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ARTICLE

Altered endocannabinoid signalling after a high-fat diet in *Apoe*$^{-/-}$ mice: relevance to adipose tissue inflammation, hepatic steatosis and insulin resistance

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Abstract
Aims/hypothesis Apolipoprotein E (ApoE) deficiency is associated with reduced fat accumulation in white adipose tissue (WAT) and high liver triacylglycerol content. Elevated levels of endocannabinoids and cannabinoid receptor type 1 (CB_1) receptors in the liver and in epididymal vs subcutaneous WAT are associated with fatty liver, visceral adipose tissue, inflammatory markers and insulin resistance.
Methods We investigated, in *Apoe*$^{-/-}$ and wild-type (WT) mice, the effect of a high-fat diet (HFD) on: (1) subcutaneous and epididymal WAT accumulation, liver triacylglycerols, phospholipid-esterified fatty acids, inflammatory markers in WAT and liver, and insulin resistance; and (2) endocannabinoid levels, and the gene expression levels of the *Cb1* receptor and endocannabinoid metabolic enzymes in liver and WAT.
Results After a 16 week HFD, *Apoe*$^{-/-}$ mice exhibited lower body weight, WAT accumulation and fasting leptin, glucose and insulin levels, and higher hepatic steatosis, than WT mice. Glucose clearance and insulin-mediated glucose disposal following the HFD were slower in WT than *Apoe*$^{-/-}$ mice, which exhibited higher levels of mRNA encoding inflammatory markers (tumour necrosis factor-α [TNF-α], monocyte chemoattractant protein-1 [MCP-1], cluster of differentiation 68 [CD68] and EGF-like module-containing mucin-like hormone receptor-like 1 [EMR1]) in the liver, but lower levels in epididymal WAT. HFD-induced elevation of endocannabinoid levels in the liver or epididymal WAT was higher or lower, respectively, in *Apoe*$^{-/-}$ mice, whereas HFD-induced decrease of subcutaneous WAT endocannabinoid and CB_1 receptor levels was significantly less marked. Alterations in endocannabinoid levels reflected changes in endocannabinoid catabolic enzymes in WAT, or the availability of phospholipid precursors in the liver.
Conclusions/interpretation Liver and adipose tissue endocannabinoid tone following an HFD is altered on *Apoe* deletion and strongly associated with inflammation, insulin resistance and hepatic steatosis, or lack thereof.

Keywords CB_1 receptor · Fasting glycaemia · Inflammation · Insulin sensitivity · Liver triacylglycerols

Abbreviations

AA	Arachidonic acid
2-AG	2-Arachidonoylglycerol

A. Bartelt and P. Orlando contributed equally to this study.

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ApoE	Apolipoprotein E
CB_1	Cannabinoid receptor type 1
DAGL	Diacylglycerol lipase
epiWAT	Epididymal WAT
FAAH	Fatty acid amide hydrolase
HFD	High-fat diet
LDLR	LDL receptor
MAGL	Monoacylglycerol lipase
NASH	Non-alcoholic steatohepatitis
PUFA	Polyunsaturated fatty acids
subWAT	Subcutaneous WAT
TNF-α	Tumour necrosis factor-α
WAT	White adipose tissue
WT	Wild-type

Introduction

As a component of triacylglycerol-rich lipoproteins, apolipoprotein E (ApoE) mediates clearance of chylomicron and VLDL remnants via interaction with LDL receptors (LDLRs), LDLR-related protein 1 (LRP1) and heparin sulphate proteoglycans [1, 2]. ApoE is also found associated with HDL and facilitates reverse cholesterol transport, a process describing the transport of extra-hepatic cholesterol to the liver for bile excretion. In addition, ApoE is involved in VLDL assembly, the regulation of vascular lipolysis and intestinal lipid absorption. Consequently, ApoE deficiency in gene-targeted mice (*Apoe*$^{-/-}$) results in the accumulation of remnant lipoproteins and low HDL levels. These mice are intensively used as a model to study the development of hyperlipidaemia, atherosclerosis and cardiovascular disease [3, 4]. Furthermore, ApoE deficiency leads to non-alcoholic steatohepatitis (NASH), whereas dietary cholesterol is an important risk factor for the progression to hepatic inflammation in diet-induced NASH in relation to ApoE [5, 6]. Although the vast majority of plasma ApoE is synthesised in the liver, ApoE is highly expressed in adipose tissue [7]. Indeed, ApoE modulates triacylglycerol content in adipocytes and might be involved in the development of insulin resistance. In general, the lack of ApoE is associated with reduced fat accumulation in white adipose tissue (WAT) during diet-induced obesity [8–10]. Thus, ApoE is primarily important for dietary and endogenous lipid transport between adipose tissues and the liver, and may also influence insulin sensitivity and tissue lipid composition.

All studies so far have focused on changes in total triacylglycerol and cholesterol content of analysed tissues but have completely neglected the importance of fatty acid composition. Yet, fatty acids released by WAT control the integrated metabolic responses in obesity and diabetes [11]. By using systemic lipid profiling, 16:1 *n*-7 palmitoleate was identified as an adipose tissue-derived lipid hormone that strongly stimulates insulin action in muscle and suppresses hepatosteatosis [12]. Furthermore, functional derivatives of fatty acids, such as the endocannabinoids, are linked to the development of insulin resistance and obesity [13–15]. In particular, levels of the two most studied endocannabinoids, *N*-arachidonoylethanolamine (also known as anandamide) and 2-arachidonoylglycerol (2-AG), and/or one of their molecular targets, cannabinoid receptor type 1 (CB_1), are upregulated in the epididymal WAT (epiWAT) and liver and downregulated in subcutaneous WAT (subWAT) of mice made obese by following a high-fat diet (HFD) [15, 16]. Since CB_1 activation contributes to de novo lipogenesis in both adipocytes and hepatocytes, dysregulation of the endocannabinoid system is likely to contribute to excess WAT accumulation with subsequent insulin resistance, and hepatic steatosis. Accordingly, CB_1 antagonism reduces fatty liver and insulin resistance in mice with HFD-induced obesity [17], and reduces intra-abdominal obesity, glucose intolerance and hepatic steatosis in abdominally obese individuals [18]. Conversely, experimental elevation of endocannabinoid levels results in impaired ApoE-mediated clearance of triacylglycerols [19], increased fasting glucose and insulin, and excess visceral and liver fat following an HFD [20]. More recent studies on the respective contribution of hepatic and adipose tissue endocannabinoid signalling in HFD-induced insulin resistance and fatty liver in mice provided somewhat conflicting results. Whilst selective *Cb1* (also known as *Cnr1*) knockout in hepatocytes prevents most HFD-induced hypertriacylglycerolaemia, low HDL-cholesterol, fatty liver and insulin resistance [21], studies with a rescue model in which CB_1 receptors are expressed only in hepatocytes over a global *Cb1* null phenotype suggest that, following an HFD, hepatic CB_1 receptors, although sufficient to produce glucose intolerance, alone cause only very little triacylglycerol accumulation in the liver [22]. On the other hand, preliminary data on adipocyte-specific *Cb1*$^{-/-}$ mice also indicate that WAT endocannabinoids play a key role in HFD-induced glucose intolerance and dyslipidaemia [23].

Based on this background, we hypothesised that, relative to wild-type (WT) mice, HFD-induced NASH in *Apoe*$^{-/-}$ mice might be accompanied, on the one hand, by reduced WAT mass, tissue-specific inflammation and improved systemic insulin sensitivity and, on the other hand, by a unique situation in which endocannabinoids in adipose tissue and liver are inversely regulated. This would represent a unique model to investigate the effects on metabolism of HFD-induced endocannabinoid overactivity in the liver concomitantly with reduced endocannabinoid tone in WAT. Therefore, we examined the association between endocannabinoid signalling and the inflammatory

response in WAT and liver of WT and *Apoe*$^{-/-}$ mice fed either a control diet or HFD.

Methods

Animals and diets Animal care and experimental procedures were performed with approval from the animal care committees of the University Medical Center Hamburg-Eppendorf. Male *Apoe*$^{-/-}$ and *Apoe*$^{+/+}$ (WT) mice on the C57BL/6J background (Jackson Laboratory) were used as described in the electronic supplementary material (ESM) Methods. The diets are described in ESM Table 1.

Experiments in isolated hepatocytes from WT and Apoe$^{-/-}$ *mice* Primary hepatocytes were harvested as described [24] and cells seeded in DMEM containing 10% FCS to a density of 200,000 cells per well in collagen-coated 12 well plates. Experiments were carried out as described in the ESM Methods.

Plasma analysis and liver histology Mice were fasted for 4 h prior to being killed at a standardised time of the day. Analyses were carried out as described in the ESM Methods. Paraffin-embedded liver samples were cut and slices were stained with haematoxylin and eosin.

Total RNA extraction and real-time quantitative PCR Total RNA was extracted as described in the ESM Methods. Real-time RT-PCR was performed, and data analysed, as described previously [25, 26] (see ESM Methods).

Liver lipid quantification General procedures are described in the ESM Methods. Total lipids from the liver were extracted as described by Folch et al. [27] and phospholipid-esterified fatty acids were measured as described [28]. Lipid separation was performed according to Hamilton and Comai [29]. Fatty acid methyl esters were prepared based on the method of Lepage and Roy [30], except that toluene was used instead of benzene.

Endocannabinoid extraction and analysis Tissues and hepatocytes were homogenised and extracted, and lipid extracts purified and analysed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionisation/mass spectrometry, as described previously [31] (see ESM Methods).

Assays of fatty acid amide hydrolase and monoacylglycerol lipase activity Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) activities in the liver, epiWAT and subWAT from *Apoe*$^{-/-}$ and WT mice were measured as described in the ESM Methods.

Statistical analysis An unpaired, two-tailed Student's *t* test was used to assess statistical significance between groups. For comparisons between groups of fatty acid and endocannabinoid levels or enzymatic activity, two-way ANOVA followed by the post hoc Bonferroni's test was used.

Results

Effect of HFD on tissue, metabolic and biochemical variables Fasting cholesterol and triacylglycerol levels were higher, and glucose levels lower, in *Apoe*$^{-/-}$ than WT mice after 16 weeks of control diet (Table 1). In both genotypes, a 16 week HFD led to increased body weight and subWAT and epiWAT accumulation, but no changes in liver weight were observed (Table 1). Fasting glucose, insulin, cholesterol and leptin levels were also augmented by HFD, whereas no changes were observed for fasting triacylglycerol and adiponectin levels (Table 1). When compared with WT mice, *Apoe*$^{-/-}$ mice following HFD exhibited lower body weight and subWAT and epiWAT accumulation, lower fasting glucose and insulin, and still higher fasting cholesterol and triacylglycerol levels (Table 1).

Effect of HFD on hepatic steatosis and insulin resistance The *Apoe*$^{-/-}$ mice exhibited more lipid droplets in the liver and higher liver triacylglycerol and cholesterol content than WT mice after 16 weeks of both control diet and, particularly, HFD (Fig. 1 a, b). As shown in Fig. 1c, a 16 week HFD led to impaired clearance of glucose after oral glucose gavage in both genotypes. However, after both a control diet and an HFD, *Apoe*$^{-/-}$ mice exhibited more rapid clearance of glucose. *Apoe*$^{-/-}$ mice also exhibited a more rapid glucose clearance after i.p. insulin injection than WT mice (Fig. 1d).

Effect of HFD on markers of inflammation in the liver and adipose tissue The *Apoe*$^{-/-}$ mice exhibited higher hepatic levels of mRNA for proinflammatory markers such as *Mcp-1* (also known as *Ccl2*), *Tnf-α* (also known as *Tnf*), *Cd68*, *Emr1* and *Cxcl10* than WT mice after 16 weeks of control diet, although differences in *Tnf-α* and *Emr1* mRNA levels were not statistically significant (Fig. 2a). Sixteen weeks of HFD caused statistically significant enhancements of *Mcp-1* and *Cxcl10* expression in both genotypes, of *Tnf-α* only in WT mice, and of *Cd68* only in *Apoe*$^{-/-}$ mice. The latter, however, exhibited higher levels of hepatic *Tnf-α, Cd68* and *Emr1* than WTs following the HFD (Fig. 2a). In epiWAT, no differences existed between genotypes in the expression of *Mcp-1*, *Tnf-α*, *Cd68*, *Emr1* and *Adipoq* following the control diet, whereas the HFD induced a strong and statistically significant elevation of *Mcp-1*, *Tnf-*

Table 1 Plasma variables and organ and body weights in WT (*Apoe*$^{+/+}$) and ApoE-deficient (*Apoe*$^{-/-}$) mice on control diet and HFD

Measurement	*Apoe*$^{+/+}$		*Apoe*$^{-/-}$	
	Control	HFD	Control	HFD
Cholesterol (mmol/l)	3.615±0.64	4.821±0.49***	11.385±1.92†††	9.641±2.44*†††
Triacylglycerols (mmol/l)	0.607±0.12	0.618±0.17	1.169±0.48†††	0.933±0.27††
Glucose (mmol/l)	7.54±1.4	9.85±1.8***	6.27±1.7†	8.42±1.6 *†
Insulin (pmol/l)	103±50	637±330***	138±100	362±190***†
Leptin (ng/ml)	6.1±6.4	26.6±9.9***	3.1±2.2	18.9±8.9***†
Adiponectin (μg/ml)	32.9±5.8	34.6±8.7	28.4±5.0	33.8±5.3
Body weight (g)	33.3±4.1	45.1±4.2***	32.7±5.0	39.6±4.8**†††
Liver weight (g)	1.45±0.1	1.61±0.2	1.49±0.3	1.32±0.3
SubWAT (g)	0.43±0.1	3.76±0.5***	0.35±0.2	1.97±0.8***†††
EpiWAT (g)	0.92±0.2	2.45±0.3***	0.66±0.3	1.91±0.6***

Data are mean±SD of WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice after 16 weeks' control diet or HFD of six to eight animals per group

Means were compared by Student's *t* test with *$p<0.1$; **$p<0.01$; ***$p<0.001$ (control *Apoe*$^{+/+}$ vs HFD and control *Apoe*$^{-/-}$ vs HFD); $^{\dagger}p<0.1$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$ (control *Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$ and HFD-*Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$)

α, *Cd68* and *Emr1* expression and a reduction of *Adipoq*, only in WT mice (Fig. 2b). A similar scenario was observed in subWAT, where, however, the HFD-induced increase of inflammatory markers in WT mice was less marked than in epiWAT (Fig. 2c).

Effect of HFD on endocannabinoid tissue concentrations No significant differences in levels of anandamide and 2-AG were observed between the two genotypes after 16 weeks of control diet. In the liver and subWAT, the levels of 2-AG were ~150- and ~240-fold higher than those of anandamide, whereas in epiWAT, 2-AG levels were only ~8-fold higher than those of anandamide (Fig. 3). In WT mice, HFD caused a strong elevation of anandamide, but not 2-AG, levels in the liver, strong enhancement of both anandamide and 2-AG concentrations in epiWAT, and a strong reduction in both anandamide and 2-AG levels in subWAT (Fig. 3). In *Apoe*$^{-/-}$ mice, HFD caused changes in neither anandamide nor 2-AG levels in the epiWAT, a reduction of only 2-AG levels in subWAT, and an increase of 2-AG, but not anandamide, levels in the liver (Fig. 3).

Effect of HFD on the expression of cannabinoid receptors and of Faah and Magl No significant difference between genotypes following the control diet, nor any statistically significant different effect of HFD, was observed in the liver for Cb_1 and *Faah*. However, *Magl* (also known as *Mgll*) was higher in WT than *Apoe*$^{-/-}$ mice under both dietary conditions, and the diet did not affect the expression of this gene in either genotype (Fig. 4a). In epiWAT, again no significant difference between genotypes was observed following the control diet, except for a ~3-fold higher expression of *Faah* in WTs. Following HFD, levels of expression of *Faah* decreased ~10-fold in WT but not in *Apoe*$^{-/-}$ mice, whereas no other changes were observed for the other genes in either genotype, except for a small decrease and increase in Cb_1 and Cb_2 (also known as *Cnr2*) mRNA expression, respectively, only in WTs (Fig. 4c). In subWAT, the only genotypic difference in expression following the control diet was observed for *Faah*, which was ~3-fold higher in *Apoe*$^{-/-}$ mice. HFD caused a strong ~5-fold decrease in Cb_1 (also known as *Cnr1*) expression, and a slight, non-statistically significant increase in *Faah* expression in WT mice, whereas in *Apoe*$^{-/-}$ mice only a small decrease in *Faah* and Cb_2 mRNA expression was observed (Fig. 4c).

Effect of HFD on FAAH and MAGL activity The results obtained in the assays of FAAH and MAGL activity did not always reflect the changes in expression, or lack thereof, observed in the quantitative PCR experiments (Table 2). In brief, in the liver, MAGL assays did not confirm the slightly higher expression of this enzyme in WT vs *Apoe*$^{-/-}$ mice, observed with quantitative PCR, irrespective of the type of diet. Regarding FAAH, in WT mice, differences were observed only with 1 and 5 μg protein (from 712±120 to 1,108±74 pmol min^{-1}[mg protein]$^{-1}$ and from 487±70 to 799±64 pmol min^{-1}[mg protein]$^{-1}$, respectively for 1 and 5 μg of protein; $p<0.05$). In *Apoe*$^{-/-}$ mice, a small decrease in FAAH activity was observed only with 15 μg protein (from 801±67 to 595±73 pmol min^{-1}[mg protein]$^{-1}$, $p<0.05$). In epiWAT, a 16 week HFD was accompanied by a strong decrease in FAAH activity in WT but not in *Apoe*$^{-/-}$ mice, whereas no HFD-induced changes in MAGL activity were observed. In subWAT, FAAH activity increased following HFD in WT mice and decreased in

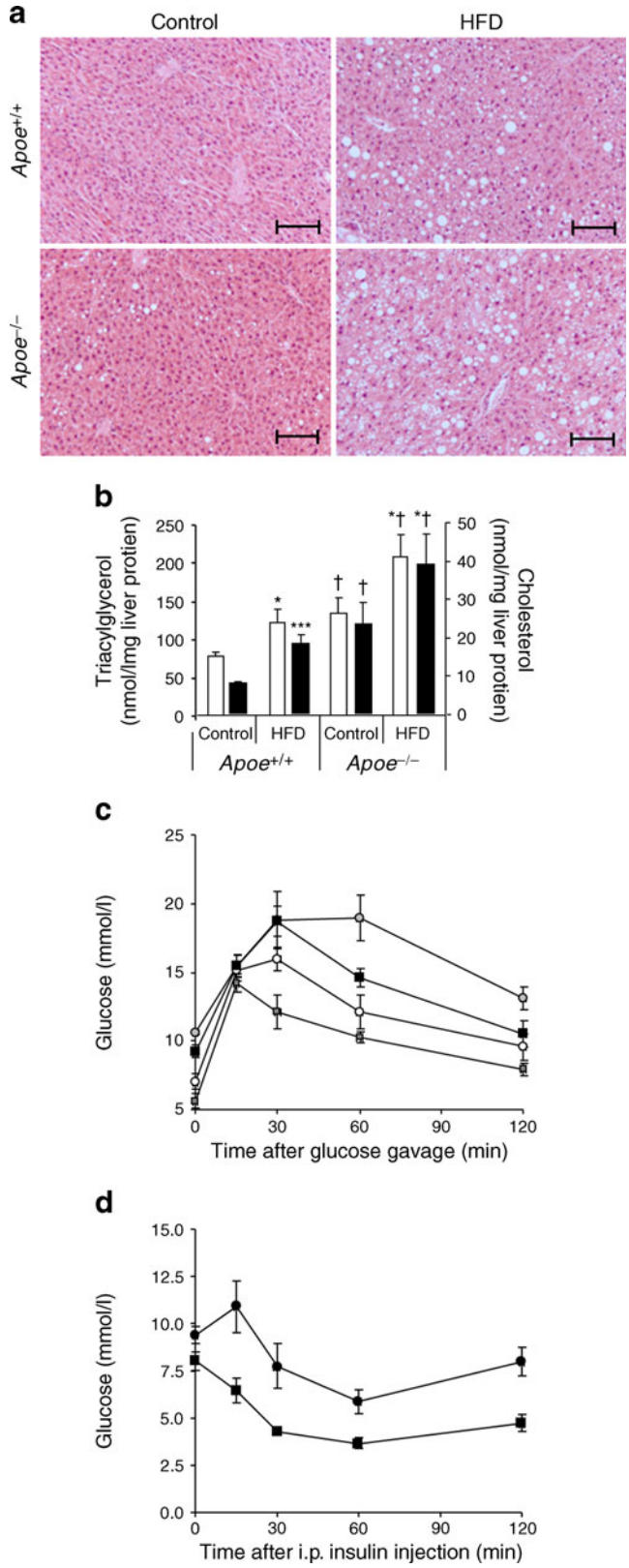

Fig. 1 Hepatic lipid concentration, oral glucose and insulin tolerance in WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice. **a** Representative pictures of haematoxylin and eosin stained liver sections of WT and *Apoe*$^{-/-}$ mice after 16 weeks control diet or HFD. Scale bar 100 μm. **b** Total triacylglycerol (white bars) and cholesterol (black bars) content per mg liver protein (n=6 per group). **c** Plasma glucose levels of WT and *Apoe*$^{-/-}$ mice after 16 weeks' control diet or HFD before and after gavage of glucose (1.5 g/kg) (white circles, *Apoe*$^{+/+}$ control; grey circles *Apoe*$^{+/+}$ HFD; grey squares, *Apoe*$^{-/-}$ control; black squares, *Apoe*$^{-/-}$ HFD). **d** Plasma glucose levels of WT (circles) and *Apoe*$^{-/-}$ (squares) mice after 16 weeks' HFD before and after i.p. injection of insulin (1 U/kg). Means±SEM were compared by Student's t test with *p<0.1; ***p<0.001; control *Apoe*$^{+/+}$ vs HFD and control *Apoe*$^{-/-}$ vs HFD; $^{\dagger}p$<0.1; control *Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$ and HFD-*Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$

Apoe$^{-/-}$ mice, again with no HFD-induced changes in MAGL activity. A small increase in MAGL activity in *Apoe*$^{-/-}$ mice, irrespective of the diet, was also observed in this tissue (Table 2).

Effect of HFD on phospholipid-esterified fatty acid composition of the liver The diet basically caused no decrease in phospholipid-esterified arachidonic acid (AA) in *Apoe*$^{-/-}$ compared with WT mice (Fig. 5), which might underlie at least in part the increased 2-AG levels following HFD observed in this genotype vs WT mice (Fig. 3). Compared with WT mice, phospholipid-esterified saturated fatty acids were decreased whereas polyunsaturated fatty acids were increased in HFD-treated *Apoe*$^{-/-}$ mice, suggesting a reduced de novo lipogenesis in *Apoe*$^{-/-}$ mice. Palmitoleate and oleate were reduced to the same extent in WT and *Apoe*$^{-/-}$ mice by HFD treatment, indicating that these phospholipid-esterified fatty acids cannot explain the difference in severity of HFD-induced NASH between WT and *Apoe*$^{-/-}$ mice.

Effect of TNFα and excess fatty acids on endocannabinoid levels in primary hepatocyte cultures We found that TNF-α stimulated anandamide, but not 2-AG, levels in WT, and 2-AG, but not anandamide, levels in *Apoe*$^{-/-}$ mice (Table 3). We also observed that oleic acid only resulted in a small reduction of anandamide, but not 2-AG, levels only in *Apoe*$^{-/-}$ mice (Table 3). Finally, AA increased 2-AG, but not anandamide, levels in *Apoe*$^{-/-}$ mice (Table 3).

Discussion

Using a diet-induced obesity model, the present study investigated the role of ApoE, first, in the development of high WAT mass and liver triacylglycerol levels and, hence, in the inflammatory status of these two organs and its most likely consequences, such as glucose intolerance and hepatic steatosis; and, second, in the WAT and hepatic tone of the endocannabinoid system, a key player in determining de novo lipogenesis and inflammatory cytokine release in rodents [32]. We report that, following an HFD, *Apoe*$^{-/-}$, compared with WT, mice develop: (1) increased fatty liver but reduced WAT; (2) a stronger inflammatory profile (i.e.

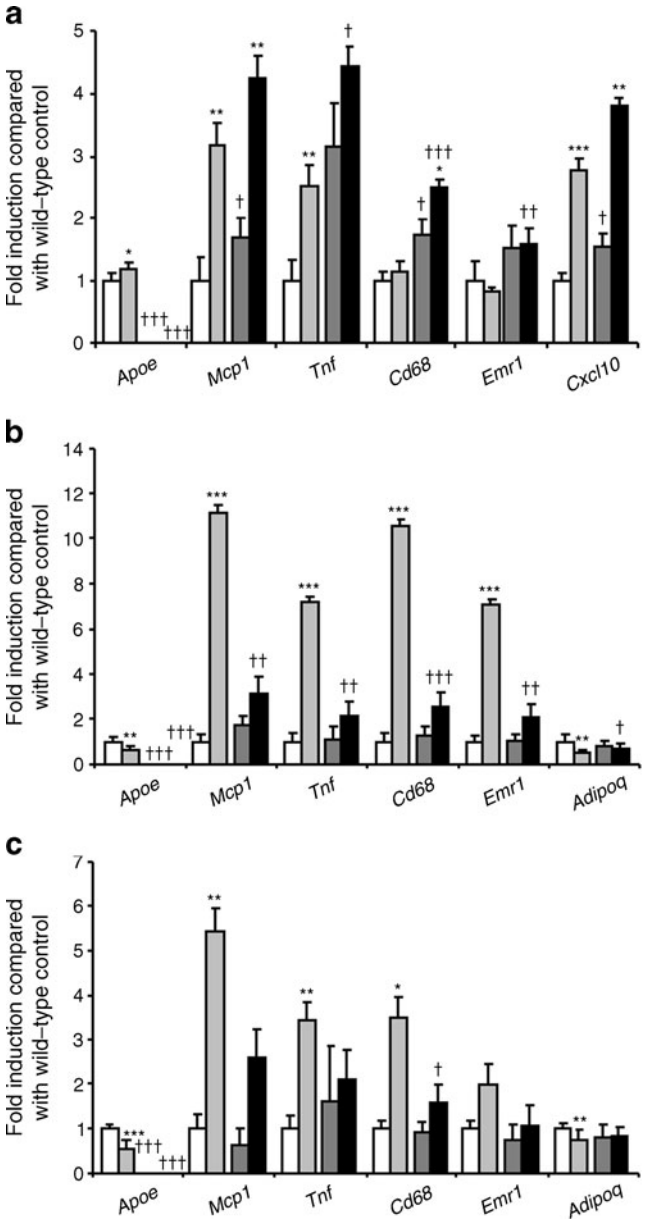

Fig. 2 Expression levels of the mRNA for *Apoe* and inflammatory genes in the liver (**a**), epiWAT (**b**) and subWAT (**c**) of WT and *Apoe*$^{-/-}$ mice after 16 weeks' control diet or HFD. In adipose tissues the expression levels of the mRNA for anti-inflammatory *Adipoq* were also determined. Data are presented as fold-change compared with WT (*Apoe*$^{+/+}$) control mice (n=6 per group). Means±SEM were compared by Student's t test with *p<0.1; **p<0.01; ***p<0.001 (control *Apoe*$^{+/+}$ vs HFD and control *Apoe*$^{-/-}$ vs HFD); $^{\dagger}p$<0.1; $^{\dagger\dagger}p$<0.01; $^{\dagger\dagger\dagger}p$<0.001 (control *Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$ and HFD-*Apoe*$^{+/+}$ vs *Apoe*$^{-/-}$). White bars, *Apoe*$^{+/+}$ control; light grey bars, *Apoe*$^{+/+}$ HFD; dark grey bars, *Apoe*$^{-/-}$ control; black bars, *Apoe*$^{-/-}$ HFD

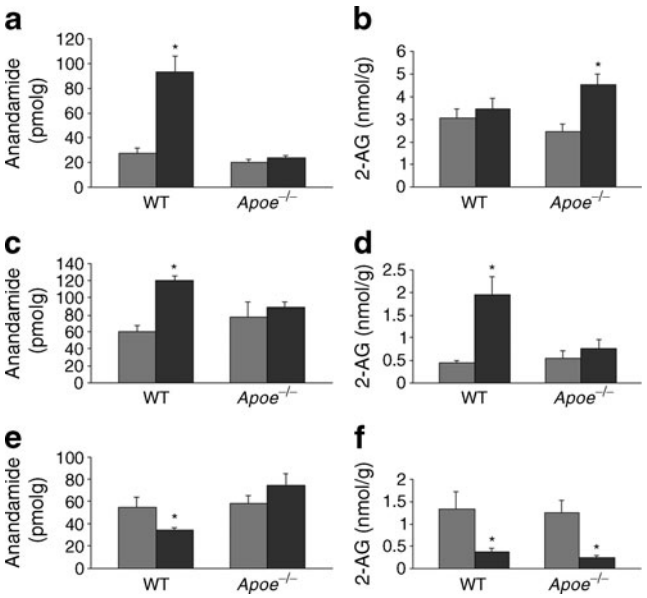

Fig. 3 Levels of the endocannabinoids anandamide (in picomoles/gram wet tissue weight) and 2-AG (in nanomoles/gram wet tissue weight) in the liver (**a**, **b**), epiWAT (**c**, **d**) and subWAT (**e**, **f**) of WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice after 16 weeks' control diet (light grey bars) or HFD (dark grey bars). Data are mean±SE (n=4). Means were compared by ANOVA followed by Bonferroni's test. *p<0.05 vs the respective control

higher expression of *Tnf-α* and *Emr1*, and of the macrophage marker *Cd68*) in the liver and a lower inflammatory profile (i.e. lower expression of *Mcp-1*, *Tnf-α*, *Cd68* and *Emr1*, and higher expression of *Adipoq*) in the epiWAT; and (3) reduced glucose intolerance and insulin resistance. Regarding endocannabinoid signalling in the three tissues analysed, we found that, following HFD, *Apoe*$^{-/-}$, compared with WT, mice develop: (1) lower levels of anandamide but higher levels of 2-AG in the liver; (2) no decreased anandamide levels and Cb_1 receptor expression levels in the subWAT; and (3) unchanged levels of both anandamide and 2-AG in the epiWAT. In view of the stimulatory effect on hepatic and WAT lipogenesis by endocannabinoids and CB_1 [17, 33, 34], and of the association between hepatic triacylglycerol accumulation and hepatic inflammatory markers, and between low epiWAT lipid accumulation/inflammation and insulin sensitivity [34], these findings suggest that the observed ApoE-deficiency-associated and HFD-induced metabolic changes might be due to corresponding changes in endocannabinoid tone in the liver and WAT. By contrast, metabolic differences between WT and *Apoe*$^{-/-}$ mice following a normal diet, observed here and previously [9], cannot be accounted for by differences in endocannabinoid levels, which were very similar in the two strains in all tissues.

Since hypertrophic adipose tissue mass is associated with an increased risk of developing insulin resistance, dyslipidaemia and cardiovascular disease [35], interest has increased in deciphering factors linking lipid metabolism directly or indirectly with insulin signalling. Gao et al. established *Apoe*$^{-/-}$ mice on a genetically obese background (the KK-Ay mice) [9] and, similarly to our results, showed that ApoE deficiency protected against diet-

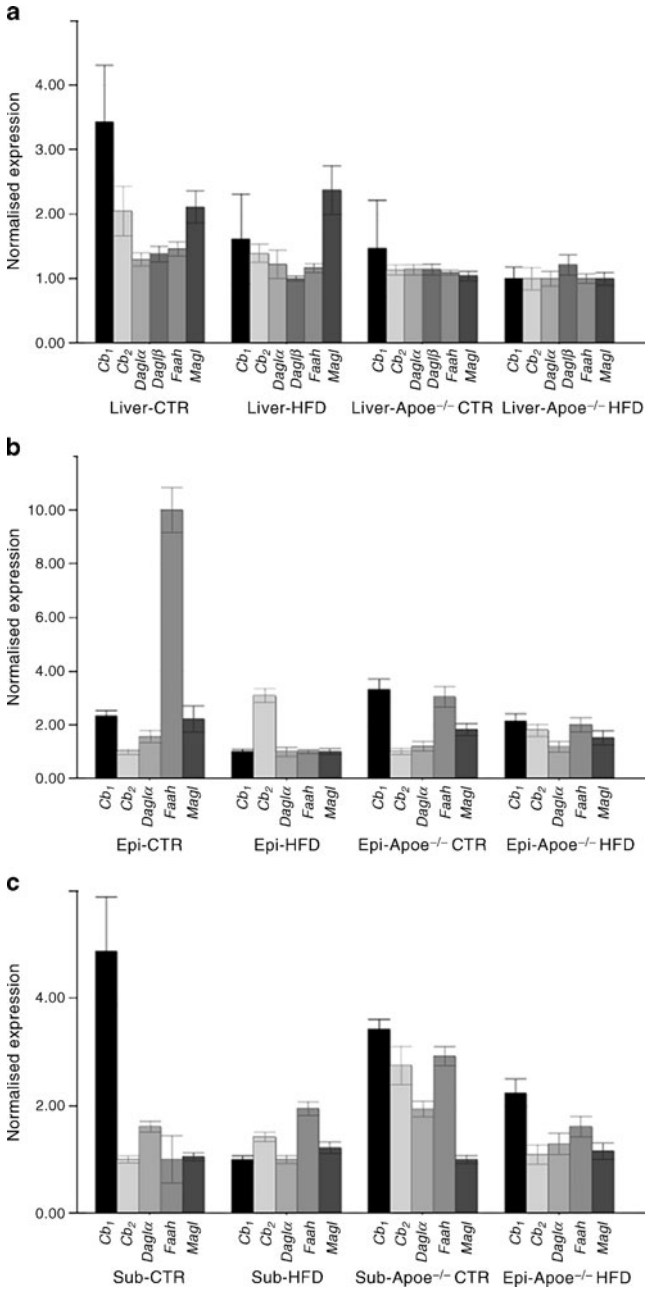

Fig. 4 Quantitative determination of the mRNA encoding Cb_1 and Cb_2 receptors, and for $Dagl\alpha$ (also known as *Dagla*), *Faah* and *Magl* obtained from WT ($Apoe^{+/+}$) or $Apoe^{-/-}$ mice fed a control diet (CTR) or an HFD. $Dagl\beta$ (also known as *Daglb*) was only quantified the liver (**a**) because only in this tissue has the enzyme been shown to play a role in 2-AG biosynthesis. In (**a**) data for the liver are shown, and no statistically significant differences were detected between genotypes or diets, except for *Magl*, which was higher in WTs under both dietary conditions ($p<0.05$). In (**b**) data from the epididymal (Epi) white adipose tissue are shown, and the only statistically significant differences ($p<0.05$) were observed with *Faah* between WT mice fed a control diet and those fed an HFD, and between WT mice fed a control diet and $Apoe^{-/-}$ mice fed a control diet, as well as with Cb_1 and Cb_2 between WT mice fed a control diet and those fed an HFD. In (**c**) data from the subcutaneous (Sub) white adipose tissue are shown, and the only statistically significant differences ($p<0.05$) following the HFD were observed in WT mice with Cb_1, and in $Apoe^{-/-}$ mice with Cb_2 and *Faah*

induced obesity, glucose intolerance and insulin resistance. However, unlike the diet-induced model in the present study, ApoE deficiency in this previous case was accompanied by lower hepatic steatosis, possibly due to reduced hepatic uptake of ApoE-deficient VLDL [9], thus emphasising how *Apoe* knockout can produce different metabolic phenotypes in different genetic backgrounds. Here, we hypothesise that, in HFD-fed $Apoe^{-/-}$ mice, worsening of triacylglycerol accumulation in the liver is due to increased de novo lipogenesis in hepatocytes, in turn caused by overstimulation of CB_1 receptors. In fact, the typical upregulation of endocannabinoid levels in the liver of mice with HFD-induced obesity [17], and of obese Zucker rats [36], is exacerbated in HFD-fed ApoE-deficient mice, in which the increased levels of the most abundant endocannabinoid, 2-AG (+2 nmol/g), might have compensated for the reduction in anandamide levels (−0.09 nmol/g), thus leading to a net higher molar concentration of hepatic endocannabinoids (+1.9 nmol/g). Indeed, both anandamide and 2-AG stimulate lipogenesis in hepatocytes via CB_1 activation, under different conditions [17, 37]. However, although 2-AG is always more abundant than anandamide in tissues, much of it (~20–50%) was estimated to constitute a non-signalling pool [38]. Therefore, the increase in 2-AG levels might not necessarily offset the reduction in anandamide levels. Recent data questioned the role of overactive hepatic CB_1 receptors as a strong determinant of fatty liver following HFD in mice [22], whereas they supported its key function in glucose intolerance [21, 22]. Thus, the absence of an HFD-induced rise in hepatic anandamide may contribute to the improved glycaemic control observed here in $Apoe^{-/-}$ mice.

ApoE levels modulate adipocyte triacylglycerol content and turnover [8] through molecular mechanisms that have not been investigated so far. We found here that the typical upregulation of endocannabinoid levels observed in the epiWAT of mice with HFD-induced obesity [34, 39], and in the visceral WAT of obese and overweight human beings [40–43], was absent in HFD-fed $Apoe^{-/-}$ mice. Conversely, downregulation of endocannabinoid levels and Cb_1 receptor expression observed in the subWAT of mice with HFD-induced obesity [16], and typical also of Zucker rats and overweight and obese humans [36, 40, 41], was attenuated by ApoE deficiency. Thus, given the proposed contribution of CB_1 overactivity in epiWAT vs subWAT to visceral fat accumulation and inflammation [33], and of the latter to insulin resistance [35], we hypothesise that differential changes in adipose tissue endocannabinoid signalling in the two genotypes following HFD underlies, at least in part, the lower epiWAT accumulation and inflammation, and the

Table 2 Enzymatic activities of FAAH and MAGL in the liver, epiWAT and subWAT of WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice after a 16 week control diet (CTR) or HFD

Tissue	Genotype	Diet	FAAH	MAGL
Liver	Apoe$^{+/+}$	CTR	223±7	1,253±60
		HFD	249±7	1,279±120
	Apoe$^{-/-}$	CTR	251±26	1,299±29
		HFD	223±10	1,254±61
EpiWAT	*Apoe*$^{+/+}$	CTR	21±2	1,252±19
		HFD	11±2*	1,284±47
	Apoe$^{-/-}$	CTR	17±2†	1,220±88
		HFD	18±1†	1,108±36†
SubWAT	*Apoe*$^{+/+}$	CTR	34±7	621±51
		HFD	52±3*	669±18
	Apoe$^{-/-}$	CTR	22±2	722±21†
		HFD	16±2*,†	723±26†

Activities are in picomoles (milligram protein)$^{-1}$ minute^{-1}. Data are mean±SD (n≥3), and were calculated using 35, 40 and 70 μg membrane proteins (FAAH) or 90, 100 and 100 μg cytosol proteins (MAGL) for subWAT, epiWAT and liver, respectively. In the liver, lower amounts of protein (1, 5 and 15 μg) were also used to carry out the FAAH assay (see Results). Means were compared by ANOVA followed by Bonferroni's test. *p<0.05 vs the respective control. $^{\dagger}p$< 0.05 vs the respective WT value

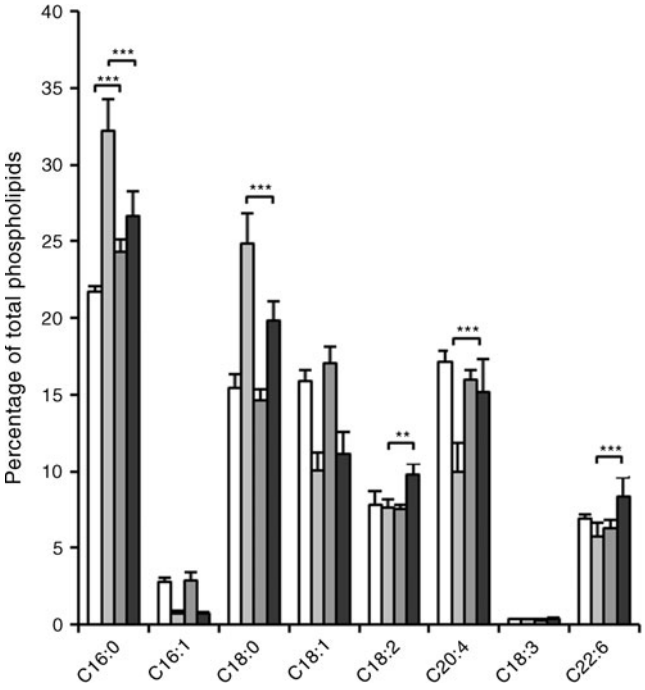

Fig. 5 Phospholipid-esterified fatty acid content of WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice after 16 weeks' control diet or HFD in livers of fasted mice. Data are presented as per cent of total phospholipid fatty acids (n=6 per group). Means were compared by two-way ANOVA followed by Bonferroni's post hoc test for multiple testing; statistical significance is only shown between means of different genotypes on the same diet; **p<0.01; ***p<0.001. A full panel of statistical analyses can be found in ESM Table 2. White bars, *Apoe*$^{+/+}$ control; light grey bars, *Apoe*$^{+/+}$ HFD; mid-grey bars, *Apoe*$^{-/-}$ control; dark grey bars, *Apoe*$^{-/-}$ HFD

reduced glucose intolerance, that HFD-*Apoe*$^{-/-}$ mice exhibit compared with HFD-WT mice.

In *Apoe*$^{-/-}$ mice on HFD we also observed higher levels of several *n*-3 fatty acids, suggesting generally higher activities of enzymes synthesising polyunsaturated fatty acids (PUFA). The higher levels of PUFA, in turn, may be responsible for the paradoxical lower levels of palmitic acid in liver phospholipids compared with WT mice, which suggests decreased de novo lipogenesis-associated fatty acids. Recently is has been shown that palmitoleate attenuates fatty acid induced insulin resistance and NASH [12, 44]. However, in our study, palmitoleate was reduced to the same extent in WT and *Apoe*$^{-/-}$ mice by HFD treatment, indicating that this lipokine cannot explain the difference in HFD-induced NASH between WT and *Apoe*$^{-/-}$ mice.

In the two WAT depots analysed here, differential changes in the production of endocannabinoid metabolic enzymes following HFD in the two genotypes, which were largely confirmed by the finding of corresponding changes in the respective enzymatic activities, are likely to account for a large part of the observed differential changes in endocannabinoid levels. In fact, in epiWAT, the strong decrease in FAAH production and activity in WT mice might have caused the increase in both anandamide and 2-AG levels in this tissue, as has also been previously suggested for obese humans [42]. The fact that such a decrease did not occur in *Apoe*$^{-/-}$ mice might explain, at least in part, why HFD did not cause elevation of either anandamide or 2-AG levels in the epiWAT of these transgenic mice. Likewise, in the subWAT of *Apoe*$^{-/-}$, but not WT, mice there was a decrease of *Faah*

Table 3 Endocannabinoid levels in isolated hepatocytes from WT (*Apoe*$^{+/+}$) and *Apoe*$^{-/-}$ mice

Mouse type	Treatment	Anandamide (pmol/2×10^5 cells)	2-AG (pmol/2×10^5 cells)
WT	Mock	0.44±0.01	1.5±0.6
	TNF-α	0.60±0.10*	1.9±0.3
	Oleic acid	0.3±0.1	1.9±0.1
	Arachidonic acid	0.34±0.03	2.1±0.3
Apoe$^{-/-}$	Mock	0.6±0.02†	0.8±0.04
	TNF-α	0.4±0.1*,†	1.3±0.2*
	Oleic acid	0.3±0.05*	0.8±0.1†
	Arachidonic acid	0.3±0.02*	1.6±0.01*

Mice were treated with either 2 ng/ml TNF-α, 100 μmol/l oleic acid or 100 μmol/l arachidonic acid for 20 h at 37°C. Data are means±SD of n=3 experiments. Means were compared by ANOVA followed by Bonferroni's test

*p<0.05 vs respective vehicle; $^{\dagger}p$<0.05 vs respective WT

expression and activity, which might have counteracted the typical HFD-induced decrease of anandamide levels in this tissue. However, we speculate that, since this decrease in FAAH expression/activity was not as strong as that observed in the epiWAT of WT mice, and since 2-AG is not a preferential substrate for FAAH, it did not lead also to higher subWAT 2-AG levels. Despite the fact that alterations in *Faah* expression after shorter periods (3–8 weeks) of HFD were previously found to underlie the elevation of anandamide levels in mouse liver [17], we found changes in neither the production nor activity of this enzyme that could account for the alterations in hepatic anandamide levels found here in HFD-fed WT mice. In HFD-fed *Apoe*$^{-/-}$ mice, FAAH activity did decrease to some extent, and this change might contribute to the observed elevation of 2-AG levels in the liver, since neither production nor activity of the other 2-AG degrading enzyme, MAGL, changed in either genotype following HFD. However, if decreased FAAH activity participated in increasing 2-AG levels in the liver, this would not explain why hepatic anandamide concentrations did not increase too. Hepatic 2-AG levels are also controlled by the biosynthetic enzymes diacylglycerol lipase (DAGL)α and β, but, again, the production of these enzymes did not change with genotype and diet. Nevertheless, we observed that HFD decreased levels of phospholipid-esterified AA in WT, but not *Apoe*$^{-/-}$, mice. Since *sn*-2-arachidonate-containing phospholipids act as ultimate biosynthetic precursors for 2-AG, we hypothesise that the elevation of 2-AG levels in the liver of *Apoe*$^{-/-}$ vs WT mice on HFD was due to the relatively higher content of phospholipid-esterified AA and, therefore, to higher availability of the biosynthetic precursors for this endocannabinoid. An analogous mechanism was previously described in isolated adipocytes [45].

In order to investigate further the differential regulation of hepatic endocannabinoid, and particularly anandamide, levels by HFD in WT and *Apoe*$^{-/-}$ mice, we prepared primary hepatocytes from the two types of mice and incubated them either with TNF-α, thus mimicking the effect of inflammation, or oleic acid and AA, to simulate the effect of HFD or of higher availability of *n*-6 PUFA, respectively [45]. In agreement with the finding of elevated hepatic anandamide, but not 2-AG, levels in HFD-WT mice, and of elevated 2-AG, but not anandamide, levels in HFD-*Apoe*$^{-/-}$ mice, we found that TNF-α, the levels of which were elevated following HFD significantly more in *Apoe*$^{-/-}$ than WT mice, stimulated anandamide, but not 2-AG, levels in WT mice, and 2-AG, but not anandamide, levels in *Apoe*$^{-/-}$ mice. This reflects the changes observed in hepatic anandamide and 2-AG levels following HFD in the two genotypes and, therefore, might suggest that HFD differentially dysregulates hepatic endocannabinoid levels in the two types of mice at least in part via TNF-α signalling. However, we also observed that oleic acid caused a small, but significant, reduction of hepatocyte anandamide, but not 2-AG, levels only in *Apoe*$^{-/-}$ mice, which might also explain why HFD did not cause anandamide elevation in the liver of these mutated animals. Finally, we observed that AA increased 2-AG, but not anandamide, levels in *Apoe*$^{-/-}$, but not WT, mice, in agreement with previous findings in adipocytes [45] and with the hypothesis that increased hepatic 2-AG levels are mostly determined by enhanced AA availability. Under no condition in vitro, however, were hepatocyte 2-AG levels higher in *Apoe*$^{-/-}$ mice than in WT mice. This suggests either that a combination of factors (i.e. higher levels of both TNF-α and AA) is necessary to cause HFD-induced elevation of hepatic 2-AG levels in *Apoe*$^{-/-}$ mice, or that cell types other than hepatocytes contribute to 2-AG levels in the liver. Indeed, 2-AG can also be produced by hepatic stellate cells and act on hepatocytes in a paracrine manner to induce lipogenesis [37]. Regarding the HFD-induced increase in hepatic anandamide levels in WT mice, found here and previously [17], although the effects of oleic acid and TNF-α on hepatocytes in vitro might underlie this phenomenon, it remains to be explained how such an increase may have occurred under our conditions, since we found no HFD-induced increase in AA-esterified phospholipids, nor could we confirm the previously observed downregulation of FAAH (detected, however, after 3 weeks of HFD [17]). It is possible that, in WT, but not *Apoe*$^{-/-}$, mice, our 16 week HFD caused an enhancement of production and/or activity of anandamide biosynthetic enzymes, which we did not evaluate because of the redundancy in anandamide anabolic pathways [33].

In conclusion, we present here unprecedented evidence that tissue-specific dysregulation of the endocannabinoid system is associated with the presence of ApoE. This finding pairs with the previously reported observation that pharmacologically induced endocannabinoid overactivity impairs ApoE function [19], and suggests that there is cross-talk between these two important regulators of metabolism and hepatic function. Furthermore, our data confirm the existence of a strong correlation between altered distribution of endocannabinoid signalling in metabolically distinct WAT depots and the accumulation of fat and the inflammatory profile of visceral WAT. This, together with dysregulated hepatic endocannabinoid signalling [17, 21, 22], plays an important role in determining glucose intolerance and insulin resistance [23, 34, 38]. Nevertheless, given the mostly correlative nature of our findings, further mechanistic studies will need to be performed in order to understand how, in response to an HFD, ApoE deficiency affects endocannabinoid levels and, hence, peripheral glucose and lipid metabolism.

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