The Receptor Binding Domain of Apolipoprotein E Is Responsible for Its Antioxidant Activity†

Thomas Pham, Ahmer Kodvawala, and David Y. Hui*

Department of Pathology and Laboratory Medicine, Genome Research Institute, University of Cincinnati College of Medicine, Cincinnati, Ohio 45237-0507

Received December 30, 2004; Revised Manuscript Received March 21, 2005

ABSTRACT: Apolipoprotein E (apoE) is a 34-kDa lipid-associated protein present in plasma and in the central nervous system. Previous studies have demonstrated that apoE has multiple functions, including the ability to transport lipids, regulate cell homeostasis, and inhibit lipid oxidation. The lipid binding domain of apoE has been localized to the carboxyl-terminal domain, whereas a cluster of basic amino acid residues within the N-terminal domain is responsible for its receptor binding activity. This study was undertaken to identify the domain in apoE responsible for its antioxidant activity. Results showed that apoE inhibits Cu^{2+}-induced LDL oxidation by delaying conjugated diene formation in a concentration-dependent manner. Reductive methylation of lysine residues or cyclohexanedione modification of arginine residues in apoE abolished its ability to inhibit LDL oxidation. Additional studies showed that a 22-kDa peptide containing the N-terminal domain of apoE3 was more effective than a similar peptide with the apoE4 sequence in inhibiting Cu^{2+}-induced LDL oxidation. In contrast, the 10-kDa peptide that contains the C-terminal domain of apoE was ineffective. Inhibition of Cu^{2+}-induced LDL oxidation can also be accomplished with a peptide containing either a single sequence or a tandem repeat sequence of the receptor binding domain (residues 141−155) of apoE. Taken together, these results localized the antioxidant domain of apoE to its receptor binding domain and the basic amino acids in this domain are important for its antioxidant activity.

Apolipoprotein E (apoE)[1] is a 34-kDa glycoprotein that circulates in plasma in association with various lipoproteins including chylomicrons, chylomicron remnants, very low density lipoproteins, and a cholesterol-rich subclass of high-density lipoproteins. ApoE is also synthesized in the brain and present in lipid transport vesicles in cerebrospinal fluid. The importance of apoE functions in normal physiology is reflected by the direct relationships between apoE genetic polymorphisms that affect apoE structure and functions and the increased risk of a number of diseases, including atherosclerosis (*1*), restenosis (*2*, *3*), Alzheimer's disease (*4*, *5*), Parkinson's disease (*6*), and impaired cognitive functions (*7*−*9*).

The most established physiological function of apoE is its ability to modulate cholesterol distribution between cells within each organ as well as between cells from different tissues (*10*, *11*). In this capacity, apoE synthesized in the liver promotes VLDL secretion (*12*, *13*). The apoE associated with VLDL mediates lipoprotein binding to the LDL receptor and its related family member proteins, thereby delivering hepatic-derived triglycerides and cholesterol to extrahepatic tissues (*14*). The apoE in circulation can also be redistributed to chylomicron remnants and transports dietary fat and cholesterol to the liver (*14*). Extrahepatic-derived apoE as well as apoE in circulation also promotes cholesterol efflux from cells, particularly the lipid-laden macrophages, and promotes reverse cholesterol transport from extrahepatic tissues back to the liver for reprocessing, storage, or excretion (*15*−*17*). The domain in apoE responsible for receptor binding, and thus mediating lipoprotein uptake by cells, resides between residues 141 and 155 (*18*, *19*). In contrast, apoE-mediated lipid egress from cells, either in promotion of VLDL assembly and secretion by the liver or facilitating cholesterol efflux from peripheral cells via ABCA1, requires the lipid binding domain located at the carboxyl-terminal two-thirds of the protein (*20*, *21*).

In addition to its cholesterol transport properties, apoE also has cholesterol transport-independent functions in protection against chronic metabolic diseases (*11*, *22*). Most of these lipid transport-independent apoE functions are related to its ability to modulate cell functions. For example, in the vessel wall, apoE may protect against vascular occlusion by inhibiting agonist-induced platelet aggregation via stimulation of nitric oxide production (*23*, *24*). ApoE also increases nitric oxide production in human macrophages (*25*), endothelial cells (*26*), and smooth muscle cells (*27*). The activation of nitric oxide synthesis in smooth muscle cells results in apoE inhibition of smooth muscle cell proliferation (*27*). Additionally, apoE also inhibits smooth muscle cell migration but in a mechanism that is independent of nitric oxide synthesis (*27*). Rather, apoE inhibits cell migration via activation of the cAMP signaling cascade (*28*). The ability of apoE to directly influence cell functions is due to its interaction with

† This work was supported by a National Institutes of Health grant awarded to D.Y.H. (HL61332). T.P. was a recipient of a Medical Student Research Fellowship award (T35 DK60444).

* To whom correspondence should be addressed. Tel: 513-558-9152. Fax: 513-558-1312. E-mail: huidy@email.uc.edu.

[1] Abbreviations: apoE, apolipoprotein E; LDL, low-density lipoproteins; VLDL, very low density lipoproteins; LRP, low-density lipoprotein receptor related protein.

10.1021/bi0472696 CCC: $30.25
Published on Web 04/23/2005

a number of different LDL receptor family proteins, including LRP, apoE receptor-2, and VLDL receptor, to activate specific cell signaling events (*29*). The domain in apoE responsible for binding to these receptors to modulate cell functions also resides in the basic amino acid cluster between residues 140 and 155 (*29*, *30*).

The ability of apoE to activate cell signaling events is also evident by its protective function against the development of Alzheimer's disease. For example, apoE protects against Alzheimer's disease by promoting neurite outgrowth and tubulin polymerization (*31*, *32*). The mechanism appears to be related to the ability of apoE3 to protect the microtubule-associated protein tau from hyperphosphorylation (*33*). Additionally, apoE also modulate intracellular calcium level in neurons (*34*, *35*). Although the precise domain in apoE responsible for neural protection has not been identified in direct assays, the requirement of apoE binding to LDL receptor or its related proteins for activation of these cellular events suggests the importance of the receptor binding domain between residues 140 and 155 in these functions (*36*, *37*).

Another important physiological function of apoE is its antioxidation properties in protection against lipid oxidation (*38*). The different antioxidant ability of apoE3 and apoE4 has been implicated as a major factor associating apoE4 gene polymorphism with increased risk of atherosclerosis (*39*) and Alzheimer's disease (*40*, *41*). However, the structural domain in apoE responsible for its antioxidant activity has not been identified to date. Thus, the purpose of this study is to identify the domain in apoE that protects LDL against lipid oxidation.

MATERIALS AND METHODS

Lipoproteins, Proteins, and Peptides. Human plasma was obtained from healthy donors at the Hoxworth Blood Center (Cincinnati, OH), and LDL was isolated by sequential ultracentrifugal flotation in KBr solutions between the densities of 1.02 and 1.063 g/mL. The isolated LDL fraction was dialyzed against phosphate-buffered saline containing 1 mM EDTA and stored at 4 °C until use. Recombinant human apoE3 was isolated from serum-free media of HEK-293 cells that have been stably transfected with a pCMV4 plasmid containing the human apoE3 cDNA (obtained from Dr. Catherine Reardon, University of Chicago), as described (*42*). Purification of the recombinant apoE3 was accomplished by affinity chromatography on a heparin−Sepharose column (*43*). The apoE3 bound to the heparin−Sepharose column was eluted with 0.75 M ammonium bicarbonate and lyophilized. Homogeneity of the purified human apoE3 was confirmed by the appearance of a single band at 34 kDa after SDS−polyacrylamide gel electrophoresis. The 22- and 10-kDa peptides corresponding to the N- and C-terminal domains of human apoE3 and apoE4 were obtained as apoE thrombin-digested fragments from Dr. Karl Weisgraber (Gladstone Institute, San Francisco). Polypeptides containing either a single copy or tandem repeat sequence of apoE residues 141−155, designated as apoE(141−155) and apoE-$(141-155)_2$, respectively, and a randomized sequence of similar amino acids, KLRPKLDLRDRLDLR, were synthesized chemically by the Synpep Co. (Hopkinton, MA). The sequence of each peptide was verified by mass spectrometry and was stored after lyophilization.

Chemical Modification of ApoE. Purified apoE3 was solubilized in phosphate-buffered saline containing 1 mM EDTA. Reductive methylation of its lysine residues was performed following the procedure described previously by Weisgraber et al. (*44*). Solution containing 1 mg/mL apoE was diluted 1.5-fold by the addition of 0.3 M sodium borate, pH 9.0, and kept on ice. One milligram of sodium borohydride was then added to the solution, followed by six additions of 1 μL each of 37% formaldehyde over a 30 min period. In separate reactions, the guanido groups on arginine residues in apoE were modified by cyclohexanedione (*45*). One milligram of apoE in 1 mL of phosphate-buffered saline containing 1 mM EDTA was mixed with 2 mL of 0.15 M 1,2-cyclohexanedione in 0.12 M sodium borate buffer (pH 8.1). The reaction was incubated for 2 h at 35 °C. The methylated apoE and cyclohexanedione-modified apoE were dialyzed exhaustively against phosphate-buffered saline without EDTA and used immediately for experiments.

Oxidation Assay. Human LDL was dialyzed against phosphate-buffered saline without EDTA overnight prior to each experiment. The oxidation assay was performed by mixing 100 μg/mL LDL with apoE or its peptides and 1 μMf $CuSO_4$ in a quartz cuvette and then incubated at 37 °C in a temperature-controlled Biomate 5 spectrophotometer. The absorbance reading at 234 nm was monitored continuously over 300 min.

RESULTS

The antioxidant property of apoE was confirmed by determining the effectiveness of increasing apoE concentration to inhibit conjugated diene formation in an in vitro incubation of LDL with $CuSO_4$. Using a standard assay condition of 100 μg/mL LDL and 1 μM $CuSO_4$, the addition of 10−50 μg/mL human apoE3 progressively delayed conjugated diene formation (Figure 1). However, maximum conjugated diene formation was achieved at all apoE concentrations tested after 300 min of incubation. When the data were analyzed to determine the amount of time required to reach 50% maximum conjugated diene formation, minimal inhibition of LDL oxidation was noted with 10 μg/mL apoE, but significant delay in LDL oxidation was notable at apoE concentrations greater than 20 μg/mL (Figure 1B). The concentrations of apoE required to inhibit LDL oxidation were 2−3 times higher than that reported previously by Miyata and Smith (*38*). The difference between the two studies is not apparent at this time but may be related to differences in the oxidative potential of various LDL preparations and/or the apoE preparation. Nevertheless, both data are consistent with the interpretation that apoE inhibits lipid oxidation by inhibiting the propagation phase of the oxidative process.

Previous studies have shown that apoE contains two independently folded domains connected by a short 26-residue protease-sensitive loop (*46*, *47*). These two major domains of apoE were obtained separately by digesting native apoE with thrombin. The resulting 22-kDa peptide, which contains residues 1−191, and the 10-kDa peptide, which contains residues 216−299 of apoE, were then tested for their ability to inhibit Cu^{2+}-induced LDL oxidation in vitro. Results showed that the N-terminal 22-kDa fragments of both apoE3 and apoE4 were capable of inhibiting LDL oxidation (Figure 2A,B). Interestingly, the inhibition of LDL oxidation

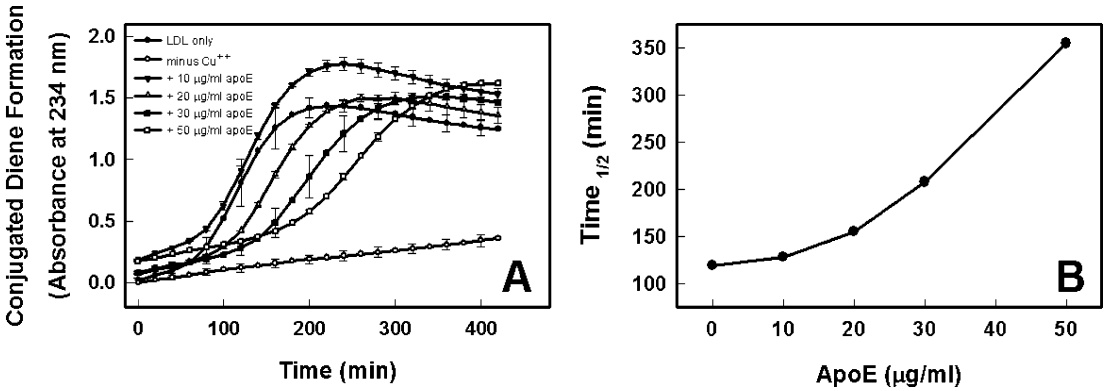

FIGURE 1: Apolipoprotein E inhibition of Cu^{2+}-induced LDL oxidation. (A) Human LDL (100 μg/mL) was incubated in phosphate-buffered saline containing 1 μM $CuSO_4$ in the absence (filled circles) or presence of apoE at 10 (filled triangles), 20 (open triangles), 30 (filled squares), or 50 (open squares) μg/mL recombinant apoE3. Oxidation of LDL was monitored on the basis of conjugated diene formation measured at 234 nm throughout the 400 min incubation period. Human LDL incubated in the absence of $CuSO_4$, and apoE (open circles) serves as the control. Results represent the mean data from three separate determinations ± SEM. Panel B shows the time required to achieve 50% maximal LDL oxidation when incubated with various apoE concentrations.

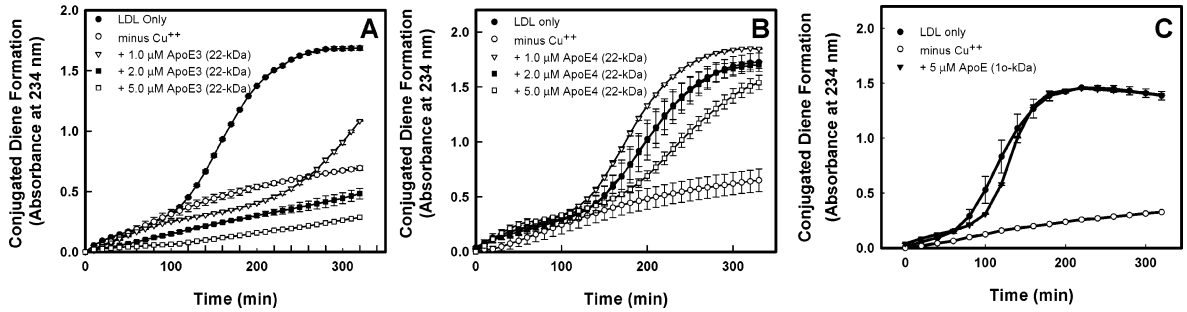

FIGURE 2: Effects of 22- and 10-kDa fragments of thrombin-digested apoE on Cu^{2+}-induced LDL oxidation. Human LDL (100 μg/mL) was incubated in phosphate-buffered saline containing 1 μM $CuSO_4$ in the absence (filled circles) or presence of the 22-kDa peptide from apoE3 (panel A) or apoE4 (panel B) at 1 μM (open triangles), 2 μM (filled squares), or 5 μM (open squares) or with the 10-kDa peptide (filled triangles, panel C) obtained after thrombin digestion of human apoE. Oxidation of LDL was monitored on the basis of conjugated diene formation measured at 234 nm throughout the 300 min incubation period. Human LDL incubated in the absence of $CuSO_4$ and apoE (open circles) serves as the control. Note that, in panel B, the lines for incubation with LDL only and with 2 μM 22-kDa apoE4 peptide overlapped each other. Results represent the mean data from three separate determinations ± SEM.

with the 22-kDa peptide of apoE4 required 5 μM peptide (Figure 2B). In contrast, the 22-kDa peptide of apoE3 was capable of inhibiting LDL oxidation at all concentrations tested, ranging from 1 to 5 μM. The higher antioxidation potency of the 22-kDa peptide of apoE3 than the apoE4 peptide is consistent with previous reports showing that whole apoE3 protein was more efficient than apoE4 in inhibiting LDL oxidation (*38*). In contrast to the data with the N-terminal 22-kDa peptide of apoE3 and apoE4, our data revealed that addition of the C-terminal 10-kDa fragment of apoE had no effect on Cu^{2+}-induced LDL oxidation even at the highest concentration tested (Figure 2C).

The 22-kDa N-terminal domain of apoE contains the basic amino acid cluster between residues 140 and 160 that is important for apoE binding to the LDL receptor and its related family member proteins (*18*, *48*−*50*). Therefore, two additional experiments were performed to determine if the basic amino acid domain in apoE is also responsible for its antioxidant activity. The first set of experiments compared native apoE with reductively methylated apoE and cyclohexanedione-modified apoE in inhibiting LDL oxidation. Both reductive methylation of lysine residues and cyclohexanedione modification of arginine residues in apoE have been shown previously to abolish its receptor binding activities (*44*, *45*). In this experiment, we showed that chemical modification of lysine or arginine residues also abolished the ability of apoE to inhibit Cu^{2+}-induced LDL oxidation. The time required to achieve maximum LDL oxidation in the presence of chemically modified apoE was similar to that observed in the absence of apoE (Figure 3). This observation suggested the possibility that basic amino acid residues required for apoE binding to receptors may also be important for its antioxidant activity in protection against LDL oxidation.

In the second set of experiments to directly examine the importance of the apoE receptor binding domain in prevention of LDL lipid oxidation, we tested the antioxidation potential of a synthetic peptide containing a tandem repeat sequence of the basic amino acid cluster at residues 141−155. This peptide, apoE$(141-155)_2$, has been shown previously to bind LDL receptor (*51*, *52*) and LRP-1 avidly (*30*, *49*). In the current experiment, apoE$(141-155)_2$ was shown to be effective in inhibiting Cu^{2+}-induced LDL oxidation in a concentration-dependent manner (Figure 4A). The monomeric apoE(141−155) peptide that does not interact with receptors but binds to heparan sulfate proteoglycans (*51*, *53*, *54*) was capable of inhibiting LDL oxidation also (Figure 4B). In contrast, a similar peptide containing identical amino acid residues as the apoE(141−155) peptide but with a scrambled sequence was ineffective in inhibiting Cu^{2+}-induced LDL oxidation (Figure 4B). Thus, these results documented that the domain in apoE responsible for its antioxi-

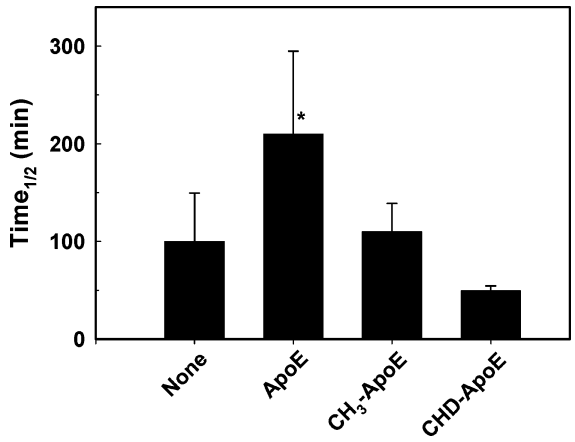

FIGURE 3: Effects of lysine and arginine modifications on apoE inhibition of LDL oxidation. Human LDL (100 μg/mL) was incubated with 1 μM $CuSO_4$ in the absence or presence of native recombinant human apoE3, reductively methylated apoE3 (CH_3-apoE), or cyclohexanedione-modified apoE3 (CHD-apoE) at 50 μg/mL. Conjugated diene formation was monitored over a 300 min period, and the time required to achieve 50% of maximal LDL oxidation was determined. The results represent the mean of three separate determinations ± SEM.

dant activity is identical to the receptor and heparan sulfate proteoglycan binding domain of this protein, residing in the basic amino acid cluster between residues 141 and 155.

DISCUSSION

Increasing experimental evidence has pointed to the role of oxidative stress in pathogenesis of vascular dysfunctions, atherosclerosis, and neurodegenerative disorders such as Alzheimer's disease (*55*−*57*). The susceptibility of individuals to develop these disorders is also modulated by their apoE genotype (*1*, *5*, *10*). The importance of apoE in limiting oxidative stress in the prevention of atherosclerosis was suggested by observations of increased oxidation-specific epitopes in atherosclerotic lesions and the presence of autoantibodies against oxidized LDL in serum of apoE-deficient mice (*58*). The direct evidence documenting apoE protection against oxidative insults was provided by Miyata and Smith (*38*). These investigators showed that physiological concentrations of apoE suppressed Cu^{2+}-induced LDL oxidation and inhibited hydrogen peroxide-induced cytotoxicity in B12 cells.

In addition to the level and presence of apoE, the structure of apoE is also an important determinant in its protection against oxidative damage. Human apoE is polymorphic with three common isoforms, apoE2, apoE3, and apoE4, encoded by their respective alleles, ϵ2, ϵ3, and ϵ4. Elevated levels of hydroxyl radicals were detected in blood and plasma of Alzheimer patients with the ϵ4 genotype in comparison to patients without the ϵ4 allele or in the control group (*41*). These observations suggested that apoE4 is the least effective in antioxidation. This hypothesis is supported by direct experimental data showing that the apoE2 isoform, with cysteine residues at residues 112 and 158, was more effective than apoE3 (with Cys-112 and Arg-158) in protection against lipid oxidation, whereas the apoE4 isoform with arginines at both residues 112 and 158 was the least effective (*38*).

The mechanism and epitope in apoE responsible for its antioxidant activity has not been determined previously. The Miyata and Smith study suggested that apoE may prevent lipid oxidation by its ability to sequester metal cations and preventing their activation of lipid peroxidation (*38*). Copper binding to proteins is predominantly mediated via interaction with cysteinyl side chains through mercaptide bonds (*59*, *60*). Although the effectiveness of the antioxidant activity in apoE2, apoE3, and apoE4 can be correlated with the number of cysteine residues present in each of these apoE isoforms, it must be noted that apoE4 (with no cysteine residue) was also capable of inhibiting lipid oxidation, albeit less effectively as apoE2 and apoE3. Thus, additional domains in apoE, other than the cysteine residues in apoE2 and apoE3, can also participate in its inhibition of LDL oxidation.

Results of the current study showed that chemical modification of arginine and lysine residues abolished the ability of apoE to inhibit Cu^{2+}-induced LDL oxidation, thus suggesting the importance of these basic amino acid residues in the antioxidant activity of apoE. Importantly, a tandem repeat peptide and a monomeric peptide containing the residue 141−155 sequence of apoE were sufficient to inhibit LDL oxidation. Thus, the antioxidant activity of apoE is localized to the same domain as its receptor binding domain. Interestingly, the sequence between residues 141 and 155 is conserved between apoE3 and apoE4, yet the 22-kDa N-terminal fragment of apoE3 was shown to be more effective than the 22-kDa fragment of apoE4 in limiting LDL oxidation. The latter observation is consistent with a previous report that apoE3 was more effective than apoE4 in protection against lipid oxidation (*38*). Therefore, the conformation of this domain within the 22-kDa fragment as well as within

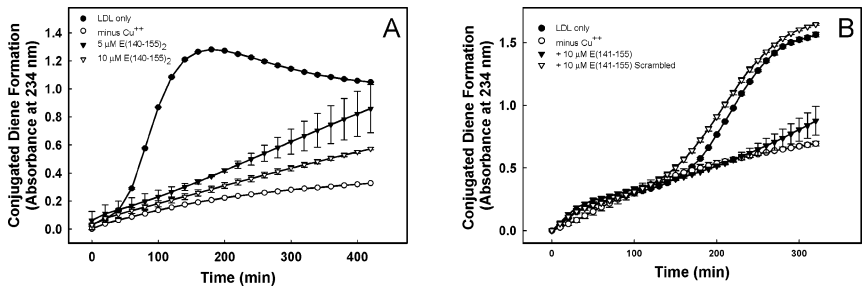

FIGURE 4: Effects of apoE receptor binding domain peptides on Cu^{2+}-induced LDL oxidation. Human LDL (100 μg/mL) was incubated in phosphate-buffered saline containing 1 μM $CuSO_4$ in the absence (filled circles) or presence of the tandem repeat peptide apoE(141−155)$_2$ at 5 μM (filled triangles) or 10 μM (open triangles) concentrations (panel A) or with 10 μM monomeric peptide apoE(141−155) (filled triangles, panel B) or a peptide with scrambled sequence of the same amino acid residues (open triangles, panel B). Oxidation of LDL was monitored on the basis of conjugated diene formation measured at 234 nm throughout the 400 min incubation period. Human LDL incubated in the absence of $CuSO_4$ and apoE (open circles) serves as the control. Results represent the mean data from three separate determinations ± SEM.

native apoE protein is also likely to play an important role in determining its ability to inhibit LDL oxidation. Previous studies have demonstrated the greater propensity of apoE4 and its 22-kDa fragment than apoE3 and its 22-kDa fragment in forming molten globule folding characteristics (*61*). The difference in molten globule formation has been implicated to influence lipid binding properties of apoE in an isoform-specific manner (*61*). The difference in molten globule formation has also been suggested to play a role in the association of apoE with neurodegeneration (*61*). The current data suggest that molten globule formation may modulate the ability of the 141−155 domain to protect LDL against lipid oxidation.

The receptor binding domain peptide did not contain any cysteine residues. Thus, the precise mechanism by which this apoE motif inhibits LDL oxidation remains unclear. The enrichment of positively charged amino acid residues in this domain makes it unlikely for direct interaction with the positively charged copper ion. In view of previous studies demonstrating the ability of positively charged amino acid domains to confer radical scavenger activity (*62*), it is possible that the positively charged receptor binding domain of apoE also serves as a radical scavenger and inhibits LDL oxidation through this mechanism. The ability of apoE to quench the enhanced luminol chemiluminescence even in the presence of EDTA and to inhibit H_2O_2-induced cytotoxicity (*38*) is consistent with this hypothesis. The possibility that apoE may serve as a free radical reservoir may also explain its cytotoxic effects at high concentrations, via the delivery of reactive oxygen species and H_2O_2 to the cells (*38*, *63*).

The current study revealing the identity between receptor binding domain and antioxidant domain in apoE has potential therapeutic implications. A tandem repeat peptide containing the receptor binding domain of apoE has been shown to interact with LDL receptor and facilitate cholesterol transport (*51*, *52*). Another similarly designed peptide has been shown to promote rapid clearance of plasma cholesterol in dyslipidemic mice (*64*). Thus, such apoE peptides may inhibit atherosclerosis via reducing the plasma cholesterol level as well as reducing oxidative stress-induced vascular events.

ACKNOWLEDGMENT

We thank Drs. Catherine A. Reardon and Godfrey S. Getz from the University of Chicago for providing us with the HEK-293 cells that express high levels of recombinant apoE3. We also appreciate the assistance of Dr. Karl H. Weisgraber (Gladstone Institute, San Francisco), who provided us with the 22- and 10-kDa thrombin-digested fragments of apoE for these studies.

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BI0472696