Apolipoprotein E4 Impairs Macrophage Efferocytosis and Potentiates Apoptosis by Accelerating Endoplasmic Reticulum Stress*

Background: Apolipoprotein E4 (apoE4) is associated with inflammatory metabolic diseases. Results: Human APOE4 gene replacement mice displayed elevated tissue inflammation. APOE4 macrophages showed impaired efferocytosis, increased apoptosis, and endoplasmic reticulum stress. Conclusion: ApoE4 structural abnormalities induce ER stress to promote inflammation. Significance: Reducing ER stress and/or apoE4 structure correctors may reduce inflammatory metabolic disease risk in human apoE4 subjects.

Apolipoprotein (apo) E is a major genetic risk factor for a wide spectrum of inflammatory metabolic diseases, including atherosclerosis, diabetes, and Alzheimer disease. This study compared diet-induced adipose tissue inflammation as well as functional properties of macrophages isolated from human APOE3 and APOE4 mice to identify the mechanism responsible for the association between apoE4 and inflammatory metabolic diseases. The initial study confirmed previous reports that APOE4 gene replacement mice were less sensitive than APOE3 mice to diet-induced body weight gain but exhibited hyperinsulinemia, and their adipose tissues were similarly inflamed as those in APOE3 mice. Peritoneal macrophages isolated from APOE4 mice were defective in efferocytosis compared with APOE3 macrophages. Increased cell death was also observed in APOE4 macrophages when stimulated with LPS or oxidized LDL. Western blot analysis of cell lysates revealed that APOE4 macrophages displayed elevated JNK phosphorylation indicative of cell stress even under basal culturing conditions. Significantly higher cell stress due mainly to potentiation of endoplasmic reticulum (ER) stress signaling was also observed in APOE4 macrophages after LPS and oxidized LDL activation. The defect in efferocytosis and elevated apoptosis sensitivity of APOE4 macrophages was ameliorated by treatment with the ER chaperone tauroursodeoxycholic acid. Taken together, these results showed that apoE4 expression causes macrophage dysfunction and promotes apoptosis via ER stress induction. The reduction of ER stress in macrophages may be a viable option to reduce inflammation and inflammation-related metabolic disorders associated with the apoE4 polymorphism.

Apolipoprotein E (apoE) is a 34-kDa protein originally discovered to be associated with several classes of plasma lipoproteins (1). Unlike other apolipoproteins that are expressed only in liver and intestine, apoE is not expressed in intestine but is expressed in liver and other tissues, including the central nervous system, vascular smooth muscle cells, adrenals, macrophages, and adipocytes. Although the main function of apoE has been attributed to cholesterol transport, accumulating evidence indicates that apoE also modulates metabolic disease progression through lipid transport-independent mechanisms (2). The human apoE gene exists with three major polymorphic alleles (ε2, ε3, and ε4) encoding the apoE2, apoE3, and apoE4 isoforms with cysteine/cysteine, cysteine/arginine, and arginine/arginine residues at positions 112 and 158 of the 299-residue protein, respectively. The various apoE isoforms have different metabolic properties with apoE2 transporting lipids to the LDL receptor less efficiently, thereby conferring ε2 carriers with increased risk of type III hyperlipoproteinemia (3). Although apoE3 and apoE4 bind to LDL receptor and other LDL receptor family proteins with similar affinity, apoE3 appears to protect against metabolic disorders, whereas carriers of the ε4 allele have increased risk of Alzheimer disease as well as cardiovascular and metabolic diseases (2–4).

The exact mechanism by which apoE3 retards and apoE4 accelerates the onset and progression of a wide spectrum of metabolic disorders is not completely understood (2, 5). Previous studies have shown that apoE3 may protect against metabolic diseases via its anti-oxidation and anti-inflammatory properties (2, 6), including its attenuation of toll-like receptor signaling and converting the pro-inflammatory M1 macrophages to the anti-inflammatory M2 macrophage phenotype (7, 8). In the vessel wall, apoE3 also induces anti-inflammatory signaling events that limit injury-induced neointimal hyperplasia and hypercholesterolemia-induced atherosclerosis (9–13). In contrast, apoE4 is pro-inflammatory and has been shown to accelerate neurodegeneration by promoting neuronal cell
death (14–16). Paradoxically, apoE4 is associated with lower body mass index compared with apoE3 carriers (17), but apoE4 carriers are more susceptible to diet-induced diabetes and coronary artery disease (4, 18–20). Recent data examining mice in which the endogenous mouse apoe gene has been replaced with the human APOE3 or APOE4 gene also showed the increased susceptibility of APOE4 gene replacement mice to diet-induced atherosclerosis and diabetes despite less adiposity and body weight gain compared with APOE3 mice (21–23). Atherosclerosis enhancement observed in APOE4 mice is at least in part due to the apoE4 expressed in macrophages (24).

The innate immune system plays a major contributory role toward the pathogenesis of cardiovascular and metabolic diseases (25–27). Accordingly, the association between the e4 allele with increased risk of cardiometabolic diseases may be due to abnormal functions of apoE4-expressing macrophages compared with macrophages expressing the normal apoE3 protein. However, how the cysteine-to-arginine substitution in residue 112 converts the anti-inflammatory apoE3 to the pro-inflammatory apoE4 has not been resolved. The goal of this study is to compare functional properties of macrophages isolated from human APOE3 versus APOE4 gene replacement mice and to identify the mechanism responsible for the abnormal functional properties of apoE4-expressing macrophages.

EXPERIMENTAL PROCEDURES

Mice and Dietary Studies—Genetically modified C57BL/6 mice in which the endogenous mouse apoe gene has been replaced with the human APOE3 or APOE4 gene at the same locus (28, 29), hereafter designated as APOE3 and APOE4 mice, were purchased from Taconic (Hudson, NY) and housed in our institutional animal care facility under a controlled environment with free access to food and water. All animal protocols were approved by the University of Cincinnati Institutional Animal Use and Care Committee. The mice were maintained on standard chow (Teklad, Madison, WI) or fed a Western-type high fat, high cholesterol diet containing 21.2% fat by weight (41% by calories) and 0.2% cholesterol (TD88137, Teklad) for 4 weeks. Body weights were measured with a Denver 300K scale, and adiposity was determined using an EchoMRI Whole Body Composition Analyzer (Echo Medical Systems, Houston, TX) and then treated overnight with staurosporine (Sigma) to induce apoptosis. The CFDA-labeled apoptotic cells were then added onto cultures of APOE3 or APOE4 macrophages for a 2-h incubation. The macrophages were washed thoroughly with phosphate-buffered saline, removed using Accutase, and then analyzed by flow cytometry. When indicated, taurosodeoxycholic acid (Sigma) was used at a concentration of 2 mM.

Western Blot Analysis—Peritoneal macrophage lysates were prepared in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, and protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixtures (Sigma). Protein concentration was determined using BCA kit (Pierce) prior to Western blot analysis. Peritoneal macrophage lysates were peroxidase-conjugated secondary antibodies (Cell Signaling) and visualized using enhanced chemiluminescence reagents (Amersham Biosciences). Quantitative measurements were performed using Image J software (National Institutes of Health).

Quantitative PCR Determination of mRNA Levels—Total RNA was extracted from adipose tissues and peritoneal macrophages in culture using TRIzol reagent (Invitrogen). cDNA was made using iScript cDNA synthesis kit (Bio-Rad), and qRT-PCR was performed on iCyclerIQ (Bio-Rad) using sequence-specific primers (Table 1). For analysis of XBP1 splicing, the XBP1 mRNA-amplified product was digested with PstI (New England Biolabs) and then analyzed by electrophoresis on 1.7% agarose gels. For quantification, all qRT-PCR analyses were made using ∆∆Ct measurements against cyclophilin mRNA as control.

Statistics—Values were expressed as mean ± S.E. Student’s t test or analysis of variance for multiple group comparisons was used to determine significance between samples (SigmaPlot version 11.0). A p < 0.05 difference between groups was considered to be statistically significant.

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The abbreviations used are: oxLDL, oxidized LDL; NOS2, inducible nitric-oxide synthase; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; CHOP, C/EBP homologous protein; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; PERK, protein kinase RNA-like ER kinase.

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**RESULTS**

Resistance to Diet-induced Adiposity and Body Weight Gain in APOE4 Mice—Mice in which the endogenous mouse apoE gene has been replaced at the same locus by the human APOE3 or APOE4 gene express human apoE3 and apoE4 in a tissue-specific and physiological manner similar to apoE expression in humans (21). Previously, Maeda and co-workers (21) have reported that the APOE3 and APOE4 mice have similar food intake and absorb dietary lipids to a similar extent, but the APOE4 mice gained significantly less weight than APOE3 mice when challenged with a Western-type high fat, high cholesterol diet. The APOE4 mice were also reported to be more glucose intolerant compared with the APOE3 mice (21). Our initial experiments in characterizing inflammatory responses in APOE3 and APOE4 mice recapitulated these data. In our experiments, 10-week-old male APOE4 mice maintained on a basal chow diet displayed higher body weight compared with 10-week-old male APOE3 mice on a similar diet (Fig. 1A), but analysis of body composition by nuclear magnetic resonance revealed similar adiposity between chow-fed APOE3 and APOE4 mice (Fig. 1B). In contrast, when the mice were fed a Western-type high fat, high cholesterol diet for 4 weeks, significant body weight gain was observed only in the APOE3 mice, and the APOE4 mice did not show body weight difference after 4 weeks of feeding the hypercaloric Western-type diet (Fig. 1A). These results confirmed results reported previously, and our data additionally showed that body fat mass was also significantly lower in Western diet-fed APOE4 mice compared with APOE3 mice (Fig. 1B). The differences in body fat accounted for the differences in body weights between APOE3 and APOE4 mice with no statistically significant differences observed in lean body mass between APOE3 and APOE4 mice fed either chow or Western-type diets (Fig. 1C). No difference in food intake, activity, or fat absorption efficiency was observed between these animals; thus their differences in diet-induced body weight gain and adiposity were due to metabolic differences between the APOE3 and APOE4 mice. These results also established that APOE3 and APOE4 gene replacement mice are suitable models to identify the mechanism underlying the differences between e3 and e4 carriers in sensitivity to diet-induced metabolic diseases despite lower body mass index.

**Similar Fasting Plasma Lipid and Glucose Levels but Differences in Fasting Insulin Levels between APOE3 and APOE4 Mice**—Fasting plasma triglyceride and cholesterol levels were similar between APOE3 and APOE4 mice (Fig. 2, A and B). Both groups of mice responded to 4 weeks of Western diet feeding with elevated fasting plasma cholesterol levels, but no genotype-specific differences were observed (Fig. 2B). When the animals were fed a bolus lipid-rich meal by gastric gavage, a slight but statistically insignificant faster rate of triglyceride-rich lipoprotein clearance was observed in the APOE4 mice compared with APOE3 mice (Fig. 2C). These data are consistent with their similarities in food intake and dietary lipid absorption and the identical ability of apoE3 and apoE4 to interact with LDL receptor family proteins to mediate plasma lipoprotein clearance (33).

Both APOE3 and APOE4 mice also displayed similar fasting glucose levels when maintained on chow diet, and both groups responded to the Western-type diet with a slight but statistically insignificant increase in fasting glucose levels after 4 weeks (Fig. 2D). The lack of significant hyperglycemia after 4 weeks of feeding the Western-type diet was consistent with previous observations that mice are resistant to short term diet-induced hyperglycemia and are similar to humans with onset of hyperinsulinemia preceding the hyperglycemia phenotype. Interestingly, measurements of fasting insulin levels revealed dramatic differences between APOE3 and APOE4 mice, with the APOE4 mice displaying ~3-fold higher fasting insulin levels compared with APOE3 mice even under basal chow diet conditions (Fig. 2E). In fact, fasting insulin levels in chow-fed APOE4 mice were similar to that observed in APOE3 mice fed the Western-type diet (Fig. 2E). Western diet feeding did not promote an additional increase in plasma insulin levels in the APOE4 mice (Fig. 2E). Calculation of homeostasis model assessment index confirmed the insulin resistance phenotype of APOE4 mice under both basal and Western diet conditions, similar to that observed in Western diet-fed APOE3 mice (Fig. 2F). These results are consistent with those reported previously showing impaired glucose tolerance and insulin resistance in APOE4 mice (21).

**Diet-induced Inflammation in Adipose Tissues of Both APOE3 and APOE4 Mice**—Despite the lower adiposity in APOE4 mice compared with APOE3 mice, the impaired glucose tolerance and insulin resistance observed in APOE4 mice suggested that they remain sensitive to diet-induced inflammation. To test this possibility, RNA was isolated from visceral adipose tissues of chow-fed and Western diet-fed APOE3 and APOE4 mice for quantitative PCR analysis. The results showed no difference in F4/80 mRNA levels between chow-fed APOE3 and APOE4 mice (Fig. 3A). Interestingly, despite their differences in adiposity in response to Western diet feeding, both groups of mice responded with similar elevation of F4/80 mRNA levels in their adipose tissues, indicative of macrophage infiltration after feeding the Western-type diet (Fig. 3A). Expression levels of inducible nitric-oxide synthase (NOS2) mRNA were also increased after Western diet feeding, thus indicating the pro-inflammatory states of the adipose tissues in both APOE3 and APOE4 adipose tissues (Fig. 3B). Additionally, both APOE3 and

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**TABLE 1**

| Gene | Common name | Sense primer | Antisense primer |
|------|-------------|--------------|-----------------|
| Cypa | Cyclophilin A | TCATGGCCAGGTTGCTGAGA | CCATCGAGCTTGGCAGTGC |
| Lmr1 | Fat mass | TCTGCTGAACTTGTGGTGGTAG | ACCGCCTGAGTCTGGCAA |
| Nos2 | NOS2 | TCACCCACCTTCTGACCTTGA | TTCCATGGTCACCTCCAACACAAGA |
| Tnf | TNF-α | ATGCCGAGCTGGAGACTGA | ACCGCCCTGAGTCTGGCAA |
| Atf6 | ATF6 | AAGAGAGTAGCAGCGCAGACT | TCTGTTTCCATGGTGGTGTT|
| Xbp1 | XBP1 | AAGACAGGTAGCCAGCAGACT | TCTTCTGGGTAGACCTGGAG |

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**RT-quantitative PCR primer sequences for experimental and reference genes**

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APOE4 mice displayed elevated expression levels of the inflammatory cytokine TNF-α after Western diet feeding (Fig. 3C). Because macrophages are responsible for the TNF-α produced in adipose tissues (34), these data indicated that despite the lower adiposity in APOE4 mice, their adipose tissues as well as their tissue macrophages were as inflamed as those observed in the more obese Western diet-fed APOE3 mice.

ApoE4 Expression Accelerates Inflammation-induced Cell Death and Decreases Efferocytosis in Macrophages—One key factor in modulating tissue inflammation is cell death and their phagocytic clearance by macrophages in a process termed efferocytosis (35, 36). Therefore, the elevated sensitivity of adipose tissue macrophages to diet-induced inflammation despite the lack of increased adiposity in APOE4 mice may be related to
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To test these possibilities, peritoneal macrophages obtained from APOE3 (A) and APOE4 (B) mice were incubated with 50 ng/ml LPS (left panels) or 100 µg/ml oxLDL. Cell death was assessed by flow cytometry based on expression of annexin V and propidium iodide. Representative flow cytometry data are shown in A and B. C shows the mean ± S.D. from four separate experiments each performed with cells obtained from at least three mice per group. Differences as noted were evaluated by Student’s t test.

In the next series of experiments, peritoneal macrophages from C57BL/6 wild type mice were fluorescently labeled with CFDA-SE and then made apoptotic by incubation with staurosporine in serum-free media overnight. The CFDA-SE-labeled apoptotic cells (1 x 10⁶ cells) were then added to freshly plated APOE3 and APOE4 macrophages in 24-well dishes for a 2-h incubation period. Efferocytosis efficiency based on uptake of fluorescently labeled apoptotic cells by APOE3 (open tracings) and APOE4 (shaded tracings) macrophages was assessed by flow cytometry, using cells with or without fluorescent labels as positive and negative markers for gating purposes as indicated. A shows representative results from one experiment, and B shows data collected from four experiments. * denotes difference from APOE3 macrophages at p < 0.01.

Tauroursodeoxycholic Acid Improves APOE4 Macrophage Survival and Function—The relationship between the impairment of efferocytosis and elevated apoptosis sensitivity of APOE4 macrophages with apoE4-induced ER stress was explored by preincubating APOE3 and APOE4 macrophages with tauroursodeoxycholic acid for 24 h prior to the addition of impaired efferocytosis and increased apoptosis susceptibility of APOE4 macrophages compared with APOE3 macrophages.
LPS and oxLDL. Results showed that this ER chaperone significantly improved the efferocytosis efficiency of APOE4 macrophages to levels observed in APOE3 macrophages (Fig. 8). The ER chaperone tauroursodeoxycholic acid also attenuated LPS- and oxLDL-induced apoptosis of APOE4 macrophages to levels observed in APOE3 macrophages. Taken together, these results documented that the impaired macrophage function and elevated susceptibility to agonist-induced apoptosis of APOE4 macrophages are due to apoE4 potentiation of ER stress.

**DISCUSSION**

Previous studies have shown a more robust innate immune response in macrophages from APOE4 mice compared with that observed in APOE3 macrophages, with elevated expression of inducible nitric-oxide synthase, TNF-α, and IL-6 after LPS activation (42). In this study, we showed that, in comparison with APOE3 macrophages, the APOE4 macrophages are also dysfunctional with impaired efferocytosis of apoptotic cells. The APOE4 macrophages are also more sensitive to LPS- and oxLDL-induced cell death. Our data revealed that one consequence of these defects is hyperinsulinemia due to the elevation of adipose tissue inflammation in response to feeding a Western-type high fat, high cholesterol diet, even in the absence of excessive adiposity. Because defective efferocytosis and macrophage apoptosis are also contributing factors to atherosclerosis (43, 44), the impairment of efferocytosis along with increased...
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Sensitivity to oxLDL-induced cell death observed in this study suggested that apoE4 instead of apoE3 expression in macrophages also provides a second hit to exacerbate atherosclerosis. Indeed, exaggerated atherosclerosis has been reported previously in diabetic Ldlr⁻/⁻ mice expressing human APOE4 instead of the APOE3 gene (23). Taken together, our results indicate that apoE4-induced macrophage dysfunction is at least one underlying cause for the elevated risk of atherosclerosis and the prevalence of diabetes, independent of obesity, in human subjects carrying the e4 allele.

Results of our study also showed that expression of apoE4 instead of apoE3 causes macrophage cell stress even under basal conditions as indicated by increased JNK phosphorylation in APOE4 macrophages compared with that observed in APOE3 macrophages. Additionally, apoE4 also provides a second hit in exacerbating cell stress in LPS- and oxLDL-stimulated macrophages. The increase in IRE-1α and PERK phosphorylation along with elevated alternative splicing of XBP1 illustrated that the increased cell stress is due to apoE4 potentiation of ER stress response. Previously, endogenous apoE4 expression has been shown to alter cell functions via induction of ER stress and/or mitochondrial dysfunction in a cell type-specific manner. Specifically, apoE4-expressing astrocytes have elevated ER stress with increased expression of CHOP, ATF4, and XBP1 compared with apoE3-expressing astrocytes (45). In contrast, the apoE4-induced neuronal dysfunction is not related to ER stress but is a result of mitochondrial dysfunctions with reduced mitochondrial respiration activities (46). The ability of the ER chaperone tauroursodeoxycholic acid to improve efferocytosis efficiency and reduce apoptosis of APOE4 macrophages indicates that the apoE4-related macrophage dysfunction is due to its elevation of ER stress. It is important to note that although this study focused on the relationship between apoE4-induced ER stress and macrophage efferocytosis and apoptosis, mitochondrial dysfunction in macrophages has also been reported to promote inflammation (47). Additionally, mitochondrial dysfunction has also been associated with increased risk of atherosclerosis (48) and diabetes (49). Thus, it is possible that apoE4 expression may also induce mitochondrial abnormalities in macrophages and promote inflammation through this additional mechanism. Whether apoE4 expression in macrophages also causes mitochondrial abnormalities independent of ER stress needs to be explored further in future experiments.

Recent data comparing tertiary structures between apoE3 and apoE4 have provided valuable insights into potential underlying mechanisms by which apoE4 causes macrophage dysfunction. X-ray crystallographic analyses revealed the cysteine to arginine substitution at position 112 alters interactions between the N- and C-terminal domains of the protein, resulting in apoE4 having a more compact conformation (50–52) that can also exist in a molten globule state that favors unfolding (53). Thus, the elevated ER stress observed in APOE4 macrophages may be due to the abnormal conformation of apoE4, compared with the normal apoE3, in triggering the ER stress response.

In contrast to human apoE3, apoE in other species contains arginine 112 similar to human apoE4. However, native apoE expressed in macrophages of other species resembles human apoE3 without the compacted domain interaction (54). Interestingly, replacement of threonine 61 in mouse apoE with arginine resulted in a mutant mouse apoE protein with a compact domain interaction similar to human apoE4 but without the molten globule state (55). Interestingly, a recent report from Raffai and co-workers (56) showed that macrophages isolated from mutant mice expressing mouse apoE with Arg-61 did not have elevated ER stress compared with wild type macrophages. The difference between their observations and this study is that the APOE4 mice expressed human apoE4 with both the compact domain interaction as well as a molten globule state that is lacking in mouse Arg-61 mutant apoE. Additionally, macrophages used in the study by Raffai and co-workers (56) were obtained from hypomorphic mutant mice with dramatically reduced apoE expression, although this study used APOE4 gene replacement mice expressing physiological levels of apoE. The normal level of apoE expression together with the molten globule state of apoE4 may account for the elevated ER stress observed in APOE4 macrophages.

Efferocytosis efficiency and apoptosis sensitivity were not reported in the macrophages of mouse Arg-61 mutant mice. Nevertheless, the ability of tauroursodeoxycholic acid to improve efferocytosis function and to reduce apoptosis sensitivity of APOE4 macrophages indicates that these abnormalities are attributable to apoE4-induced ER stress. It is important to note, however, that even in the absence of elevated ER stress, the macrophages from hypomorphic Arg-61 mutant mice were also more inflamed, and the mice also displayed accelerated atherosclerosis in comparison with wild type mice (56). Thus, based on the results of our study together with reports from Raffai and co-workers (56), we propose that apoE4 may promote macrophage inflammation and dysfunction in accelerating atherosclerosis through multiple mechanisms. We speculate that expression of the molten globule state of apoE4 promotes ER stress leading to impaired efferocytosis and increased apoptosis sensitivity while the compacted domain interaction in apoE4 also contributes to elevated inflammation. Whether the molten globule- and ER stress-dependent mechanism is similar to the apoE4-induced mitochondrial dysfunction in neurons requires additional studies.

In summary, this study shows that endogenous apoE4 expression in macrophages impairs efferocytosis and provides a second hit to accentuate agonist-induced apoptosis. The mechanism is related to apoE4 potentiating ER stress. Because abnormal macrophage functions and inflammation are important contributors to a wide spectrum of metabolic diseases, including atherosclerosis, diabetes, and Alzheimer disease, the association between apoE4 with the same spectrum of metabolic diseases may be due in large part to its role in promoting macrophage dysfunction. Moreover, apoE4 structure correctors that have been proposed for Alzheimer disease modulation (57, 58) may also be tested for efficacy in reducing the risk of atherosclerosis, diabetes, and other inflammatory diseases associated with the e4 genotype.
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