreated mice and did not increase further even by 24 h after the injury (Fig. 3). Qualitatively similar results were obtained with the 900-mV anodic currents, but the magnitude of the head trauma-induced changes and of the differences between the two groups of mice were less pronounced (Fig. 4). Measurements of the levels of anodic currents of homogenates of brain areas located away from the site of the head injury revealed for the contralateral cortical hemisphere a pattern similar to that described above, except that the magnitudes of the effects were much reduced and there were no detectable changes in the cerebellum (not shown).

The diminished ability of apoE-deficient mice to respond to brain injury by a surge of brain-reducing compounds may be related to direct or indirect antioxidant activity of apoE, which, of course, is lacking in the knockout mice. To address this issue, we set up an in vitro model system in which the antioxidant capacity of apoE was evaluated. Incubation of Cu^{2+} with β-VLDL results in a lengthy lag period that is followed by an exponential surge in lipid peroxides (Fig. 5). Addition of recombinant human apoE3 (0.3–0.6 μg/ml) to the reaction mixture resulted in prolongation of the lag period and thus delayed Cu^{2+}-mediated β-VLDL peroxidation.

DISCUSSION

This study revealed that prior to head injury, apoE-deficient and control mice have similar levels of antioxidant reducing compounds but that their levels differ markedly following closed head injury. In control mice, closed head injury results in an initial decrease and in a subsequent surge of antioxidant reducing substances, whereas in the apoE-deficient mice, the initial decrease and particularly the subsequent increase are much less pronounced. Below we discuss possible mechanisms that may underlie these neurochemical differences and the extent to which they are related either to the neuronal and cognitive deficits of the apoE-deficient mice (9,11) or to their diminished ability to recover from head trauma (4,16).

The initial decrease in the levels of antioxidant reducing materials occurs within minutes after injury and represents consumption of reducing agents by the resulting oxidative burst (5). The subsequent increases in the levels of reducing compounds, which evolve over hours (Fig. 3), are most likely due to induction of brain antioxidant repair mechanisms or to the recruitment of antioxidants from the brain (1). Accordingly, the present findings suggest that apoE-deficient mice are impaired in their ability to employ basal antioxidant reducing compounds to counteract the brain injury-induced oxidative burst as well as in their ability to activate antioxidant brain repair mechanisms. Cyclic voltammetry, the technique employed in this study, enables simultaneous measurements of the levels of groups of reducing compounds each of which have distinct oxidation potentials. Thus, although the chemical identities of the brain reducing compounds of the two mice groups are not known, it is possible that changes in brain levels of compounds such as uric acid and vitamin C, whose oxidation potentials are similar to those of the first anodic wave (~400 mV), are responsible for the observed effects. Further analyses are required to chemically identify the brain reducing compounds of the two groups of mice and to determine the extent to which they are affected by head injury.

The inability of apoE-deficient mice to respond to brain injury by a surge of brain reducing agents may be due either

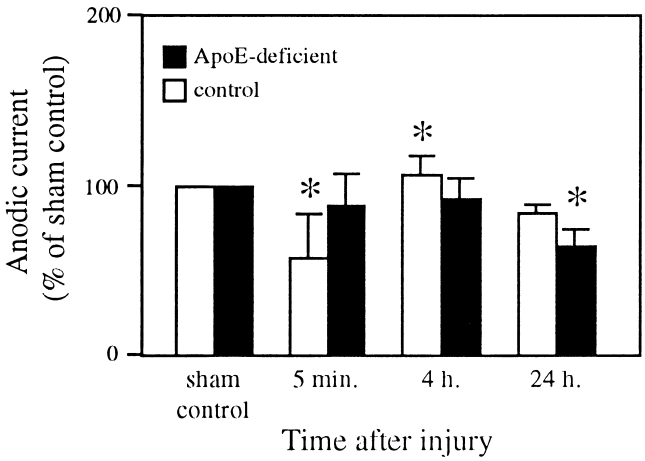

FIG. 4. Effects of closed head injury on cortical levels of reducing compounds with an oxidation potential of 900 mV in apoE-deficient and control mice. Twenty-eight mice in each group were subjected to closed head injury; seven mice of each group were then sacrificed at the indicated time points and their levels of 900-mV antioxidant reducing compounds (second anodic wave) were measured by cyclic voltammetry. Results presented for each group (mean + SD) are in percent relative to results for the corresponding untreated group. The 900-mV reducing currents for the noninjured control and apoE-deficient mice were, respectively, 0.36 ± 0.02 and 0.43 ± 0.05 μA/mg protein. *Significant difference ($p < 0.01$) from the corresponding sham control.

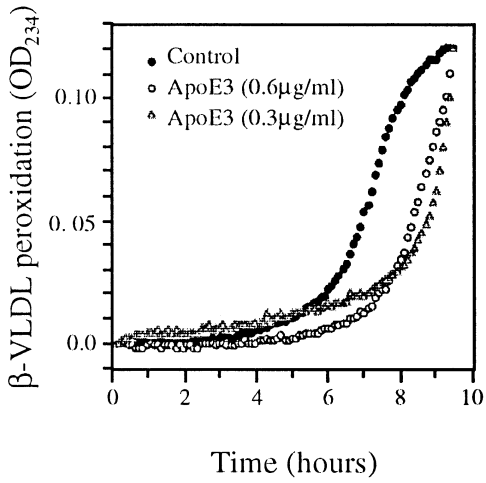

FIG. 5. Effects of apoE on Cu^{2+}-induced β-VLDL peroxidation. β-VLDL (6 μg/ml) was oxidized by Cu^{2+} (5 μM $CuCl_2$) in the presence and absence of the indicated concentrations of recombinant human apoE3. ApoE3 was added to the reaction mixture 30 min prior to Cu^{2+}, after which the resulting peroxidation of β-VLDL was monitored spectrophotometrically at 234 nm. Results shown are representative of four repeat experiments.

to direct antioxidant activity of apoE or to an alternative mechanism that is not related to either the oxidative or the reducing properties of apoE. The present *in vitro* experiments, which revealed that apoE can affect and retard Cu^{2+}-induced lipid peroxidation (Fig. 5), suggest that apoE can indeed act as an antioxidant. Thus, it is possible that at least part of the observed in vivo effects are due to the absence of the antioxidant capacity of apoE in apoE-deficient mice. It is important, however, to stress that further mechanistic studies are needed to determine the extent of applicability of the in vitro findings to the situation *in vivo*. This includes studies of the extent to which the *in vitro* effects of apoE are due either to metal chelation or to direct antioxidation, as well as examination of the possibility that under appropriate conditions distinct apoE3 isoforms vary in their antioxidative capacity.

Antioxidative mechanisms are believed to play an important role in the defense response following injury (13). Thus, it is tempting to suggest that the presently observed reduction in the ability of the apoE-deficient mice to generate cortical antioxidants after head injury is causally related to the subsequent neurological and cognitive impairments of these mice (4,16). However, although the differences in antioxidant levels occur within minutes and hours following closed head injury (see Figs. 3, 4), whereas the neurological and behavioral impairments of the apoE-deficient mice persist for at least 4 weeks after head injury (4,16), we cannot exclude the possibility that these effects are not related. The basal levels of reducing cortical antioxidants of the two mice groups are similar (Fig. 2). However, recent measurements of oxidation products in noninjured mice revealed higher levels of oxidized proteins in brains of apoE-deficient mice (18), whereas we have found no differences in the extent of lipid peroxidation in the brains of the two groups of mice (in prep.). Thus, it remains to be determined whether the basal neurochemical and cognitive derangements of noninjured apoE-deficient mice (9,11) are related to antioxidant deficiencies. Further experiments in which apoE-deficient and control mice will be treated with specific antioxidants (e.g., tocopherol and vitamin C) before and after closed head injury will help to resolve this issue.

In conclusion, this study revealed a markedly lower brain antioxidative response in apoE-deficient mice following closed head injury than in control mice and suggests that this phenomenon may play a role in the impaired ability of the apoE-deficient mice to recover from head injury. Further animal model studies and in vitro oxidation experiments using distinct apoE isoforms are required for unraveling the role of oxidative mechanisms in the neurobiology of apoE and the extent to which they mimic similar processes in AD.

ACKNOWLEDGEMENTS

We thank Prof. D. Lictenberg for his advice and generous help. This work was supported in part by grants to D. M. M. from the Joseph K. & Inez Eichenbaum Foundation, the Simon Revah-Kabelli Fund, the U.S. Israel Binational Foundation, and the Israel Science Foundation, and to L. L. from the Robinowitch Fund.

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