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The redox status of cysteine thiol residues of apolipoprotein E impacts on its lipid interactions

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Abstract: Redox-mediated modulation of cysteine (Cys) thiols has roles in various pathophysiological functions. We recently found that formation of disulfide-linked complexes of apolipoprotein (apo) E3 prevented apoE3 from irreversible oxidation. In this report, the influence of modification of Cys thiols in apoE2 and apoE3 on interactions with lipids was investigated. The apoE redox status was examined by a band-shift assay using a maleimide compound, and interactions with lipids were evaluated by a kinetic assay using dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and non-denaturing polyacrylamide gel electrophoresis. A reduction in DMPC clearance activity of apoE2 and apoE3 but not apoE4 was observed. Although hydrogen peroxide-induced oxidation decreased the clearance activity of the isoforms, apoE2 showed the greatest residual activity. Both Cys thiol masking and dimerization decreased the activity of apoE2 and apoE3 but not apoE4. In contrast, apoAII preincubation markedly increased the activity (apoE2 > apoE3 > apoE4), in accordance with the formation of apoE-AII and apoAII-E2-AII complexes. ApoAII preincubation also reduced the particle size of apoE-DMPC liposome complexes, especially for apoE2. Redox-mediated modification of Cys thiols of apoE2 or apoE3, especially disulfide bond formation with apoAII, affects lipid metabolism and consequently may be responsible for the diverse isoform specificity of apoE.

Keywords: Alzheimer's disease; apoAII-E2-AII complex; apoE-AII complex; cardiovascular disease; disulfide bond; DMPC clearance.

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Introduction

Human apolipoprotein (apo) E, a plasma protein consisting of 299 amino acids with a molecular mass of 35 kDa, participates in both plasma and cerebral lipid transport and metabolism as a component of lipoproteins (Pitas et al., 1987; Mahley, 1988). ApoE has three major isoforms, apoE2, apoE3 and apoE4, which are produced by three independent alleles at a single genetic locus (Mahley, 1988).

The cysteine (Cys)-arginine (Arg) interchange at residues 112 and/or 158 is a major characteristic that distinguishes the three isoforms (E2, Cys112/Cys158; E3, Cys112/Arg158; E4, Arg112/Arg158). Although these differences in amino acids are quite simple, this interchange impacts strongly on the structure and function of the isoforms and has pathological consequences (Huang and Mahley, 2014; Mahley, 2016). A large number of studies have suggested that apoE and its polymorphism are closely involved in the pathogenesis of various diseases, such as Alzheimer's disease (AD) and cardiovascular disease (CVD) (Mahley, 2016). It is well known that apoE4 is an identified risk factor of sporadic late-onset AD (Corder et al., 1993) and apoE2 is a causative factor of type III hyperlipoproteinemia (Innerarity et al., 1986). Previous studies have demonstrated that a homodimer and heterodimers [apoE-AII (Weisgraber and Mahley, 1978) and apoAII-E2-AII complexes (Tozuka et al., 1992), respectively] generated through disulfide bond formation via the Cys thiols of apoE2 and apoE3 have an impact on the synthesis of lipoproteins (Gong et al., 2002; Minagawa et al., 2009). Thus, understanding the role of isoform-specific differences in the development of disorders may provide insight into the precise pathophysiology of apoE.

Cys thiol, a highly reactive residue in proteins, is one of the main targets of post-translational redox-mediated modifications (Thomas et al., 1995; Cooper et al, 2002). It has been suggested that modulation of Cys thiols participates in various physiological functions (e.g. regulatory switches in signal pathways, modulation of transcription and protein expression, maturation of proteins, control of cell death, and protection from oxidative stress) and the pathogenesis of various diseases (Go and Jones, 2013; Groitl and Jakob, 2014; Ulrich and Jakob, 2019).

Redox-mediated alterations of Cys thiols can be categorized as either reversible or irreversible. Irreversible modifications to sulfonic acid (SO_3H) cause a loss of function leading to the degradation of the modified protein (Mera et al., 2005; Kawakami et al., 2006). In contrast, reversible modifications, represented by disulfide bond formation, do not cause irreversible detrimental changes to the protein (Anraku et al., 2013).

It is hypothesized that the redox status of the Cys thiol in apoE affects the physiological functions of apoE and the pathology of related diseases. We have previously focused on disulfide-linked complexes, which correspond to the reversible oxidized forms of apoE2 and apoE3. Although many studies have focused on apoE4, investigation of the other two isoforms and their redox status is necessary to uncover the pathophysiology of apoE. We recently found that the apoE3-AII complex is beneficial for maintaining the apoE3 redox status by preventing the change to the irreversible oxidized form (Yamauchi et al., 2019). We believe that this finding provides a basis for understanding the pathological mechanisms of apoE-related diseases caused by the contribution of oxidative stress and its associated functional loss of apoE.

In this study, we examined the fundamental differences in the redox status of the Cys thiols of apoE2 and apoE3 by using our previously devised band-shift assay and the photocleavable maleimide-conjugated polyethylene glycol (PM). On the basis of the obtained results, we also investigated the influence of the redox status of the Cys thiols in apoE, especially focusing on their reversible oxidized forms, on the clearance of phospholipid liposomes and lipoprotein formation to clarify the influence of the redox status on lipid metabolism under oxidative stress conditions.

Results

Redox status of Cys thiol residues of apoE

As previously described (Yamauchi et al., 2017), a 40-kDa band corresponding to a single PM adduct [apoE-(PM)] was observed as a consequence of the binding of one PM molecule with one Cys thiol of both recombinant apoE2 and apoE3. When fresh recombinant apoE2 was labeled with PM, a 45-kDa band corresponding to apoE2-$(PM)_2$ was also observed by conjugation of PM to two Cys thiols. In addition, homodimer-(PM) [$(apoE2)_2$-$(PM)_1$] and homodimer-$(PM)_2$ [$(apoE2)_2$-$(PM)_2$] were detected at the high molecular region around the apoE2 homodimer (Figure 1A). When serum samples containing apoE3/E3 or apoE2/E3 were used, a 40-kDa band corresponding to one PM adduct was observed after PM labeling, which is similar to the results for recombinant apoE3. In addition, a 43-kDa band representing the apoE-AII complex was detected in the proximity of the band from one PM adduct. In contrast, unlike with recombinant apoE2, the band for apoE2-$(PM)_2$ was not observed after PM labeling of the intact serum containing apoE2/E3. However, when the serum was pretreated with tris (2-carboxyethyl) phosphine (TCEP), a specific reducing agent for Cys thiols, apoE2-$(PM)_2$ could be detected between the two bands of the apoE-AII and apoAII-E2-AII complexes (58 kDa); however, the band for the homodimer adduct with two PM molecules was not as clear when compared with the results for recombinant apoE2. The PM labeling of serum containing TCEP-treated apoE2 also generated several bands from the reaction with either of or both the anti-apoE and anti-apoAII antibodies (Figure 1B).

Influence of hydrogen peroxide oxidation on the Cys thiol residues of apoE

To investigate the influence of oxidative stress on the Cys thiol residues of apoE, we incubated TCEP-treated recombinant apoE2 or apoE3 with serially diluted hydrogen peroxide (H_2O_2). When recombinant apoE2 was oxidized with H_2O_2, the intensities of bands corresponding to the PM adducts, apoE2-$(PM)_2$ and $(apoE2)_2$-$(PM)_2$, were markedly decreased. In particular, the band corresponding to $(apoE2)_2$-$(PM)_2$ had completely vanished under oxidizing conditions with 0.3 mM H_2O_2. In contrast, the single PM adducts of apoE2 [apoE2-$(PM)_1$ and $(apoE2)_2$-$(PM)_1$], the non-reduced form (nr)-apoE2, detected at the position of monomeric apoE2, and the apoE2-homodimer showed no particular trend in the fluctuations of their band intensities. The redox changes of recombinant apoE3 under oxidizing conditions were simpler than those of recombinant apoE2; that is, H_2O_2 oxidation decreased the apoE3-homodimer and increased nr-apoE3 in a dose-dependent manner. Similar to apoE2-$(PM)_1$, alterations to apoE3-$(PM)_1$ showed no particular trend (Figure 2A,B).

Influence of the redox status of apoE on DMPC clearance

We examined the influence of the apoE redox status on the solubilization of liposome particles using a dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) clearance assay. Briefly, we pre-incubated each recombinant apoE

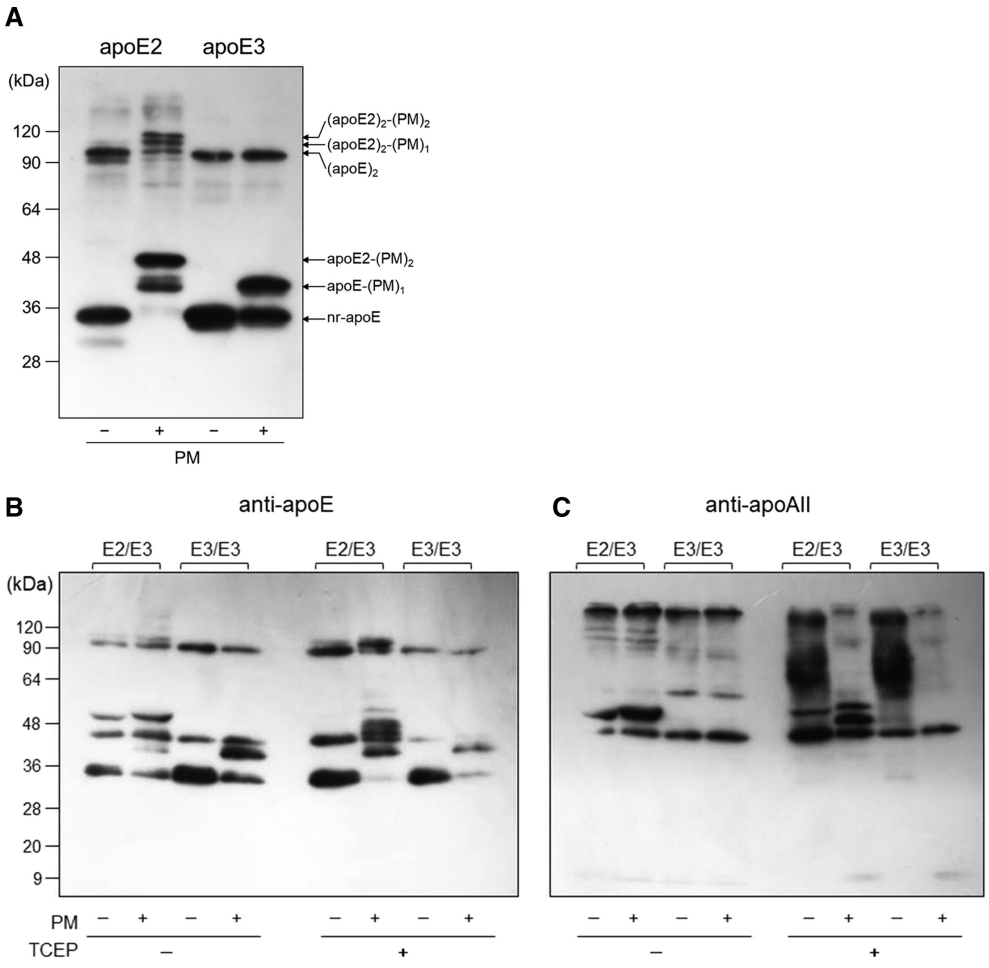

Figure 1: Analysis of the redox status of apoE2 and apoE3 by the band-shift assay using PM.
Recombinant apoE2 and apoE3 (10 mg/l) (A), and pooled serum with the apoE phenotype apoE3/E3 (17 mg/l) and apoE2/E3 (20 mg/l), pretreated with TCEP at a final concentration of 2.5 mM for 30 min (B), were incubated with PM followed by the band-shift assay. In accordance with our previous study (Yamauchi et al., 2017), the monomeric form (35-kDa unlabeled apoE) remaining in the presence of PM was termed the non-reduced form of apoE (nr-apoE).

isoform with or without TCEP at a final concentration of 2.5 mM for 30 min at 25°C, or subjected the samples to oxidation with H_2O_2. We then added each of these mixtures into a solution of DMPC multilamellar vesicles (mLV) and monitored changes in turbidity at 325 nm (Figure 3A–C). Without pre-incubation with TCEP, apoE4 had a greater ability to clear DMPC mLV when compared with that of apoE2 (1.8-fold) and apoE3 (4.7-fold). TCEP treatment significantly enhanced the clearance activity of apoE2 and apoE3 (approximately 1.8-fold when compared with the TCEP-untreated compounds), but had no effect on the clearance activity of apoE4. H_2O_2 oxidation reduced the clearance activity of all the apoE isoforms in a dose-dependent manner. However, the residual activity of apoE2 under oxidizing conditions with both 0.01 and 0.3 mM H_2O_2 was significantly greater than for apoE3 and apoE4 (Supplementary Figure 1). The *p*-values for the between-group relative-clearance comparisons are shown in Supplementary Table 1.

Influence of Cys thiol modification of apoE on DMPC clearance

First, to investigate the influence of Cys thiol masking on DMPC clearance, we incubated TCEP-treated recombinant apoE isoforms with N-ethylmaleimide, followed by the clearance assay (Figure 4A–C). The activity of apoE2 and apoE3 to clear DMPC mLV after incubation with 0.03 mM N-ethylmaleimide decreased by approximately 40% and 27%, respectively, when compared with non-N-ethylmaleimide-treated apoE2 and apoE3 (controls). The residual activity of apoE2 was approximately 3.3-fold greater than that of apoE3. Incubation with 0.1 mM N-ethylmaleimide

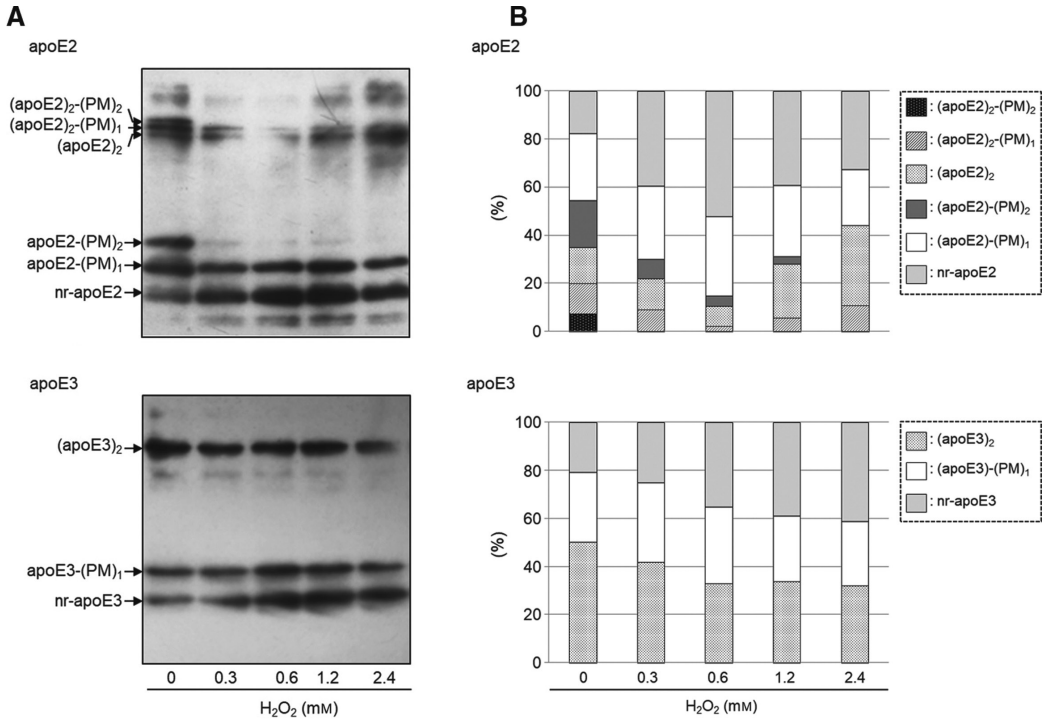

Figure 2: Influence of H_2O_2 oxidation on the Cys thiols of apoE2 and apoE3.
Recombinant apoE2 and apoE3 (10 mg/l) were treated with TCEP at a final concentration of 2.5 mM for 30 min at 25°C and subsequently incubated with serially diluted H_2O_2 at final concentrations of 0, 0.3, 0.6, 1.2 and 2.4 mM for 15 min at 37°C followed by the band-shift assay (A). The band intensity of each fraction was determined by densitometry using Image J 1.45. The values are the mean obtained from duplicate measurements of each sample (B).

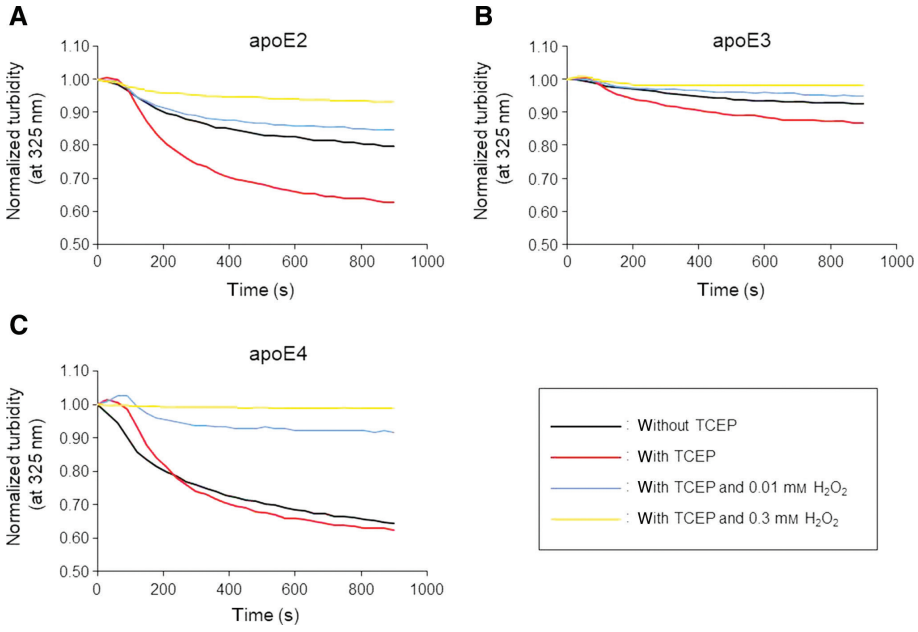

Figure 3: Influence of the redox status of apoE on its DMPC clearance activity.
Recombinant apoE2, apoE3 and apoE4 (10 mg/l) with or without treatment with TCEP at a final concentration of 2.5 mM for 30 min at 25°C were incubated with or without H_2O_2 at final concentrations of 0.01 and 0.3 mM for 15 min at 37°C. Each of these mixtures was added into an equal volume of DMPC mLV solution to a final DMPC:apoE molar ratio of approximately 500:1, and changes in turbidity were monitored at 325 nm. We performed three independent experiments. Turbidity values were normalized at time zero. Time-courses for DMPC mLV clearance by apoE2 (A), apoE3 (B) and apoE4 (C) were expressed using mean values from three independent experiments. The results of the statistical analysis are summarized in Supplementary Figure 1 and Supplementary Table 1.

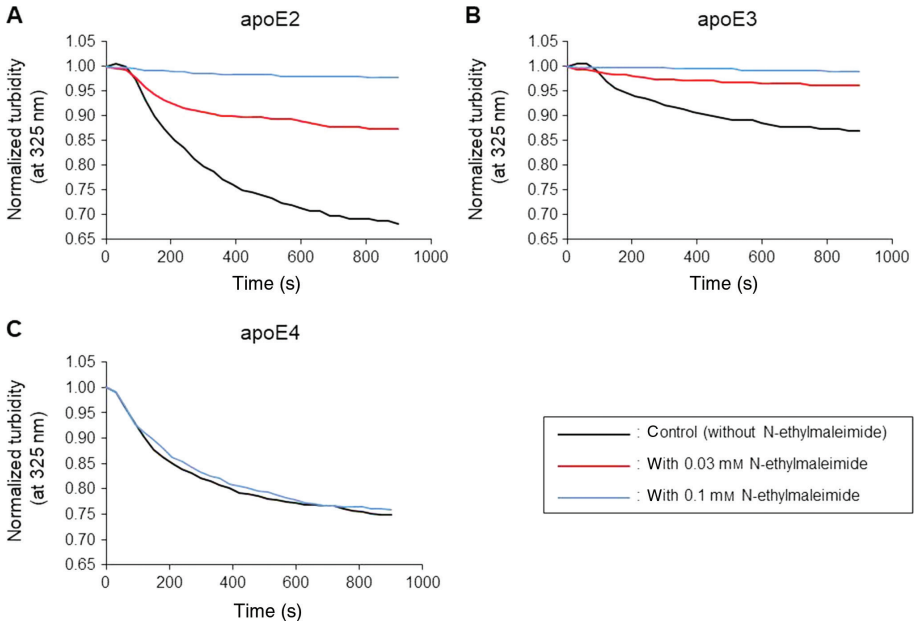

Figure 4: Influence of Cys thiol masking of apoE on its DMPC clearance activity.
Recombinant apoE2, apoE3 and apoE4 (10 mg/l) were treated with TCEP at a final concentration of 2.5 mM for 30 min at 25°C and were subsequently incubated with N-ethylmaleimide at final concentrations of 0 (control), 0.03 and 0.1 mM for 30 min at 37°C followed by the clearance assay. Turbidity values were normalized at time zero. Time-courses for DMPC mLV clearance by apoE2 (A), apoE3 (B) and apoE4 (C) were expressed using mean values from three independent experiments. The results of the statistical analysis are summarized in Supplementary Figure 2.

almost completely abolished the clearance activity of both apoE2 and apoE3. In contrast, N-ethylmaleimide had no impact on the DMPC clearance by apoE4 (Supplementary Figure 2).

Next, to investigate the influence of disulfide bond formation on DMPC clearance, we incubated TCEP-treated recombinant apoE isoforms with N,N,N′,N′-tetramethylazodicarboxamide (diamide), a thiol-oxidizing agent. As previously demonstrated (Yamauchi et al., 2017), diamide treatment induces homodimer formation of apoE2 and apoE3, but not apoE4, by facilitating disulfide bond formation (Supplementary Figure 3). In accordance with homodimer formation, diamide-treated apoE2 and apoE3, but not apoE4, showed a decrease in the ability to clear DMPC mLV (Figure 5A–C). The clearance activities of apoE2 and apoE3 treated with 0.3 mM diamide were reduced by approximately 20% when compared with controls. The residual clearance activity of apoE2 was approximately 1.9-fold greater than that of apoE3. Pretreatment with 1.0 mM diamide almost completely abolished the clearance activity of apoE2 and apoE3. In contrast, diamide had no impact on the DMPC clearance of apoE4 (Supplementary Figure 4).

Furthermore, to investigate the influence of the interaction between the Cys thiols of apoE and apoAII on DMPC clearance, we incubated TCEP-treated recombinant apoE isoforms with recombinant apoAII for 24 h at 37°C. In addition to control-1 (treatment with TCEP only), we prepared apoE isoforms that were incubated in the absence of apoAII for 24 h at 37°C as control-2 to exclude the influence of long-term incubation on the Cys thiol of apoE. As previously demonstrated (Yamauchi et al., 2000), apoAII incubation caused apoE2 and apoE3, but not apoE4, to form an apoE-AII complex. In addition, the formation of an apoAII-E2-AII complex was induced by the incubation of apoE2 with apoAII (Supplementary Figure 5). Although 24-h incubation in the absence of apoAII (control-2) decreased the ability of the apoE isoforms to clear DMPC mLV (approximately 19% of control-1), in accordance with the formation of disulfide-linked complexes, the clearance activities of apoE2 and apoE3, but not apoE4, were dose-dependently augmented by apoAII incubation (Figure 6A–C). Compared with control-2, the clearance activities of apoE2 and apoE3 increased by 1.7-fold and 2.5-fold in the presence of 30 mg/l apoAII, and increased by 7.7-fold and 7.5-fold in the presence of 300 mg/l apoAII, respectively. The clearance activity of apoE2 in the presence of 30 and 300 mg/l apoAII was 1.4-fold and 2.2-fold, respectively, which was greater than that of apoE3. ApoAII incubation also had an effect on apoE4; however, the observed effect was not significant when compared with that of apoE2 and apoE3 (Supplementary Figure 6).

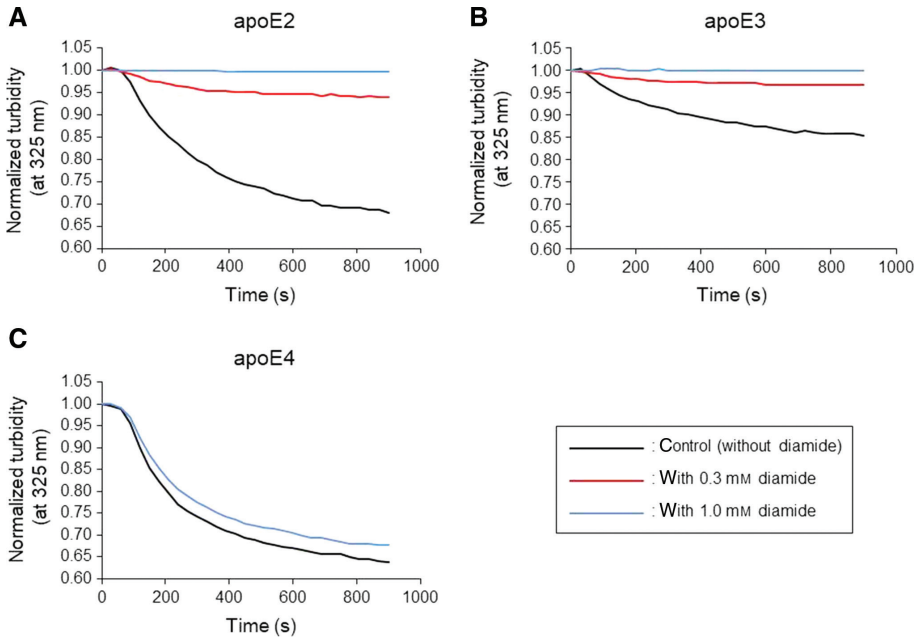

Figure 5: Influence of disulfide bond formation of apoE on its DMPC clearance activity.
Recombinant apoE2, apoE3 and apoE4 (10 mg/l) were treated with TCEP at a final concentration of 2.5 mM for 30 min at 25°C and were subsequently incubated with diamide at final concentrations of 0 (control), 0.3 and 1.0 mM for 30 min at 37°C followed by the clearance assay. Turbidity values were normalized at time zero. Time-courses for DMPC mLV clearance by apoE2 (A), apoE3 (B) and apoE4 (C) were expressed using mean values from three independent experiments. The results of the statistical analysis are summarized in Supplementary Figure 4.

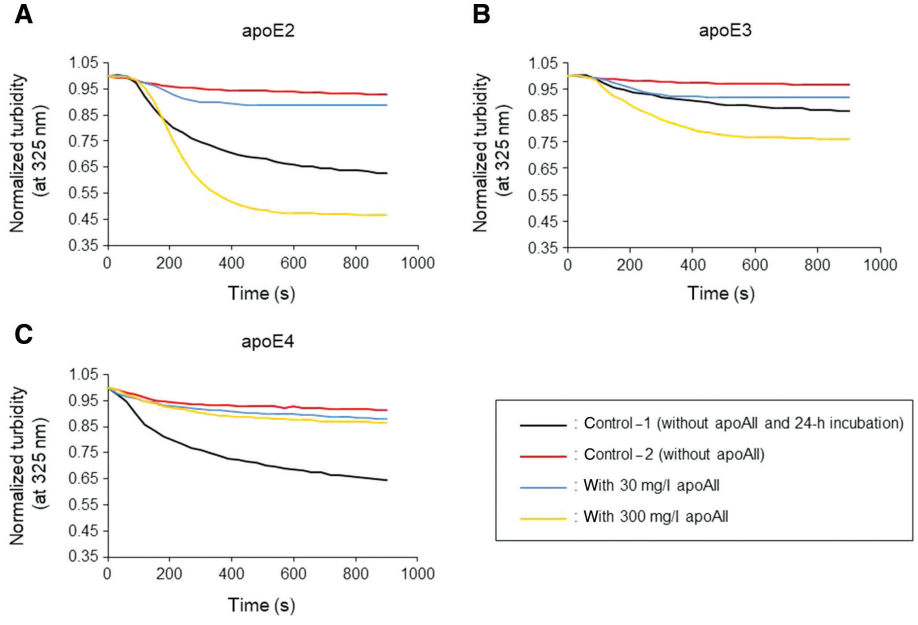

Figure 6: Influence of apoAII on the DMPC clearance activity of apoE.
Recombinant apoE2, apoE3 and apoE4 (10 mg/l) were treated with TCEP at a final concentration of 2.5 mM for 30 min at 25°C and were subsequently incubated with recombinant apoAII at final concentrations of 0 (control-2), 30 and 300 mg/l for 24 h at 37°C followed by the clearance assay. Control-1 was the apoE isoforms treated only with TCEP. Turbidity values were normalized at time zero. Time-courses for DMPC mLV clearance by apoE2 (A), apoE3 (B) and apoE4 (C) were expressed using mean values from three independent experiments. The results of the statistical analysis are summarized in Supplementary Figure 6.

Influence of apoAII on the formation of apoE-DMPC liposome complexes

We examined the influence of apoAII on the formation of an apoE-containing lipoprotein, using samples prepared by incubating each apoE isoform with DMPC mLV in the absence or presence of apoAII, by non-denaturing polyacrylamide gel electrophoresis (PAGE). The addition of apoAII resulted in apoE-DMPC liposome complexes (apoE-Lp) with smaller particle sizes, regardless of the apoE isoform. However, the particle sizes of apoE-Lp that showed apoAII-immunoreactivity were different between the apoE isoforms. The particle sizes of the main apoE2-Lp, which showed immunoreactivity against both apoE and apoAII, were smaller (8.1–10.4 nm) than those of apoE3-Lp (10.4–12.2 nm). In the presence of apoAII, apoE4-Lp had approximately the same particle sizes as apoE2-LP, but did not show apoAII immunoreactivity (the particle sizes of the main apoAII-Lp ranged from 7.5 to 8.1 nm, Figure 7).

Discussion

We hypothesized previously that the thiol groups of two Cys residues of apoE2 could not be conjugated with PM simultaneously because the site would be irreversibly modified (Yamauchi et al., 2017). However, the present study showed that if a dissolved recombinant apoE2 molecule was immediately supplied to a PM solution and incubated, two PM adducts of an apoE2 monomer [apoE2-$(PM)_2$] and one or two PM adducts of an apoE2 homodimer [$(apoE2)_2$-$(PM)_1$, and $(apoE2)_2$-$(PM)_2$] were apparently induced by the reaction of both the Cys thiols of apoE2 with PM. However, 1 day after dissolution, we could not detect these two PM adducts of recombinant apoE2 (Supplementary Figure 7). These findings suggest that the majority of the Cys thiols of a just-dissolved recombinant apoE2 molecule may be reversibly modified and may be easily irreversibly oxidized. In addition, the PM reactivity of the Cys thiols of recombinant apoE2 and apoE3 may intrinsically be low because part of the recombinant protein would already be oxidized through the process of bacterial production and/or purification, as described previously (Jolivalt et al., 2000). The PM reactivity of serum with the apoE phenotype E3/E3 was approximately the same as that of recombinant apoE3, except for the presence of the apoE-AII complex. In contrast, unlike recombinant apoE2, serum containing intact apoE2 did not form PM adducts through the conjugation of two Cys thiols with PM, even if fresh serum was supplied for analysis. However, PM incubation with TCEP-pretreated serum yielded these adducts and the intensity of the band for the apoAII-E2-AII complex was prominently reduced, suggesting that one of the two Cys thiols of serum apoE2 is easily reversibly oxidized and thus apoE2 mainly forms an apoAII-E2-AII complex. Under non-reducing conditions, apoAII may have higher affinity for apoE2 than for maleimide, thereby preventing PM

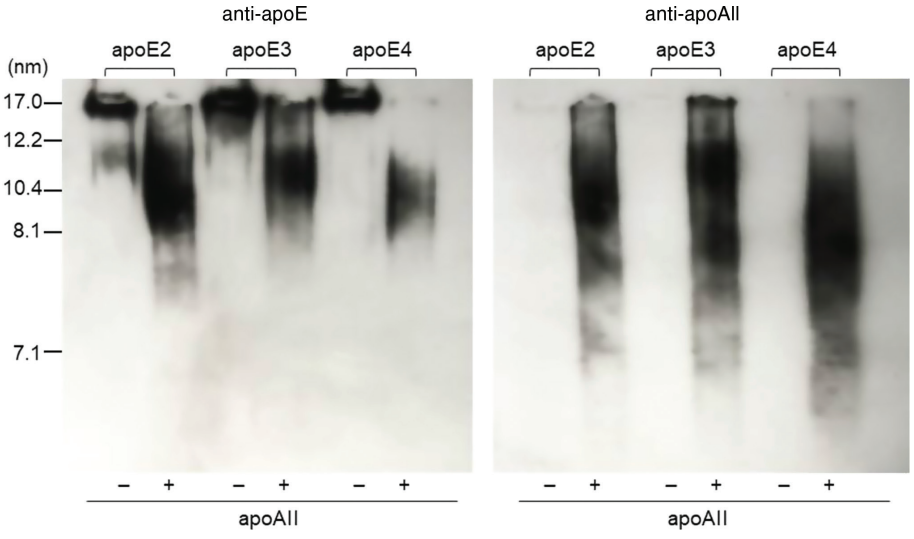

Figure 7: Influence of apoAII on the formation of apoE-DMPC liposome complexes.
ApoE-DMPC liposome complexes, prepared as described in the Materials and methods section, were separated by non-denaturing PAGE using a 4–12% gradient gel, followed by detection with anti-apoE and anti-apoAII antibodies. The Stokes diameters of the complexes were determined from a calibration curve, which was constructed by plotting mobility against the corresponding Stokes diameters of a commercial size marker.

from binding to the Cys thiols of apoE2, particularly to one of the Cys thiols. Pretreatment with TCEP facilitated the substitution of apoAII for maleimide and allowed PM to bind with the Cys thiols of apoE2. Hence, we could detect several bands corresponding to complexes reacted with either or both of the anti-apoE and anti-apoAII antibodies. Use of serum containing an apoE2 homozygote would allow this substitution to be observed more clearly and allow the detection of apoE2 in which both Cys thiols are in the reduced form, which may be present at low concentrations in the serum.

Reversible modification of the Cys thiols of proteins, represented by disulfide bond formation, is well known to protect proteins from irreversible detrimental changes (Mera et al., 2005; Kawakami et al., 2006). We have also reported that disulfide bond formation in apoE3 is beneficial for maintaining the redox equilibrium of apoE3 by preventing irreversible oxidization (e.g. sulfination or sulfonation), in response to oxidative stress (Yamauchi et al., 2019). The fact that H_2O_2-mediated oxidation markedly reduced the presence of the two PM adducts of apoE2, but had no prominent influence on the single adducts of apoE2 and apoE3, led us to the notion that Cys158 of apoE2 is more susceptible to oxidation than Cys112 of apoE2 and apoE3. Cys112 of apoE2 may be protected from further oxidation by the formation of disulfide-linked complexes via Cys158 of one or more apoE2 molecules. However, one limitation of our study was the use of H_2O_2 at non-physiological (i.e. high) concentrations. Hence, further experiments under near-physiological conditions are necessary to confirm the pathophysiological effects of oxidative stress. In addition, subsequent studies will be necessary to clarify the differences in susceptibility to oxidation between Cys158 and Cys112 of apoE2 and their detailed redox status by means of liquid chromatography-mass spectrometry (LC-MS) analysis.

To gain insight into the functional role of the apoE redox status, we evaluated the influence of oxidation on apoE interaction with lipids using a DMPC clearance assay. Garai et al. (2011) have demonstrated previously that dissociation of the self-assembled apoE is required for binding with DMPC mLV, and that the dissociation rate of apoE4 oligomers is faster when compared with that of apoE2 oligomers. Our data also showed that apoE4 was more able to efficiently clear DMPC mLV when compared with that of the other isoforms. The nature of apoE4, which contains no Cys residues and exists only in a monomeric form, may favor binding and clearance of DMPC mLV. The fact that TCEP treatment significantly enhanced the clearance activity of apoE2 and apoE3, but had no effect on that of apoE4, strongly supports this notion. In addition, the differences in the clearance activity of apoE2 and apoE3, regardless of TCEP treatment, led us to assume that the number of Cys residues also has an impact on the clearance activity. Our finding that apoE2 had a high clearance activity even after H_2O_2-mediated oxidation can also be attributed to the effect of the number of Cys residues. It is not clear why apoE3 showed the lowest clearance activity, regardless of TCEP treatment. Nonetheless, one possible reason is that apoE3 may be more susceptible to self-association when compared with that of apoE2 and apoE4. From this point of view, it appears that Cys158 may contribute to the dissociation of self-assembled apoE2 and the binding of apoE2 with lipids.

H_2O_2 oxidation is not specific to Cys thiols, and thus also influences the clearance activity of apoE4, which does not contain a Cys residue. Hence, we set out to modify the Cys thiols of apoE2 and apoE3 with N-ethylmaleimide, diamide and apoAII, as an alternative for specifically oxidizing the thiols. Iodoacetamide is generally used as a Cys thiol-alkylating reagent but shows cross reactivity with nucleophilic amino acids such as Lys and His (Boutureira and Bernardes, 2015). Thus, in the present study, we selected maleimide as a masking agent. Although maleimide treatment is simply a chemical modification, the result of such a treatment could aid our understanding of the biological effects of Cys thiol reversible modifications, such as S-sulfenylation, S-nitrosylation, S-glutathiolation and S-cysteinylation. Maleimide treatment markedly decreased the clearance activity of apoE2 and apoE3, but not apoE4, providing compelling evidence that the Cys thiol is involved in the interaction with lipids. The fact that a large amount of dimer, caused by diamide treatment, prominently impaired clearance activity also supports this notion. Considering these findings, we expected that disulfide bond formation between apoE and apoAII would also cause a reduction in clearance activity. Interestingly, contrary to our expectations, the clearance activity of apoE2 and apoE3, particularly apoE2, increased considerably after the formation of apoE-AII or apoAII-E2-AII complexes. Alternatively, these findings are consistent with a previous finding that the apoE-AII complex preferentially distributes with high-density lipoprotein (HDL), a phospholipid-rich lipoprotein (Weisgraber, 1990). Natural oxidation (after a 24-h incubation in the absence of apoAII) impaired the clearance activity of all the apoE isoforms; however, the positive effects of the apoE-AII and apoAII-E2-AII complexes may greatly exceed the adverse effect of natural oxidation. ApoAII is a highly lipophilic apolipoprotein (Bassett et al., 2012) and functions by clearing DMPC liposomes (Jayaraman et al., 2005). Thus, we have to take into account the effect of unbound (free)

apoAII. However, a mixture of apoE4 and apoAII showed only a slight increase in clearance activity. Overall, our data suggest that the formation of apoE-AII and apoAII-E2-AII complexes may enhance the basal clearance activity levels of apoE2, apoE3 and apoAII.

Non-denaturing PAGE analysis indicated that the presence of apoAII might also have an advantage in the formation of compact lipoprotein particles, such as HDL. Based on previous evidence that apoAII exists predominantly in HDL (Blanco-Vaca et al., 2001) and the apoE-AII complex prefers HDL to low-density lipoproteins (Weisgraber, 1990), the present finding is certainly reasonable. Our finding that the particle sizes of apoE2-Lp were smaller than those of apoE3-Lp led us to the idea that Cys158 or the apoAII-E2-AII complex may be involved in the formation of apoE-containing lipoprotein particles. Of interest, apoAII also reduced the particle sizes of apoE4-Lp, although apoE4 cannot form a disulfide-linked complex with apoAII. The large particles observed for all apoE isoforms in the absence of apoAII were considered to be complexes consisting of naturally oxidized apoE and DMPC liposomes, because the 24-h incubation caused apoE oxidative stress. Taken together, these results indicate that, as previously proposed (Yamauchi et al., 2019), apoAII may play a critical role in the formation of apoE2- or apoE3-containing lipoproteins by regulating the redox status through disulfide bond formation. Additionally, we speculate that apoAII may also assist in the formation of apoE-containing lipoproteins, irrespective of the isoform, by other effects (e.g. an inhibitory effect on the self-association of apoE molecules or an anti-oxidative effect other than through disulfide bond formation). Although apoAII is known to be fully lipidated during the intrahepatic processing of lipoprotein particles, and only after secretion do the particles fuse with apoE-containing particles (Clay et al., 2000; Gillard et al., 2009), the phase after the fusion of the particles may also be important for the formation and modulation of apoE-containing lipoproteins.

It is generally believed that the C-terminal domain of apoE contains the major lipid-binding site. Segall et al. (2002) suggested that the interhelical flexibility of the α-helices in the C-terminal domain determines the binding ability of apoE and lipids, and that the binding ability of the C-terminal domain decreases by the covalent linkage of the remainder of apoE, such as the hinge region. They also proposed that the binding of apoE with lipids occurs not only through the C-terminal domain but also by opening of the N-terminal helix bundle, whereby helix-helix interactions converted to helix-lipid interactions. They interpreted that apoE2 showed the lowest DMPC clearance activity among apoE isoforms because its N-terminal domain of apoE2 is most resistant to thermal and chemical denaturation. On the contrary, our data suggest that the formation of the apoE-AII and apoAII-E2-AII complex probably increases the interhelical flexibility of the α-helices in the C-terminal domain or accelerates the opening of the N-terminal helix bundle of apoE2 and apoE3.

In conclusion, alterations in the redox states of apoE2 and apoE3 caused by various Cys thiol modifications, especially the interaction of the Cys158 of apoE2 with apoAII, may be involved in lipid metabolism, and consequently may be responsible for the specificity of the isoforms of apoE, such as that found in the pathology of apoE-related diseases.

Materials and methods

Materials

Recombinant apoE isoforms (apoE2, apoE3 and apoE4) were purchased from BioVision, Inc. (Milpitas, CA, USA), and recombinant apoAII was supplied by Athens Research & Technology, Inc. (Athens, GA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-apoE polyclonal antibody and goat anti-apoAII polyclonal antibody were supplied by Academy Bio-medical Company, Inc. (Houston, TX, USA). HRP-conjugated anti-goat IgG was supplied by MBL Co., Ltd. (Nagoya, Japan). N-ethylmaleimide and PM [molecular weight (MW), 2736 Da] were purchased from FUJIFILM Wako Pure Chemical, Co. (Osaka, Japan) and Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), respectively. DMPC was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All other chemicals used were of the highest grade.

Serum samples

Sera were obtained from healthy volunteers who had provided written informed consent. The apoE phenotype was determined by isoelectric focusing and immunoblot analysis as described previously (Yamauchi et al., 1999). We selected the serum with apoE phenotypes E2/E3 ($n=6$) and E3/E3 ($n=13$) and pooled them according to the apoE phenotype. This study was approved by the Tsukuba University Ethics Committee.

Determination of apoE redox status

The redox status of apoE was analyzed with a band-shift assay using PM, according to our previous study (Yamauchi et al., 2017). Briefly, PM was added to a sample at a final concentration of 1.0 mmol/l. The mixture was incubated for 30 min at 37°C and then treated with non-reducing Laemmli buffer (Laemmli, 1970), followed by loading onto

a 10% sodium dodecyl sulfate (SDS)-PAGE gel. After electrophoresis, the gel was ultraviolet (UV)-irradiated for 15 min to eliminate the polyethylene glycol moiety, and the separated proteins were transferred onto a polyvinylidene fluoride membrane. The blots were probed with an anti-apoE or anti-apoAII polyclonal antibody. The specific bands were developed using an enhanced chemiluminescence (ECL) detection kit (Nacalai Tesque, Inc., Kyoto, Japan). Three independent experiments were carried out and one representative blot was analyzed in duplicate using the ImageJ 1.45 software from the National Institutes of Health.

Preparation of DMPC vesicles

DMPC mLV were prepared by the method described by Segall et al. (2002). Briefly, 1.0 g/l of DMPC chloroform solution was dried under a nitrogen steam to a thin film on the wall of a round bottom flask followed by desiccation using an evaporator overnight. The dried lipid film was dispersed in 5 ml of Tris-buffered saline (TBS; 0.01 M Tris-HCl, pH 7.4, containing 0.1 M NaCl) and was then sonicated at 25°C to form DMPC mLV.

DMPC turbidity clearance assay

The solubilization of DMPC mLV by apoE proteins was determined as described previously (Segall et al., 2002). Briefly, recombinant apoE treated with TCEP, H_2O_2, N-ethylmaleimide, diamide or apoAII was added into an equal volume of DMPC mLV solution to a final DMPC:apoE molar ratio of approximately 500:1 in a quartz cuvette, which was preincubated at 24°C in a spectrophotometer with water-circulated temperature control. The contents were mixed within 10 s by repeated pipetting. The solubilization of DMPC mLV was monitored as a decrease in absorbance at 325 nm. Three independent experiments were carried out for each treatment.

Formation of apoE-DMPC liposome complexes

The dried DMPC film was resuspended in 5 ml TBS supplemented with 19 mM sodium deoxycholate, and this suspension was preincubated at 37°C until a clear solution was obtained. The treated recombinant apoE was then added to the solution to reach a DMPC:apoE molar ratio of approximately 100:1, and the mixture was incubated for 24 h at 25°C. After incubation, the deoxycholate was removed by dialysis against a 1000-fold excess of TBS for 3 days at 4°C with three buffer changes using a Slide-A-Lyzer™ MINI Dialysis Device (10 K MW cut-off, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Analysis of apoE-DMPC liposome complexes

The formation of apoE-DMPC liposome complexes was confirmed by non-denaturing PAGE analysis using a 4–12% gradient gel. The Stokes diameters of the complexes were determined from a calibration curve, constructed by plotting mobility against the corresponding Stokes diameters of a commercial size marker.

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References

Anraku, M., Chuang, V.T., Maruyama, T., and Otagiri, M. (2013). Redox properties of serum albumin. Biochim. Biophys. Acta *1830*, 5465–5472.

Bassett, G.R., Gillard, B.K., and Pownall, H.J. (2012). Cholesterol determines and limits rHDL formation from human plasma apolipoprotein A-II and phospholipid membranes. Biochemistry *51*, 8627–8635.

Blanco-Vaca, F., Escolà-Gil, J.C., Martín-Campos, J.M., and Julve, J. (2001). Role of apoA-II in lipid metabolism and atherosclerosis: advances in the study of an enigmatic protein. J. Lipid. Res. *42*, 1727–1739.

Boutureira, O. and Bernardes, G.J. (2015). Advances in chemical protein modification. Chem. Rev. *115*, 2174–2195.

Clay, M.A., Pyle, D.H., Rye, K.A., and Barter, P.J. (2000). Formation of spherical, reconstituted high density lipoproteins containing both apolipoproteins A-I and A-II is mediated by lecithin:cholesterol acyltransferase. J. Biol. Chem. *275*, 9019–9025.

Cooper, C.E., Patel, R.P., Brookes, P.S., and Darley-Usmar, V.M. (2002). Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. Trends. Biochem. Sci. *27*, 489–492.

Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L., and Pericak-Vance, M.A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science *261*, 921–923.

Garai, K., Baban, B., and Frieden, C. (2011). Dissociation of apolipoprotein E oligomers to monomer is required for high-affinity binding to phospholipid vesicles. Biochemistry *50*, 2550–2558.

Gillard, B.K., Lin, H.Y., Massey, J.B., and Pownall, H.J. (2009). Apolipoproteins A-I, A-II and E are independently distributed among intracellular and newly secreted HDL of human hepatoma cells. Biochim. Biophys. Acta *1791*, 1125–1132.

Go, Y.M. and Jones, D.P. (2013). The redox proteome. J. Biol. Chem. *288*, 26512–26520.

Gong, J.S., Kobayashi, M., Hayashi, H., Zou, K., Sawamura, N., Fujita, S.C., Yanagisawa, K., and Michikawa, M. (2002). Apolipoprotein E (ApoE) isoform-dependent lipid release from astrocytes prepared from human ApoE3 and ApoE4 knock-in mice. J. Biol. Chem. *277*, 29919–29926.

Groitl, B. and Jakob, U. (2014). Thiol-based redox switches. Biochim. Biophys. Acta *1844*, 1335–1343.

Huang, Y. and Mahley, R.W. (2014). Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. Neurobiol. Dis. *72*, 3-12.

Innerarity, T.L., Hui, D.Y., Bersot, T.P., Mahley, R.W. (1986). Type III hyperlipoproteinemia: a focus on lipoprotein receptor-apolipoprotein E2 interactions. Adv. Exp. Med. Biol. *201*, 273–288.

Jayaraman, S., Gantz, D.L., and Gursky, O. (2005). Kinetic stabilization and fusion of apolipoprotein A-2:DMPC disks: comparison with apoA-1 and apoC-1. Biophys. J. *88*, 2907–2918.

Jolivalt, C., Leininger-Muller, B., Bertrand, P., Herber, R., Christen, Y., and Siest, G. (2000). Differential oxidation of apolipoprotein E isoforms and interaction with phospholipids. Free Radic. Biol. Med. *28*, 129–140.

Kawakami, A., Kubota, K., Yamada, N., Tagami, U., Takehana, K., Sonaka, I., Suzuki, E., and Hirayama, K. (2006). Identification and characterization of oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions. FEBS J. *273*, 3346–3357.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Mahley, R.W. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science *240*, 622–630.

Mahley, R.W. (2016). Apolipoprotein E: from cardiovascular disease to neurodegenerative disorders. J. Mol. Med. (Berl.) *94*, 739–746.

Mera, K., Anraku, M., Kitamura, K., Nakajou, K., Maruyama, T., and Otagiri, M. (2005). The structure and function of oxidized albumin in hemodialysis patients: its role in elevated oxidative stress via neutrophil burst. Biochem. Biophys. Res. Commun. *334*, 1322–1328.

Minagawa, H., Gong, J.S., Jung, C.G., Watanabe, A., Lund-Katz, S., Phillips, M.C., Saito, H., and Michikawa, M. (2009). Mechanism underlying apolipoprotein E (ApoE) isoform-dependent lipid efflux from neural cells in culture. J. Neurosci. Res. *87*, 2498–2508.

Pitas, R.E., Boyles, J.K., Lee, S.H., Hui, D., and Weisgraber, K.H. (1987). Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. J. Biol. Chem. *262*, 14352–14360.

Segall, M.L., Dhanasekaran, P., Baldwin, F., Anantharamaiah, G.M., Weisgraber, K.H., Phillips, M.C., and Lund-Katz, S. (2002). Influence of apoE domain structure and polymorphism on the kinetics of phospholipid vesicle solubilization. J. Lipid Res. *43*, 1688–1700.

Thomas, J.A., Poland, B., and Honzatko, R. (1995). Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. *319*, 1–9.

Tozuka, M., Hidaka, H., Miyachi, M., Furihata, K., Katsuyama, T., and Kanai, M. (1992). Identification and characterization of apolipoprotein (AII-E2-AII) complex in human plasma lipoprotein. Biochim. Biophys. Acta *1165*, 61–67.

Ulrich, K. and Jakob, U. (2019). The role of thiols in antioxidant systems. Free Radic. Biol. Med. *140*, 14–27.

Weisgraber, K.H. and Mahley, R.W. (1978). Apoprotein (E--A-II) complex of human plasma lipoproteins. I. Characterization of this mixed disulfide and its identification in a high density lipoprotein subfraction. J. Biol. Chem. *253*, 6281–6288.

Weisgraber, K.H. (1990). Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine-arginine interchange at residue 112. J. Lipid Res. *31*, 1503–1511.

Yamauchi, K., Tozuka, M., Hidaka, H., Hidaka, E., Kondo, Y., and Katsuyama, T. (1999). Characterization of apolipoprotein E-containing lipoproteins in cerebrospinal fluid: effect of phenotype on the distribution of apolipoprotein E. Clin. Chem. *45*, 1431–1438.

Yamauchi, K., Tozuka, M., Hidaka, H., Nakabayashi, T., Sugano, M., Kondo, Y., and Katsuyama, T. (2000). Effect of apolipoprotein AII on the interaction of apolipoprotein E with beta-amyloid: some apo(E-AII) complexes inhibit the internalization of beta-amyloid in cultures of neuroblastoma cells. J. Neurosci. Res. *62*, 608–614.

Yamauchi, K., Ebihara, Y., and Kawakami, Y. (2017). Redox status of serum apolipoprotein E and its impact on HDL cholesterol levels. Clin. Biochem. *50*, 777–783.

Yamauchi, K., Iwasaki, S., and Kawakami, Y. (2019). Redox equilibrium of serum apolipoprotein E3: a buffering effect of disulfide-linked complexes against oxidative stress on apolipoprotein E3-containing lipoproteins. Biosci. Rep. *39*, BSR20190184, doi:10.1042/BSR20190184.

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