Research paper

Platelet cytochrome oxidase and citrate synthase activities in APOE ε4 carrier and non-carrier Alzheimer's disease patients

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ABSTRACT

A degradation product of APOE ε4-encoded apolipoprotein E protein targets mitochondria and inhibits cytochrome oxidase (COX), and autopsy brains from young adult APOE ε4 carriers show reduced COX activity. To further explore relationships between APOE alleles and COX, we measured platelet mitochondria COX activity in AD subjects with (n = 8) and without (n = 7) an APOE ε4 allele and found the mean COX activity, when normalized to sample total protein, was lower in the APOE ε4 carriers (p < 0.05). Normalizing COX activity to citrate synthase (CS) activity eliminated this difference, but notably the mean CS activity was itself lower in the APOE ε4 carriers (p < 0.05). COX and CS protein levels did not appear to cause the lower APOE ε4 carrier COX and CS Vmax activities. If confirmed in larger studies, these data could suggest mitochondria at least partly mediate the well-recognized association between APOE alleles and AD risk.

1. Introduction

Apolipoprotein E (APOE) variants associate with Alzheimer's disease (AD), with the APOE ε2, ε3, and ε4 alleles respectively defining reduced, intermediate, and increased risk [1–6]. Different mechanisms may mediate this, including mechanisms that involve mitochondrial function [1,7–12]. Relevant to this possibility, APOE-mitochondria relationships occur outside the context of an AD diagnosis. For instance, Valla et al. reported autopsy brains from young adult APOE ε4 carriers show reduced cytochrome oxidase (COX) activity, at a point that predates differences in beta amyloid protein [13,14]. Fluorodeoxyglucose positron emission tomography (FDG PET) studies also show reduced glucose utilization in asymptomatic, middle-aged APOE ε4 carriers, which could reflect mitochondria-related bioenergetic differences [15,16].

From an epidemiologic perspective, two studies report APOE genotypes and mitochondrial DNA (mtDNA) haplogroups actually interact to define AD risk [17,18]. This interaction would presumably converge at the level of mitochondrial function. Also, the TOMM40 gene, which encodes a critical mitochondrial translocase, borders the APOE gene and as a result TOMM40 and APOE allele signatures show linkage disequilibrium [2,19–21]. This raises the possibility that APOE alleles may relate indirectly to mitochondrial function through TOMM40 linkage disequilibrium. Even if an indirect association such as this does exist, though, APOE genotypes may still directly influence mitochondrial function via the production of apolipoprotein E isoform-dependent degradation products. Specifically, cleavage of the APOE ε4-derived apolipoprotein E isoform (but not other isoforms) generates a mitochondrial targeting sequence and this mitochondria-targeted peptide inhibits COX [8,9,22].

A mechanistic link between APOE ε4 and COX seems particularly intriguing, as AD subject groups show lower mean COX activities than age-matched control groups [23]. The AD COX deficit manifests across several tissues, including platelets [24]. mtDNA contributes to the AD COX activity reduction [25], but this does not rule out the possibility that other modifiers or potential modifiers of mitochondrial function, including TOMM40 or APOE, play a role.

In this study, we measured platelet mitochondria COX activities in APOE ε4 carrier and non-carrier AD patients. To test whether APOE allele types relate to COX activity, we compared the total protein and A

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; COX, cytochrome oxidase; COX4I1, cytochrome oxidase subunit 4 isoform 1; CS, citrate synthase; FDG PET, Fluorodeoxyglucose positron emission tomography; mtDNA, mitochondrial DNA; SD, standard deviation; SEM, standard error measurement; SNP, single nucleotide polymorphism; TOMM40, translocase of the outer mitochondrial membrane.

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citrate synthase (CS)-referenced inter-group COX activity means.

2. Materials and methods

2.1. Approvals and Human Subjects

This study in part includes a secondary analysis of data acquired during the course of the S-equol in Alzheimer’s Disease (SEAD) trial (ClinicalTrials.gov NCT02142777; manuscript in review). The Kansas University Medical Center Human Subjects Committee approved the SEAD protocol and all participants provided informed consent prior to enrolling. This study was conducted in accordance with the Code of Ethics of the World Medical Association (the Declaration of Helsinki).

We enrolled fifteen female participants (12 Caucasians and 3 African Americans), all met McKhann et al. AD diagnostic criteria [26], and all had clinical dementia rating (CDR) scores of 0.5 or 1 [26]. Participants were excluded if they reported any potentially confounding, serious medical risks such as type 1 diabetes, cancer, or a recent cardiac event such as a heart attack or angioplasty. Each subject had a study partner who was engaged in their daily social and medical care. Over the course of the trial participants underwent three 40 ml phlebotomies, which occurred at two week intervals, and we determined platelet mitochondria COX and CS activities for each phlebotomy (see below). The first phlebotomy occurred during a placebo lead-in period, the second occurred two weeks after initiating the study drug, and the third occurred after a two-week treatment wash-out period.

2.2. Phlebotomy and mitochondrial isolation

Forty ml of blood was collected in tubes containing acid-citrate-dextrose anticoagulant. One ml of whole blood was removed and stored at −80 °C for genotyping; the rest was used for platelet harvesting and mitochondrial enrichment.

Briefly, 15 ml of Histopaque 1077 was centrifuged in an AccuSpin tube for 1 min at 1700 × g. Blood was layered on top of the AccuSpin tube frit, and centrifuged for 15 min at 400 × g. Platelet-rich plasma was resuspended in MSHE buffer and subjected to nitrogen cavitation for a second time (1200 psi for 20 min). Both supernatents were combined and centrifuged at 12,000 × g for 10 min, 4 °C. The first occurrence of a platelet pellet occurred during a placebo lead-in period, the second occurred two weeks after initiating the study drug, and the third occurred after a two-week treatment wash-out period.

2.3. COX and CS Vmax Assays

We added aliquots of the enriched mitochondrial suspensions to cuvettes and spectrophotometrically determined each suspension’s COX and CS Vmax activities. For the COX Vmax, we followed the conversion of reduced cytochrome c to oxidized cytochrome c, and calculated the pseudo-first order rate constant (msec⁻¹). For the CS Vmax, we followed the formation of 5-thio-2-nitrobenzoate (nmol/min) [27]. The COX rate was normalized to mg protein (msec⁻¹/mg protein) or to the CS rate (yielding a value with units of msec⁻¹/nmol/min), which we herein refer to simply as COX/CS. The CS rate was normalized to mg protein to yield a final activity with units of nmol/min/mg protein.

2.4. Immunochemistry

For mitochondrial suspensions that contained, following the Vmax assays, adequate amounts of protein we used a Western blot approach to measure COX subunit 4 isoform 1 (COX4I1) and CS protein levels.

Briefly, equal protein amounts were resolved by SDS-PAGE (Criterion TGX gels, BioRad) and proteins were tranfered to nitrocellulose membranes (ThermoFisher). Immunoblots against COX4I1 (Cell Signaling, 1:500) and CS (Cell Signaling, 1:500) were analyzed by densitometry. To visualize bands, we used WestFemto Super Signal HRP Substrate (ThermoFisher) and a ChemiDoc XRS imaging platform.

2.5. APOE and TOMM40 genotyping

We used a single nucleotide polymorphism (SNP) allelic discrimination assay to determine APOE genotypes. This involved adding 5 µl of blood to a Taqman Sample-to-SNP kit (ThermoFisher). Taqman probes to the two APOE-defining SNPs, rs429358 (C_3084793_20) and rs7412 (C_904973_10) (ThermoFisher), were used to identify APOE ε2, ε3, and ε4 alleles.

Introner 6 of the TOMM40 gene contains a variable length poly-T region that defines the rs10524523 SNP (referred to herein as TOMM40 ‘523) [21]. To obtain the ‘523 poly-T length, we used a DNeasy kit (Qiagen) to prepare genomic DNA from 100 µl of whole blood and PCR-amplified the DNA with the following primers: 5'-6FAM-CTGACCTCAGCTGTCCCTC-3' (forward) and 5'-GAGGCTGAAGGAGGATT-3' (reverse). The PCR reaction contained 0.2 µM of each primer, Takara EX Taq polymerase (Takara Bio Inc), and 5% DMSO. The PCR parameters were 1 cycle of 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 65 °C, and 30 s at 72 °C; and 1 cycle of 5 min at 72 °C. After using a QIAquick PCR purification kit to remove nucleotides and primers, we submitted the amplicons to Genewiz, Inc. for fragment analysis. The TOMM40 poly-T length was calculated by subtracting the size of the PCR product from 150 (the expected size of the PCR product lacking the poly-T region) as previously described [28]. In preliminary experiments, we showed this assay identified the TOMM40 poly-T length to within +/-1 nucleotides. We used the parameters defined by Roses et al. to place each allele into one of three groups, in which a short (S) allele represents a poly-T length of <19, a long (L) allele represents a poly-T length of 20–29, and a very long (VL) allele represents a poly-T length of >30 [19,20,28].

2.6. Data and statistical analyses

Our enzyme activity analysis did not include measurements from platelets obtained during the active S-equol treatment period. Whenever possible we averaged the two enzyme activity values from the pre-treatment lead-in and post-treatment wash-out periods to yield a single final value: 14 of the 15 participants had complete data sets. One participant had only one set of valid CS and COX/CS values, and that participant’s CS and COX/CS activities represent single rather than averaged measurements.

We organized the data from the 15 participants into two groups, one in which participants had at least one APOE ε4 allele, and one in which they did not. We also grouped the participants by their TOMM40 L status, to define TOMM40 L carrier and non-carrier groups. We summarized group COX (per total protein), CS (per total protein), and COX/CS activities by means, standard deviations (SD), and standard error of the means (SEM) and compared means by two-way Student’s T-tests with significance defined as p < 0.05.

3. Results

The APOE ε4 carrier (n = 8; all heterozygous) and non-carrier (n = 7) group mean ± SEM ages were, respectively, 70.9 ± 2.2 and 76.4 ± 3.4 (p = 0.17), and the group CDR ± SEM scores were 0.75 ± 0.09 and 0.86 ± 0.09 (p = 0.43). The APOE ε4 carrier group COX activity, when normalized to total protein, was 27% lower than that of the APOE ε4 non-carrier group (p = 0.039). The mean CS activity in the APOE ε4 carriers was 34% lower than that of the APOE ε4 non-carriers (p = 0.049). The COX/CS activities were comparable (Fig. 1). Adequate
residual samples were available to analyze protein levels in APOE ε4 carrier and 5 APOE ε4 non-carrier participants. The groups showed comparable COX4I1 protein levels. CS protein levels were also statistically equivalent, although we note a possible trend towards reduced CS protein in the APOE ε4 non-carriers (Fig. 1).

Caucasians show very frequent (98%) but not exclusive TOMM40 L523L and APOE ε4 allele linkage disequilibrium, while African Americans do not [21,29]. Three of the 15 participants (20%) did not show APOE ε4-TOMM40 L linkage disequilibrium; two of these were African American. After regrouping our participants by TOMM40 L carrier (n = 7) or non-carrier (n = 8) status, we did not observe COX, CS, or COX/CS activity differences. The TOMM40 L groups showed comparable COX4I1 and CS protein levels, although the CS protein level trended lower in the TOMM40 L non-carriers (n = 6) as compared to the TOMM40 L carriers (n = 7) (p = 0.06) (Fig. 2).
4. Conclusions

Within a small sample of AD patients, the APOE ε4 carriers had lower platelet mitochondria COX and CS Vmax activities than the APOE ε4 non-carriers. Normalizing COX activities to their corresponding CS activities eliminated the COX activity difference, which likely reflects division of the lower APOE ε4 carrier COX numerators by the lower CS denominators. COX4I1 (and by predicted extension the COX holoenzyme) and CS protein levels did not appear to mediate the lower APOE ε4 carrier COX and CS Vmax activities. Indeed, for unclear reasons CS protein levels paradoxically trended lower in the APOE ε4 non-carriers.

Due to our small study size these findings are preliminary and not...
definitive. A consideration of several points that arise from this study could, nevertheless, assist in the planning of more definitive studies. Obviously, our data can inform power calculations. If COX activity serves as the primary outcome, normalizing the COX activity to the CS activity could potentially produce a false negative result.

Future studies should also consider potential mechanisms (or artifacts) that may produce a positive result. While our data could reflect a direct interaction of APOE ε4-derived apolipoprotein E protein and mitochondria, or between an APOE ε4-derived apolipoprotein E peptide derivative and mitochondria [8,9,22], we did not determine platelet apolipoprotein E protein levels. Even if platelets turn out not to contain apolipoprotein E protein, though, they do host apolipoprotein E receptors [30]. Signaling through these receptors reportedly affects platelet activation and aggregation states, which could indirectly influence mitochondrial function or possibly create sample preparation artifacts. Regarding the possibility that TOMM 40L alleles might actually mediate differences in mitochondrial function, and APOE ε4 alleles simply indicate the presence of TOMM 40L alleles, our data argue against this but we must again note these remain preliminary findings and our small sample size precludes definitive conclusions.

Our sample lacked APOE ε4 homozygous participants. This occurred by chance, not intent. We therefore do not know whether APOE ε4 homozygosity associates with lower COX or CS activities than APOE ε4 heterozygosity. Addressing this could provide insight into the clinical significance of the AD-APOE-mitochondria nexus. To this point we further note that even if APOE genotypes do in fact associate with lower mitochondrial enzyme activities, the extent to which this mechanistic association underlies APOE-determined AD risk remains unclear. Because APOE-related changes in mitochondrial function presumably manifest during early-life [14], and AD arises during late-life, one might argue APOE status primarily influences AD risk by reducing reserve enzyme capacity, thereby increasing the likelihood that age-related declines in mitochondrial function eventually surpass functional and clinically relevant thresholds.

Despite these caveats and considerations, our data suggest platelet mitochondria COX and CS activities in APOE ε4 non-carriers may exceed those of APOE ε4 carriers. Viewed within the context of the report of Valla et al., which found brains from young APOE ε4 carriers showed a lower COX activity than brains from young APOE ε4 non-carriers [14], our data are consistent with the possibility that mitochondria mediate the association between APOE ε4 and increased AD risk [31].

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