Probucol suppresses enterocytic accumulation of amyloid-β induced by saturated fat and cholesterol feeding.

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Abbreviations:

AD	Alzheimer's disease
Aβ	Amyloid-β
Apo B	Apolipoprotein B
HF	High-fat
LF	Low-fat
TAG	Triacylglycerol(s)

Abstract

Amyloid-β (Aβ) is secreted from lipogenic organs such as intestine and liver as an apolipoprotein of nascent triacylglycerol rich lipoproteins. Chronically elevated plasma Aβ may compromise cerebrovascular integrity and exacerbate amyloidosis, a hallmark feature of Alzheimer's disease (AD). Probucol is a hypocholesterolemic agent that reduces amyloid burden in transgenic amyloid mice, but the mechanisms for this effect are presently unclear. In this study the effect of Probucol on intestinal lipoprotein-Aβ homeostasis was explored. Wild-type mice were fed a control low-fat diet and enterocytic Aβ was stimulated by high-fat (HF) diet enriched in 10% (w/w) saturated fat and 1% (w/w) cholesterol for the duration of 1 month. Mice treated with Probucol had the drug incorporated into the chow at 1% (w/w). Quantitative immunofluorescence was utilised to determine the intestinal apo B and Aβ abundance. We found that apo B is found in both perinuclear region of the enterocytes and the lacteals in all groups. However, HF feeding and Probucol treatment increased secretion of apo B into the lacteals without any changes in the net villi abundance. On the other hand, HF induced enterocytic perinuclear Aβ was significantly attenuated by Probucol. No significant changes of Aβ were observed within the lacteals. The findings of this study support the notion that Probucol suppresses dietary fat induced stimulation of Aβ biosynthesis and attenuate availability of apo B lipoprotein-Aβ for secretion.

Keywords: Apolipoprotein B; Amyloid-β; Probucol; Small intestine; Saturated fat; Cholesterol.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by neuronal cell loss and amyloid-beta (Aβ) deposition on extracellular matrices and within the cerebrovasculature [1]. Amyloid-β is present at sub-nanomolar levels in most biological fluids, such as cerebrospinal fluid and plasma [2] and at physiological levels, regulates cell growth [3-5]. An hydrophobic protein, Aβ may undergo oligomerization when it becomes disassociated from chaperone proteins that ordinarily facilitate kinetics and metabolism. It is the fibrillar form of Aβ which is thought to trigger pro-inflammatory pathways that compromise neuronal integrity [6, 7].

The origin of cerebrovascular Aβ deposits in AD is presently unclear. Amyloid-β may be generated as a consequence of proteolytic processing of the amyloid-precursor-protein, expressed in significant quantities on the plasma membrane of neuronal cells [8]. However, biogenesis of Aβ is not increased in sporadic and late onset AD, the most common phenotype of AD [9, 10]. Rather, accumulating evidence suggests that enhanced blood-to-brain delivery relative to efflux, or via Aβ degradative pathways within the choroid plexus results in extracellular retention of Aβ and thereafter, inflammatory sequelae [11, 12].

Significant Aβ in blood is associated with apolipoprotein B (apo B) lipoproteins, particularly those enriched in triacylglycerol (TAG). Subjects with AD have greater apo B lipoprotein-Aβ relative to age matched controls [13] and in transgenic amyloid mice, onset and progression of cerebral amyloidosis is strongly associated with the secretion into and concentration of plasma apo B lipoprotein-Aβ [14]. Apolipoprotein B immunoreactivity is evident in parenchymal amyloid plaque from human cadaver specimens [15] and in Aβ-transgenic mice, cerebral apo B distribution and abundance strongly colocalise with extracellular deposits of Aβ [16], observations consistent with a vascular contribution to disease aetiology.

A range of lipoprotein (lipid)-lowering agents commonly used in clinical practice for the prevention and treatment of cardiovascular disease and may reduce AD risk by reducing cerebrovascular exposure to apo B lipoprotein-Aβ. The hydroxy-methyl-glutaryl coenzyme A reductase inhibitors lower plasma cholesterol by enhancing apo B lipoprotein clearance via high affinity receptor pathways and inhibiting apo B lipoprotein biogenesis [17-19]. Similarly, fibrates reduce plasma TAG by suppressing lipogenesis, a driver for the secretion of apo B lipoproteins [20]. Population and clinical studies generally support a risk reduction

for all forms of dementia in subjects taking lipid-lowering agents [21, 22], although the mechanisms for this association are not clear.

Probucol is an older generation cholesterol-lowering agent that reduces plasma cholesterol by enhancing uptake via receptor pathways [23, 24]. However, other properties of Probucol make this a particularly interesting lipid lowering agent relative to the plasma kinetics and metabolism of apo B-lipoprotein-Aβ. Probucol is hydrophobic and secreted into blood incorporated within the nascent TAG rich apo B lipoproteins, a phenomenon which may influence Aβ association with and secretion of these macromolecules [23, 25]. In addition, lipoproteins that contain Probucol are almost exclusively cleared from circulation by the liver and consequentially vascular retention is substantially reduced [23]. A small clinical study suggested that Probucol reduced cognitive decline in subjects with mild cognitive impairment [26, 27] and consistent with the human findings, studies in transgenic amyloid mice showed that Probucol reduced the severity of amyloidosis [26]. In the latter, enhanced Aβ-efflux was put forward as one possible mechanism for the Probucol induced effects.

The putative effects of Probucol on Aβ biogenesis and lipoprotein synthesis in lipogenic organs have not been reported. This study utilized an *in vivo* high-fat (HF) feeding model previously shown to stimulate enterocytic abundance of Aβ, to determine if Probucol modulates the secretion of apo B lipoprotein-Aβ from absorptive epithelial cells of the small intestine, a major site of Aβ biosynthesis [28-30].

Methods and Materials

Animals and diet conditions

The Curtin University Animal Experimentation and Ethics Committee approved the animal housing, handling and experimental procedures described. Seven-week-old female wild-type mice (C57BL/6J) were housed in groups and randomized into the diet or drug treatment groups (n = 8 mice per group). All mice were maintained on a 12 h light and dark cycle room, at 22^{o}C and with free access to water and food. The low-fat control diet was standard AIN93M rodent chow containing <4% (w/w) fat as polyunsaturates, with <1% total digestible energy as lipids and was free of cholesterol (Glen Forrest Stockfeeders, Perth Western Australia). To stimulate enterocytic Aβ production, the control feed was replaced

with a HF diet enriched in saturated fats 10% (w/w) and 1% (w/w) cholesterol (Glen Forrest Stockfeeders). The principal fatty acid types in the HF treatment group were palmitic (16:0) and stearic (18:0) (total of 13% w/w) and oleic acid (18:1 n9, 6% w/w). Mice treated with Probucol (Sanofi-Aventis, Paris) had the drug incorporated into the chow at 1% (w/w) at the time of feed manufacture in order to achieve an estimated dose rate of 30mg/day [23].

Tissue collection and sample preparation

Mice were maintained for 32 days on the indicated diets and weighed weekly. Thereafter, mice were anesthetised with pentobarbitone (45 mg/kg i.p.) and exsanguinated by cardiac puncture. Blood was collected into heparin tubes and stored in ice. Plasma was separated by short speed centrifugation at 4^{o}C and stored at -80°C.

A 2 cm segment of the small intestine duodenum at the proximal end was isolated, flushed with chilled phosphate buffer saline (PBS, pH 7.4) and fixed in 10% buffered formal saline for a minimum of 24 h. The tissues were then processed and longitudinal segments embedded in paraffin wax. Serial sections of 5 μm thick were cut and mounted on silanised slides for histology and immunofluorescence.

Amyloid-β and apolipoprotein B Immunofluorescence

Intestinal Aβ and apo B were detected by immunoflourescent amplification method as previously described [31]. Intestinal tissue sections (5 μm) were deparaffinised, rehydrated and antigen-retrieval was carried out in boiling deionised water for 15 min. Briefly, all sections were permeabilised in PBS and incubated in blocking serum (20% goat serum).

For Aβ staining, polyclonal rabbit anti-human $A\beta_{1\text{-}40/42}$ antiserum (AB5076, Chemicon Temecula, CA), diluted to 1:2000 in PBS was incubated overnight at 4°C. The specificity of the antibodies was previously established [28]. Sections were washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1:2000 dilution) (E0432, DAKO, Carpentaria, CA) at room temperature for 1 h, followed by incubation with Streptavidin-Alexa Fluor ®546 (1:300 dilution) (S11225, Invitrogen, Victoria, Australia) for another hour in the dark for amplification. The nuclei were counterstained with DAPI (1:1000 dilution) (Invitrogen, Victoria, Australia) for 5 min. The sections were then mounted with antifade mounting medium.

Enterocytic apo B was determined essentially as described for Aβ detection. Polyclonal rabbit anti-mouse apo B (ab20737, Abcam, Cambridge, UK) as primary and the biotinylated goat anti-rabbit secondary antibody was used at 1:2250 dilutions.

Imaging

Digital images for photo microscopy were acquired through AxioCam HRm camera (Zeiss Germany) with an AxioVert 200M inverted microscope by Zeiss (Germany) at ×200 magnification (Plan Neofluar x20 objective, 1.3 numerical aperture). Excitation and emission were achieved by using filters 43 (Ex BP545/25, beam splitter FT570 and Em BP605/70) and filter 49 (Ex G365, beam splitter FT395 and Em BP445/50) to determine fluorescence of Alexa Fluor ®546 and DAPI, respectively. Individual channels are free from fluorescence from other emission sources and are therefore clear of overlap. Each image was captured under identical exposure times utilising AxioVision software (version 4.7.1) to avoid artificial modification in pixel intensity.

Quantitative immunofluorescent imaging and analysis

Images were collected at ×200 magnification and approximately 30-50 images were captured per group showing at least 4 villi in each image (1388 x 1040 pixels per image). Pixel intensity for each fluorescent dye was obtained by calculating the densitometric sum by Automatic Measurement Program in AxioVision (Software version 4.7.1). Densitometric sum was calculated for each image staining intensity of Aβ, apo B and DAPI (nuclei).

For each image, either apo B or Aβ pixel intensities were standardised with total DAPI pixel intensity to normalise for cell number in the image, and expressed as per DAPI. Staining intensity in the perinuclear region within the enterocytes was calculated and expressed as perinuclear intensity per total DAPI for the image (perinuclear apo B/total DAPI; perinuclear Aβ /total DAPI). Lacteal staining intensity was obtained by subtracting perinuclear staining intensity from the whole villi intensity (lacteal apo B/total DAPI; lacteal Aβ/total DAPI). The data were then collated and final results are expressed as mean intensity ± standard error of mean per area unit.

Plasma cholesterol and triacylglycerol analysis

Plasma Cholesterol and TAG were determined in duplicate by enzymatic assays (Randox Laboratories LTD, UK) according to manufacturers' instructions.

Statistical analysis

All data was analysed by either parametric or non-parametric one-way Analysis of Variance to assess the main effects of dietary fat and Probucol treatment and their two-way interactions. Post-hoc comparison of means was done if the associated main effect or interaction was statistically significant within the Analysis of Variance procedure. *P*-values < 0.05 were considered to be statistically significant.

Results

The distribution and abundance of immunoreactive apo B, an exclusive marker for nascent chylomicrons, was determined by quantitative immunofluorescent microscopy as previously described. Perinuclear enterocytic and lacteal abundance were utilized as surrogate markers of production and secretion, respectively. In all groups, the majority of immunoreactive apo B (approximately 80%) was located within the lacteals (Figure 1, 2a), indicative of the efficient packaging and secretory pathway of dietary lipids with chylomicrons. Provision of an HF diet for 32 days resulted in a 60% increase of secreted apo B commensurate with decreased perinuclear apo B (Figure 1, 2a), but there was no significant change in net villi apo B abundance (perinuclear + lacteal). Incorporation of Probucol in the LF diet like the HF diet stimulated secretion of apo B. However, there did not appear to be a synergistic effect of HF + P. The findings of similar net villi abundance of apo B between treatment groups and a strong negative association between the perinuclear- versus lacteal-apo B distribution (Figure 2c), is consistent with studies which suggest that enterocytic apo B is constitutively synthesized, whereas the secretion of the nascent lipoproteins is modifiable in response to the availability of dietary lipids [32].

The perinuclear and lacteal distribution of Aβ was qualitatively identical to apo B. Indeed, co-localization analysis confirms that Aβ secreted from enterocytes was associated with chylomicrons. However, there were substantial differences in the relative villi abundance of Aβ and apo B and in the pattern of secretion between of the two proteins. In

contrast to apo B, approximately 70-80% of total intestinal villi Aβ was observed within the baso-lacteal nuclear region of the absorptive epithelial cells, suggesting that only small quantities of the total enterocytic Aβ pool were being secreted (Figure 1, 2b). The HF enriched diet doubled intestinal villi Aβ abundance, but in contrast to apo B, this was predominantly reflected in increased enterocytic abundance with virtually no change in the secreted component (Figure 2b). Incorporation of Probucol in the HF diet normalized enterocytic Aβ to levels that were comparable to the LF control, in the absence of a reduction in lacteal Aβ. The latter suggests that Probucol reduced enterocytic Aβ primarily as a consequence of lower rates of biosynthesis. However, correlation analysis of perinuclear versus lacteal Aβ identified a relatively weak but nonetheless positive association (Figure 2d), suggesting that increased rates of Aβ production also lead to modest increases in apo B lipoprotein-Aβ secretion.

The effects of the HF or Probucol supplemented diets on plasma cholesterol, plasma TAG and body weight gain for each group of mice is given in table 1. The mice fed the HF enriched diet had a more than two-fold increase in plasma cholesterol compared to the LF control, however the incorporation of Probucol completely abolished this effect. Indeed, the HF + P group had comparable plasma cholesterol to the LF + P treated mice. Probucol also significantly reduced plasma cholesterol in LF mice. In contrast, there was no appreciable effect of HF feeding, or Probucol, on plasma TAG in any treatment group.

Mice maintained on the HF diet were found to have a greater rate of body weight gain compared to mice maintained on the LF diet. Probucol had a synergistic stimulatory effect on body weight gain. Mice on LF + P were similar in weight to mice maintained on the HF diet, and mice on the HF + P were significantly greater in weight than mice on HF alone.

Discussion

In absorptive epithelial cells of the small intestine, dietary fats profoundly regulate enterocytic abundance of Aβ, reflecting either changes in Aβ biogenesis, or in the secretion of lipoproteins containing Aβ [28, 29]. Several lipid lowering agents including statins and fibrates have been shown to suppress apo B secretion [17, 18, 20], however Probucol may have pleiotropic benefits post-secretion including enhanced hepatic clearance apo B lipoprotein-Aβ and anti-oxidant activity.

In this study, an established *in vivo* murine model was used to investigate if Probucol modulates the effects of a HF diet on enterocytic Aβ and its secretion thereafter, with apo B lipoproteins. The study confirms that a HF diet substantially increases enterocytic perinuclear abundance of Aβ. Apolipoprotein B lipoprotein secretion is enhanced by HF feeding, but without evidence of a concomitant increase in lacteal Aβ staining. Therefore, the HF mediated effect on enterocytic Aβ abundance is likely to be a consequence of greater rates of Aβ synthesis, rather than diminished rates of secretion. Previous studies reported that a HF induced accumulation of enterocytic Aβ is progressively depleted in the post-absorptive state, or once food is withdrawn [28]. Hence, a dietary fat induced stimulation in Aβ biogenesis with constitutive rates of secretion as suggested in this study, would result in extended post-prandial amyloidemia. Clinical studies in normal healthy subjects consuming a mixed lipid meal are consistent with a transient single meal effect [34]. A phenomenon of extended exposure may be important in modulation vascular function. Co administration of Probucol with the HF diet completely abolished the HF induced effect on enterocytic Aβ abundance in the absence of a significant stimulatory effect on apo B lipoprotein-Aβ secretion. The findings are consistent with Probucol normalizing enterocytic Aβ biogenesis, rather than promoting enterocytic secretion of Aβ. Indeed, whilst Probucol was found to stimulate apo B lipoprotein secretion in LF fed mice, there was no evidence that this translated into significantly increased enterocytic release of Aβ.

The HF diet utilized in this study contained both SFA and cholesterol, provided together in the context that it is physiologically relevant in comparison to commonly consumed atherogenic diets. The effects of Probucol on Aβ synthesis and secretion reported in this study must therefore be considered in the context of a mixed dietary lipid setting. Regulation by Probucol may change depending on the interactive effects of dose and duration of dietary lipids.

Several studies have shown synergistic stimulatory effects of fatty acids and cholesterol on apo B lipoprotein secretion [35, 36]. Therefore, the finding of increased apo B lipoprotein secretion shown in this study in HF fed mice is to be expected. However, previous studies in the same strain of mice fed SFA, or cholesterol, found stimulatory and suppressive effects, respectively, on enterocytic abundance of Aβ [29, 33]. Saturated fatty acids were shown to have a profound stimulatory effect on enterocytic Aβ abundance, whereas dietary cholesterol was inhibitory. The reasons for the paradoxical differences between SFA and cholesterol have not been determined but may include differential regulation of Aβ

biogenesis, transfer and association of Aβ with apo B lipoproteins and/or changes in intracellular degradation of Aβ or apo B lipoproteins.

In this study, saturated fats presented at 20% of digestible energy combined with 1% (w/w) cholesterol increased enterocytic abundance by approximately 75% above control mice given the LF diet alone. Hence, it would appear that the effects of SFA on enterocytic Aβ homeostasis were substantially greater than that of dietary cholesterol. How SFA influence Aβ biogenesis and association with apo B lipoproteins is not known. One possibility is increased lipidation of Aβ, a process found to protect other lipophylic apoproteins from proteolytic degradation.

Several studies suggest that the intracellular distribution between free cholesterol may be important in modulating Aβ homeostasis and intracellular kinetics. Inhibition of cholesterol trafficking in neuronal cells decreased β-secretase but enhanced γ-secretase processing of Aβ precursor protein [37]. The substantial increase in γ-secretase resulted in an increased intracellular concentration of Aβ [37]. Whilst in enterocytes Aβ biogenesis does not appear to occur at the plasma membrane, the subcellular distribution of cholesterol might nonetheless induce critical changes in the cell membranes of intracellular compartments such as within the endoplasmic reticulum and Golgi or re-localise enzymes responsible for Aβ synthesis, or its association with primordial lipoproteins. The notion that Probucol regulates enterocytic biogenesis of Aβ or association with apo B lipoproteins via modulation of intracellular pools of cholesterol is supported by the findings of Tawara et al. [38], who reported that Probucol stimulates cholesterol biosynthesis in absorptive epithelial cells of the small intestine, a process which would suppress Aβ biogenesis.

The HF diet resulted in greater body weight gain compared to LF fed mice, presumably as a consequence of increased caloric intake and somewhat surprisingly, Probucol also enhanced body weight gain in the LF fed mice. However, there was no evidence that body weight was associated with perinuclear or lacteal abundance of Aβ, or of apo B lipoprotein-Aβ, so it is unlikely there is a causal association.

Clinical and animal studies suggest that Probucol may reduce AD risk and attenuate amyloidosis [26, 27, 39]. Suggested mechanisms include enhanced cerebrovascular efflux of soluble Aβ and neuro-protection as a consequence of suppression of oxidative pathways. Other indirect lines of evidence suggest that Probucol could confer AD protection by reducing vascular exposure to cytotoxic compounds including exaggerated plasma cholesterol, fatty acids or a reduction in inflammatory proteins including Aβ. The findings of

this study support the latter notion and show that Probucol appears to suppress dietary fat induced stimulation of Aβ biosynthesis.

The one month dietary intervention study described in this study did not identify any significant increase in secretion of apo B lipoprotein-Aβ per se and hence may reflect a localized phenomenon that is not particularly relevant to AD risk. Clearly, longer term feeding studies with an emphasis on the effects of Probucol on blood-brain-barrier integrity and plasma Aβ homeostasis are warranted.

Acknowledgements

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Conflicts of Interest

The authors have no conflicts of interest to declare in relation to this article.

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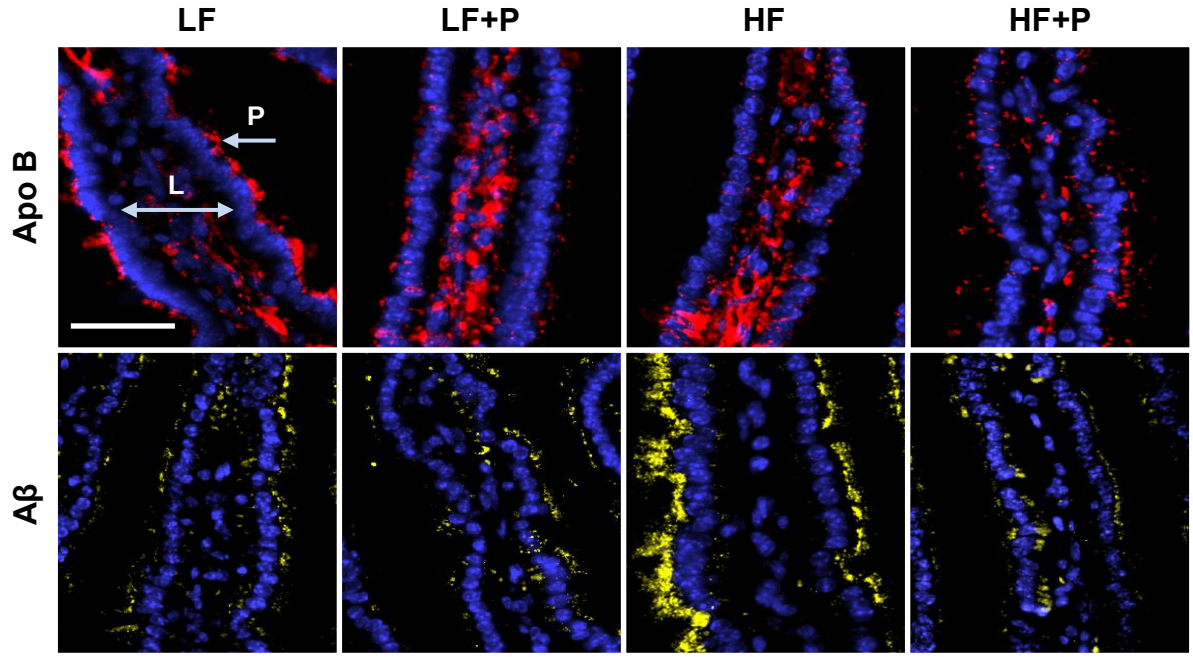
Figure Legends

Figure 1

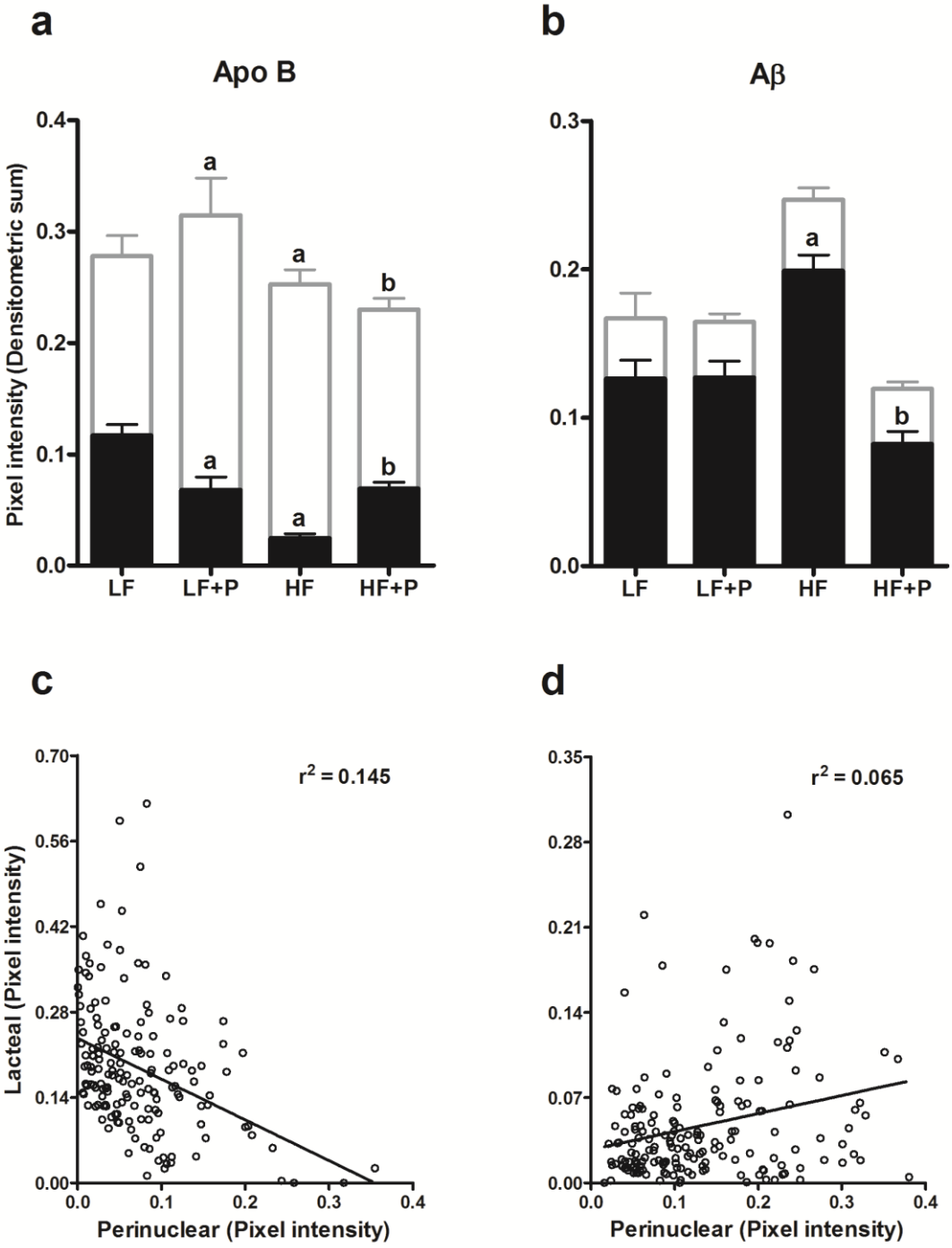


The images show apo B (red) and Aβ (yellow) in the intestinal villi. The nuclei are stained blue. The villi are lined with a single layer of absorptive epithelial cells. Amyloid-β staining is concentrated at the perinuclear region of the enterocytes (P arrow) with very little staining in the lacteals (L arrow). In contrast, high concentration of the apo B is found within the lacteals of all the groups.

Scale bar = 30 μm

Abbreviations: LF, low-fat; HF, high-fat; P, probucol.

Figure 2



Bar graphs (2a and 2b) demonstrate quantitative analysis of perinuclear (■) and lacteal (□) apo B and Aβ staining intensities. Pixel intensity is expressed as densitometric sum as a ratio of nuclei intensity to normalise for nuclei number.

Scatter plots (2c and 2d) show the distribution of apo B and Aβ between the perinuclear and the lacteal regions of all groups. Pearsons correlation analysis (r^2 values) for apo B and Aβ was 0.145 ($P < 0.0001$) and 0.065 ($P = 0.0006$), respectively.

a, statistically significant in comparison to the LF group ($P < 0.05$).

b, statistically significant in comparison to the HF group ($P < 0.05$).

Abbreviations: LF, low-fat; HF, high-fat; P, probucol.

Table 1: Effect of various feedings regimes on the average weight, plasma lipids and IL-6 in wild-type mice (C57BL/6J).

Feeding Regime	Mean ± SEM			
	Body weight (g)		Plasma lipids (mM)	
	Final	Weight gain	TC	TAG
LF (n = 8)	20.56 ± 0.27	2.00 ± 0.16	1.86 ± 0.06	0.55 ± 0.05
LF+P (n = 8)	22.23 ± 0.36[a]	4.55 ± 0.16[a]	0.83 ± 0.04[a]	0.65 ± 0.06
HF (n = 8)	22.48 ± 0.33[a]	4.36 ± 0.19[a]	2.88 ± 0.17[a]	0.63 ± 0.10
HF+P (n = 8)	23.58 ± 0.32	5.82 ± 0.19[b]	0.67 ± 0.05[b]	0.39 ± 0.04

Wild-type mice (C57BL/6J) were randomised to four different feeding regimens (n = 8 mice per group) and were fed their respective diets for 32 days. Weights, total serum cholesterol and triglyceride levels at the end of the experiment were compared between the groups with Post-hoc comparison of means within the Analysis of Variance procedure and $P < 0.05$ was considered significant.

The average final body weights (per mouse) for LF+P and HF groups were significantly greater in comparison to the LF group, however HF+P group final body weight was similar to that of the HF group. There was significant weight gain by all the groups (vs LF) and there was also weight gain by the HF+P treated mice in comparison to the HF group.

Total plasma cholesterol level was significantly reduced by probucol treatment alone (vs LF), also by the HF+P treated group in comparison to the HF group. Fat feeding significantly have increased the circulating cholesterol level. On the other hand, either the probucol treatment or fat feeding did not affect the triglyceride concentration.

Data represented as mean ± standard error of the mean (SEM), numbers (n) indicate total number of samples used.

“a” and “b” indicates statistical significance in comparison with LF and HF groups, respectively, where $P < 0.05$.

Abbreviations: LF, low-fat; HF, high-fat; P, probucol; TC, total serum cholesterol; TAG, serum triglyceride.