Expression of human apolipoprotein E4 reduces insulin-receptor substrate 1 expression and Akt phosphorylation in the ageing liver

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ABSTRACT

The diabetic drug rosiglitazone was reported to improve glucose tolerance in insulin-resistant ApoE3 but not ApoE4 knock-in mice. We therefore examined whether apolipoprotein E (ApoE) has genotype-specific effects on liver insulin function. At 12 weeks, no difference in liver insulin signaling was detected between fasting ApoE3 and ApoE4 mice. At 72 weeks however, ApoE4 mice had lower IRS-1 and PI3K expression, and reduced Akt phosphorylation. This decline was associated with lower insulin and higher glucose in ApoE4 mouse liver. Liver cholesterol was not affected. These results show that ApoE4 expression reduces liver insulin signaling and insulin levels, leading to higher glucose content.

1. Introduction

Human apolipoprotein E (ApoE) exists in 3 isoforms, E2, E3 and E4 [1,2]. These isoforms differ by amino acid substitutions at two positions (residues 112 and 158) [3].

ApoE is synthesized in various organs [4] and high expression is detected in the liver [5]. This protein is extensively studied as a group of lipid carrier molecules vital to the cholesterol homeostasis of the body [5].

However, emerging studies suggest that ApoE has other functions beyond cholesterol metabolism [1,6]. ApoE4 carriers have shown to experience greater age-related reduction in cerebral glucose metabolism [7–9], and intra-nasal insulin improves cognition in ApoE3 [10,11] but not ApoE4 non-demented elderly subjects [12]. This shows an isoform-dependent role in brain glucose metabolism and insulin function.

In non-diabetic subjects, the presence of ApoE4 did not affect fasting glucose level [13]. But, the effect of ApoE4 on fasting glucose and insulin levels in diabetic subjects is unknown. However, obese men with the ApoE4 genotype have higher insulin and glucose levels than obese men without the ApoE4 genotype [14].

In diet-induced insulin resistant ApoE3 and ApoE4 knock-in (KI) mice, the insulin sensitizer rosiglitazone only improved glucose tolerance in ApoE3 KI mice [15,16]. This suggests that ApoE could have a genotype-dependent effect on peripheral insulin function.

To understand ApoE role on peripheral insulin function, we have conducted this study to examine the age-related effects of ApoE genetic polymorphism on insulin signaling in the liver of ApoE KI mice.

2. Methods

2.1. Animals

The experimental protocol (#009/10) involving the laboratory mice used in this study was approved by the Institutional Animal Care and Use Committees (IACUC) at the National University of Singapore. The human apolipoprotein E3 and E4 knock-in (KI) mice were created as described [17] and were purchased from Taconic. Briefly, the endogenous mouse ApoE gene was replaced by the human APOE genomic fragments via homologous recombination. The mice were kept on 2018 Teklad Global 18% Protein Rodent Diet (Harland Laboratories). Mice were fasted for ∼12 h prior to experiments. All experiments were performed on at least three ($n \geqslant 3$) fasted female homozygous ApoE3 and ApoE4 mice at 12 and 72 weeks of age.

2.2. Preparation of liver homogenates

The method used to homogenize the mouse liver tissues is the same as described in our earlier study [18]. Briefly, mouse liver tissues were snapped frozen in liquid nitrogen when harvested and the wet weight of the tissues (in mg) were determined using an electronic balance. Twenty percent (w/v) liver homogenates were prepared with 1× cell lysis buffer (Cell Signaling Technology) with protease inhibitors cocktail (Roche Diagnostic).

The cell lysis buffer contains sodium orthovanadate, pyrophosphate and glycerophosphate, which can acts as phosphatase inhibitors. Lysates were then homogenized using a hand held motorized pestle (Sigma–Aldrich, St. Louis, USA) for 30 s on ice. Tissue lysates were subsequently centrifuged at 30,000g for 30 min under 4C. The soluble portion of the lysates was collected for analysis.

2.3. Protein quantification of lysates

The method used to quantify the mouse liver homogenates is the same as described in our earlier study [18]. Briefly, tissue lysates were quantified using the Pierce™ MicroBCA assay kit (ThermoFisher Scientific, Waltham, USA) in a 96-well microplate format. Lysates were diluted in PBS and the working reagent was prepared and added in accordance to the manufacturer's instructions. Samples were then incubated at 37 °C for 30 min before reading the absorbance values at 562 nm. Protein concentrations of samples were calculated based on a standard curve constructed from a range of BSA standards. The liver tissue lysates were aliquoted and stored at −80 °C.

2.4. Immunoblot analysis

The method used to perform immunoblotting is the same as described in our earlier study [18]. Soluble liver proteins from lysate samples were heated at 95 °C for 5 min. Protein samples were then centrifuged at 14,000g for 2 min on a bench top centrifuge before they were loaded on a 7.5–10% Tris–glycine polyacrylamide gel. The Precision Plus protein™ standard (Bio-Rad Laboratories, Hercules, California USA) was used as a molecular weight standard and run together with the samples on the same piece of gel.

The separated proteins were transferred onto a nitrocellulose membrane, probed with the respective antibodies and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies. The reactive protein bands were visualized by chemiluminescence on the Image Station 4000R (Carestream Health Inc) using the SuperSignal® West Dura Substrate (Pierce) system.

Immunoblotting of β-actin using a rabbit polyclonal antibody that binds to the C-terminal of β-actin (Sigma) was included in all western blot analysis to ensure comparable protein loading. The primary antibodies used include anti-huApoE (Santa Cruz Biotechn, Cat# 13521), anti-IRS1 (Cell Signaling Technology, Cat# 2382), anti-PI3K/p85 (Cell Signaling Technology, Cat#4257), anti-PI3K/p110 (Cell Signaling Technology, Cat# 4249), anti-Akt (Cell Signaling Technology, Cat# 4691), anti-pAkt(S473) (Cell Signaling Technology, Cat# 4060), and anti-pAkt(T308) (Cell Signaling Technology, Cat# 2965).

Densitometry analysis was performed [18] by measuring the optical densities of the targeted protein bands relative to the endogenous β-actin level from the same liver sample. For protein phosphorylation, the optical densities of the phosphorylated protein bands were measured relative to the targeted total protein level from the same liver sample. The analysis was performed using the NIH ImageJ software.

2.5. Glucose assay

The method used to measure tissue glucose level is the same as described in our recent study [19]. Total liver glucose content was measured using the amplex red glucose assay kit (Life Technologies) following the instructions provided by the manufacturer. Briefly, liver lysate sample was mixed with equal volume of amplex red working reagent, and the reaction mixture was then incubated for 30 min at room temperature in the dark. The fluorescence values were read at an excitation wavelength of 545 nm and an emission wavelength of 590 nm. A series of glucose standards were prepared and run alongside the mouse liver samples.

2.6. Insulin assay

The method used to measure tissue insulin level is the same as described in our recent study [19]. Total liver insulin content was measured using the sandwich ELISA mouse insulin assay system (Millipore) following the instructions provided by the manufacturer. Briefly, liver lysates were added to microtiter plate well pre-coated with anti-insulin antibody. After incubation and washing, a biotinylated anti-insulin antibody was added. This biotinylated antibody reacts against a distinctive epitope to that of the coated anti-insulin. The reaction was incubated for 15 min and the absorbance was read at 370 nm. The stop solution provided by the assay kit was added to the sample when the absorbance was read at 1.8. Immunoreactivity was immediately determined by measuring the absorbance at 450 nm and 590 nm.

2.7. Statistical analysis

Significant differences were analyzed using the same method described in our recent study [19]. Statistical analysis was performed using two-tailed Student's t-test. A p value of <0.05 is considered significant.

3. Results

3.1. Lower liver insulin receptor substrate protein and PI3K expression in aged ApoE4 KI mice

While ApoE role in liver cholesterol metabolism is well characterized [1], the effects of this protein (and its polymorphism) on liver insulin signaling has not been examined. We therefore decided to determine if ApoE polymorphism can affect hepatic insulin signaling in ageing ApoE3 and ApoE4 knock-in (KI) mice.

At 12 weeks, insulin receptor substrate 1 (IRS1) expression does not differ between fasting ApoE3 and ApoE4 mice (Fig. 1A). At 72 weeks however, IRS1 expression is non-detectable in the fasting ApoE4 KI mice.

We then examined the expression of the catalytic subunit (p110) and the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K). At 12 weeks, PI3K/p110 and PI3K/p85 expression were comparable between ApoE3 and ApoE4 mice. In the aged (72 weeks) fasting ApoE4 mice however, PI3K/p110 and PI3K/p85 expression were reduced by 70% (Fig. 1C) and 44% (Fig. 1D) respectively as compared to fasting ApoE3 mice.

3.2. Reduced Akt phosphorylation in the liver of ApoE4 mice

We next determined if the aberrant IRS1 and PI3K expression (Fig. 1) affects downstream Akt expression and phosphorylation (Fig. 2). Liver Akt expression did not differ between ApoE3 and ApoE4 mice at 12 weeks and at 72 weeks (Fig. 2B). Akt phosphorylation at Serine-473 (S473) and at Threonine-308
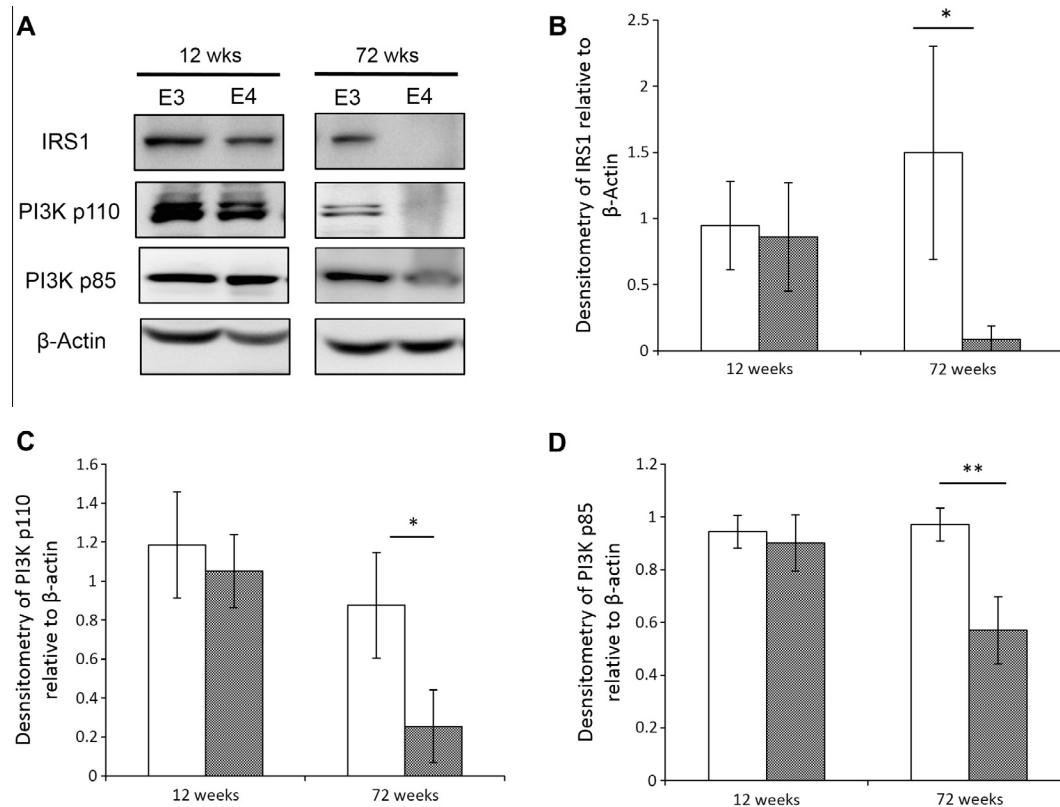
Fig. 1. Hepatic insulin receptor substrate and PI3K proteins expression in ApoE knock-in mice. (A) Western blot analysis of insulin receptor substrate-1 (IRS1), PI3K/p85 and PI3K/p110 levels in the liver of ApoE3 and ApoE4 mice at 12 and 72 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. The blot is a representative of three independent experiments. Densitometry analysis of total (B) IRS1, (C) PI3K/p110 and (D) PI3K/p85 level relative to β-actin level in 12 and 72 weeks old ApoE3 (white bar) and ApoE4 (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample (*n* = 3 at each time point for each mouse line). Lower IRS1, PI3K/p85 and PI3K/p110 levels were detected in ApoE4 mice at 72 weeks of age as compared to ApoE3 mice of similar age. (*$p < 0.03$; **$p < 0.008$, using Student's *t*-test.)

(T308) were also unaffected in 12 weeks old ApoE3 and ApoE4 mice (Fig. 2A).

However, in 72 weeks fasting ApoE4 mice, Akt phosphorylation at S473 and at T308 were significantly reduced by 44% and 53% respectively as compared to fasting ApoE3 mice at similar age (Figs. 2C and D).

3.3. Liver insulin and glucose in ApoE mice

Changes in IRS1 expression can affect insulin and glucose contents [20–22]. We therefore measured the content of liver glucose and insulin in the young and aged ApoE3 and ApoE4 mice. In the 12 weeks old fasting ApoE KI mice, we did not detect any significant difference in liver insulin and glucose level between the two mouse lines (Fig. 3). However, the liver insulin of the 72 weeks old fasting ApoE4 mice (dotted line) was 37% lower than the fasting ApoE3 mice at similar age (bold line). Liver glucose content was also significantly affected in the aged fasting ApoE mice. The liver glucose in the aged fasting ApoE4 mice (grey bar) was 27% higher than the fasting aged ApoE3 mice (white bar).

3.4. Liver cholesterol in ApoE mice

ApoE is a major player in cholesterol metabolism [1]. However, cholesterol level is only elevated when the ApoE mouse lines are kept on high-fat diet [17,23,24]. Hence, we did not observe any significant change in liver cholesterol content between the fasting ApoE3 and ApoE4 mice when kept on normal rodent diet (Fig. 4).

3.5. Liver ApoE expression

Reduced ApoE levels have been reported in the brain of huApoE4 KI mice [25,26], but ApoE expression in the peripheral tissues including liver has not been examined.

We therefore immunoblotted for ApoE in the liver of fasting ApoE3 and ApoE4 mice at 12 and 72 weeks of age. As show in Fig. 5, lower ApoE content was detected in the liver of 12 and 72 weeks old ApoE4 mice as compared to ApoE3 mice at similar ages. There was a 23% reduction observed in the young ApoE4 mice and this was further reduced to 71% in the aged ApoE4 mice when compared to ApoE3 mice at similar ages.

4. Discussion

In diet-induced insulin resistant ApoE3 and ApoE4 KI mice, the insulin sensitizer rosiglitazone only improves glucose tolerance in ApoE3 mice [15,16]. In this study using fasting ApoE mice, we are reporting an ApoE genotype-dependent effect on liver insulin signaling during ageing. In aged mice, we observed that lower ApoE4 expression was linked to lower liver brain insulin signaling and insulin content, and higher liver glucose level. Liver cholesterol content however was not affected since the mice were not kept on high-fat diet [17].

In this study, mice were fasted to trigger a catabolic state when examining peripheral insulin signaling [27]. This process is meant to reduce variability in baseline insulin signaling activation during experiments.
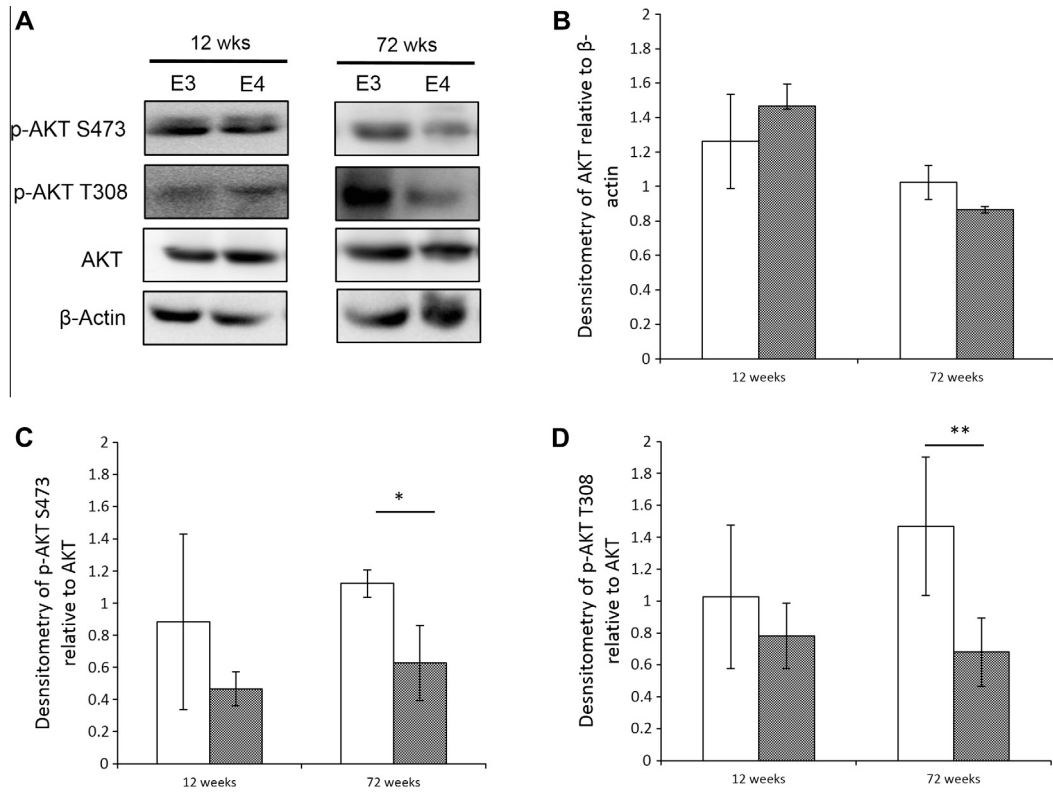
Fig. 2. Akt expression and phosphorylation in the liver of fasting ApoE mice. (A) Immunoblotting of total Akt, phosphorylated Akt (S473) and phosphorylated Akt (T308) in the liver of ApoE3 and ApoE4 mice at 12 and 72 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. The blot is a representative of three independent experiments. Densitometry analysis of (B) total Akt level relative to β-actin level, (C) phosphorylated Akt(S437) and (D) phosphorylated Akt(T308) level relative to total Akt level, in 12 and 72 weeks old ApoE3 (white bar) and ApoE4 (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample ($n = 3$ at each time point for each mouse line). Lower Akt phosphorylation at S473 and T308 were detected in 72 weeks ApoE4 mice as compared to ApoE3 mice at similar age. ($*p < 0.02$; $**p < 0.04$, using Student's t-test.)
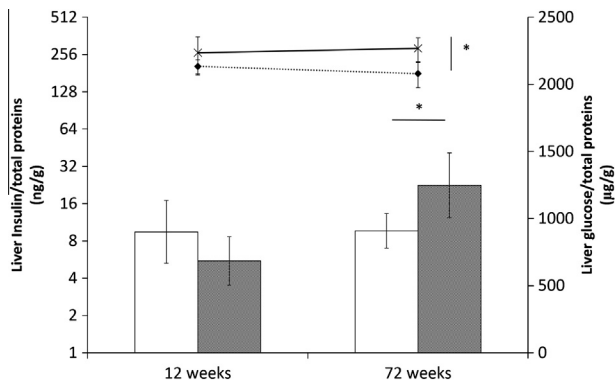

Fig. 3. Analysis of liver insulin and glucose contents in fasting ApoE mice. Higher glucose (grey bar) and lower insulin (dotted line) were detected in the liver of 72 weeks ApoE4 mice as compared to ApoE3 (white bar and bold line) of similar age. Each value represents the mean ± SEM of duplicate assays for individual samples ($n = 5$). ($*p < 0.02$ using Student's t-test.)
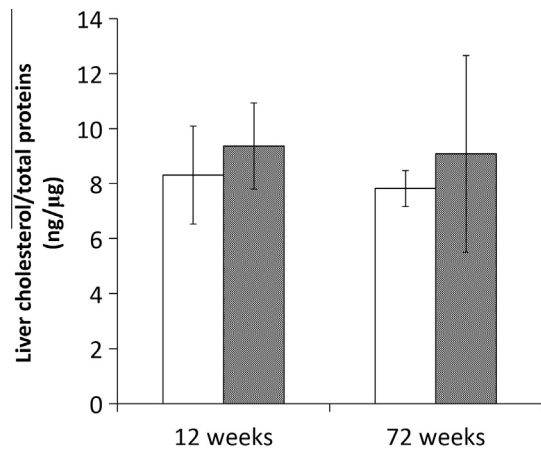

Fig. 4. Analysis of liver cholesterol content in fasting ApoE mice. No significant different in liver cholesterol contents between ApoE3 (white bar) and ApoE4 (grey bar) mice at 12 and 72 weeks of age was detected. Each value represents the mean ± SEM of duplicate assays for individual samples ($n = 5$).

In 72 weeks old fasting ApoE4 mice, IRS1 expression was almost non-detectable. Changes in IRS1 had been shown to affect PI3K/Akt signaling [28,29]. As compared to the aged fasting ApoE3 mice, lower levels of the catalytic subunit (p110) and the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K), and Akt phosphorylation were detected in the liver of aged ApoE4 mice.

One possible mechanism linking IRS1 and PI3K/Akt activation is ApoE expression. Studies have shown that increasing ApoE expression will elevate Akt phosphorylation [30]. Hence, the lower Akt

phosphorylation in ApoE4 mice could be due to lower ApoE level. This interaction may also lead to reduced IRS1 expression.

Changes in IRS1 expression can affect insulin and glucose contents [20–22]. The change in liver glucose and insulin levels were only detected between aged ApoE4 and aged ApoE3 mice. In the 72 weeks old fasting ApoE4 KI mice, lower liver insulin content was correlated with lower IRS1 and PI3/Akt expression and phosphorylation as compared to the aged fasting ApoE3 mice. However,
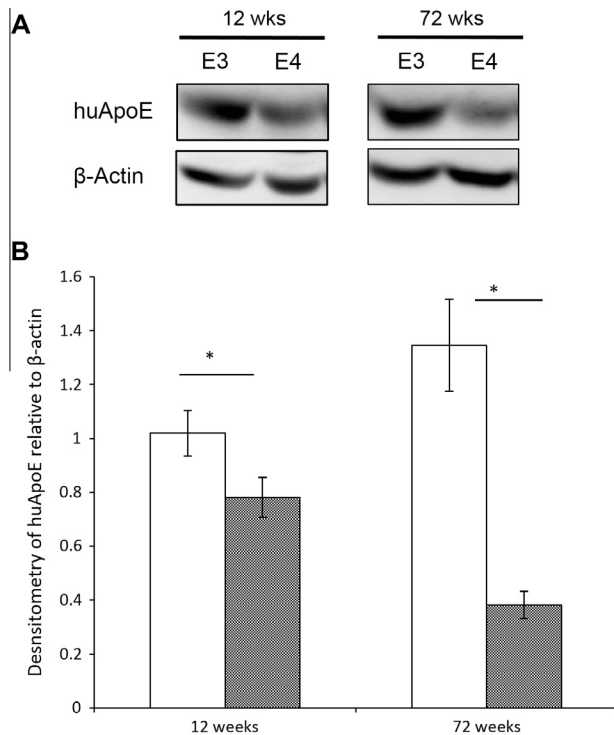
Fig. 5. Lower human apolipoprotein E (ApoE) level in the liver of ApoE4 knock-in mice. (A) Western blot and (B) densitometric analysis of liver ApoE levels in ApoE3 (white bar) and ApoE4 (grey bar) knock-in mice at 12 and 72 weeks of age. (A) The blot is a representative of three independent experiments. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. (B) Densitometric analysis was performed the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample ($n = 3$ at each time point for each mouse line). Liver ApoE level was significant reduced in ApoE4 mice as compared to ApoE3 mice of similar age. (*$p < 0.02$ using Student's t-test.)

the mechanism underlying the cause of the lowering liver insulin content and insulin signaling is unknown. Furthermore, it is unclear how the lower insulin content contributes to the higher glucose level detected in the liver of the aged ApoE4 mice as compared to ApoE3 mice.

ApoE is extensively studied as a group of lipid carrier molecules that is vital in the cholesterol homeostasis of the body [5]. However, emerging studies suggest that ApoE has other functions beyond cholesterol metabolism [1,6]. The results in this study have shown an interplay between ApoE and liver insulin signaling. Previous study has also detected higher fasting insulin and glucose levels in obese male ApoE4 carriers as compared to obese men without the ApoE4 genotype [14]. Taken together, it is possible that ApoE4-induced impairment in metabolic functions can modulate atherosclerosis and cardiovascular disease progression [1].

Conflict of interest

The authors declare no competing financial interests.

Author contributions

Q.R.O. and M.L.L. performed the experiments. Q.R.O. and B.S.W. conceived and designed the experiments, and analyzed the data. Q.R.O., E. S.C. and B.S.W. wrote the paper.

Acknowledgements

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