Identification of *ALOX5* as a gene regulating adiposity and pancreatic function

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Abstract

Aims/hypothesis We previously used an integrative genetics approach to demonstrate that 5-lipoxygenase (5-LO) deficiency in mice (*Alox5*^{−/−}) protects against atherosclerosis despite increasing lipid levels and fat mass. In the present study, we sought to further examine the role of 5-LO in adiposity and pancreatic function.

Methods *Alox5*^{−/−} and wild-type (WT) mice were characterised with respect to adiposity and glucose/insulin metabolism

using in vivo and in vitro approaches. The role of *ALOX5* in pancreatic function in human islets was assessed through short interfering RNA (siRNA) knockdown experiments.

Results Beginning at 12 weeks of age, *Alox5*^{−/−} mice had significantly increased fat mass, plasma leptin levels and fasting glucose levels, but lower fasting insulin levels ($p < 0.05$). Although *Alox5*^{−/−} mice did not exhibit insulin resistance, they had impaired insulin secretion in response to a bolus glucose injection. Histological analyses revealed

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that *Alox5*^{−/−} mice had increased islet area, beta cell nuclear size, and numbers of beta cells/mm² islet ($p<0.05$), indicative of both hyperplasia and hypertrophy. Basal and stimulated insulin secretion in isolated *Alox5*^{−/−} islets were significantly lower than in WT islets ($p<0.05$) and accompanied by a three- to fivefold decrease in the expression of the genes encoding insulin and pancreatic duodenal homeobox 1 (*Pdx1*). Direct perturbation of *ALOX5* in isolated human islets with siRNA decreased insulin and *PDX1* gene expression by 50% and insulin secretion by threefold ($p<0.05$).

Conclusions/interpretation These results provide strong evidence for pleiotropic metabolic effects of 5-LO on adiposity and pancreatic function and may have important implications for therapeutic strategies targeting this pathway for the treatment of cardiovascular disease.

Keywords Beta cell function · Insulin secretion · Leukotrienes · 5-Lipoxygenase · Obesity · Inflammation · Type 2 diabetes

Abbreviations

CVD	cardiovascular disease
IPGTT	intraperitoneal glucose tolerance test
IPITT	intraperitoneal insulin tolerance test
KRB	Krebs Ringer bicarbonate
5-LO	5-lipoxygenase
LT	leukotriene
NMR	nuclear magnetic resonance
PP	pancreatic polypeptide
PDX1	pancreatic duodenal homeobox 1
siRNA	short interfering RNA
WT	wild-type

Introduction

The 5-lipoxygenase (5-LO) pathway, which generates pro-inflammatory leukotrienes (LTs) from arachidonic acid, has recently attracted a great deal of attention for its potential role in traits related to cardiovascular disease (CVD). This stems from a series of recently conducted studies, which have collectively provided strong evidence for the proatherogenic role of LTs [1, 2]. Importantly, multiple lines of evidence support this concept, including biochemical, genetic and pharmacological studies in both mice and humans [2]. For example, we demonstrated that deficiency of the gene encoding 5-lipoxygenase (*Alox5*) in mice protects against the development of aortic lesions [3, 4], and that promoter variants of the human *ALOX5* gene are associated with increased carotid intima-media thickness [5]. Other groups have also reported findings that support the involvement of the 5-LO/LT pathway in CVD traits [6–13].

More recently, we used an integrative genomics approach with inbred mouse strains, gene-targeted mice and microarray profiling to demonstrate that 5-LO also contributes to metabolic phenotypes, such as body fat, bone density and lipid levels [14]. These observations raise important questions regarding the potential pleiotropic effects of the 5-LO pathway on components of the metabolic syndrome. To further explore these inter-relationships, we characterised *Alox5*^{−/−} mice for traits related to obesity and type 2 diabetes.

Methods

Animal husbandry *Alox5*^{−/−} mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and backcrossed onto a C57BL/6J (B6) background for more than ten generations. Wild-type (WT) B6 mice were either bred in house or purchased from Jackson Laboratories. Since no differences were observed between WT mice from either source, the data from both types of control mice were combined. All animals were housed four per cage at 25°C on a 10 h dark/14 h light cycle and maintained on a chow diet (No. 5015; Purina, Richmond, IN, USA). All procedures were in accordance with the current National Research Council Guide for the care and use of laboratory animals, and were approved by the UCLA Animal Research Committee.

Plasma measurements Mice were bled retro-orbitally under isoflurane anaesthesia or through the tail vein while restrained and conscious. Glucose levels were determined using commercially available kits from Sigma (St Louis, MO, USA) or the OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Milpitas, CA, USA). We previously established the accuracy of glucose values using this glucometer compared with the Sigma kit (M. Mehrabian, unpublished data). Insulin and leptin levels were measured using commercial ELISA kits from Crystal Chemical (Chicago, IL, USA) and R&D Systems (Minneapolis, MN, USA), respectively. All measurements were performed in duplicate or triplicate according to the manufacturer's instructions.

Body composition Whole body fat, fluids and lean tissue mass of isoflurane-anaesthetised mice were determined using a Bruker Optics Minispec nuclear magnetic resonance (NMR) analyser (The Woodlands, TX, USA) according to the manufacturer's recommendations. The mass of individual fat depots (retroperitoneal, epididymal, subcutaneous, and omental) was determined by dissecting and weighing each fat pad separately after the mice were killed.

Intraperitoneal glucose and insulin tolerance tests Intraperitoneal insulin tolerance tests (IPITTs) were performed

by administering an intraperitoneal injection (0.75 U/kg body weight) of recombinant human insulin (Novolin; Novo Nordisk, Bagsværd, Denmark) to conscious mice fasted for 5 h. Plasma glucose levels were measured in blood samples obtained through the tail vein using a glucometer, as described above. For intraperitoneal glucose tolerance tests (IPGTTs), mice were fasted for 12 h overnight and injected with a bolus (1 mg/g body weight) of glucose (10% [wt/vol.] in sterile H₂O) into the peritoneal cavity. Blood samples were obtained from conscious mice through the tail vein or from isoflurane-anaesthetised mice through the retro-orbital plexus at 0, 15, 30, 60, 90 and 120 min post-injection. Plasma glucose and insulin levels were measured as described above.

Immunohistochemistry After the animals were killed, the pancreas was dissected, weighed, fixed in formalin and embedded in paraffin. Tissue sections were deparaffinised, rehydrated and incubated with guinea pig anti-insulin antibody (Dako, Glostrup, Denmark) followed by detection with a fluorescein-conjugated rabbit anti-guinea pig antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The samples were subsequently labelled for glucagon with rabbit anti-glucagon or with a mixture of rabbit anti-somatostatin and anti-pancreatic polypeptide antibodies (Dako), followed by detection with donkey anti-rabbit indocarbocyanine (Cy3)-conjugated antibody (Jackson ImmunoResearch Laboratories). Beta cell mass was analysed using Openlab software (Improvision, Waltham, MA, USA). The relative area of beta cells (green fluorescence) was determined by calculation of the cross-sectional beta cell area divided by the cross-sectional area of total tissue. The beta cell mass per pancreas was estimated as the product of the relative cross-sectional area of beta cells per total tissue and the weight of the pancreas. Beta cell number per mm² of islet, nuclear size, nuclear distance and islet size were quantified using an inverted system microscope (IX70; Olympus America, Melville, NY, USA) and Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA). The number of beta cells per islet was calculated by multiplying islet area in mm² by the number of beta cells per mm². Ten representative 20 µm sections from each pancreas (spanning the width of the pancreas) were used in these analyses, and the results are from ten male mice of each genotype, from three litters, at 12 weeks of age.

Islet isolation and culture Human islets were isolated from the pancreas of five healthy organ donors at the University of Illinois at Chicago, as described previously [15]. All donors gave informed consent, and institutional review board approval was obtained from the participating institutions. Mouse islets were isolated by bile duct

perfusion and collagenase digestion, as described elsewhere [16]. The islets were cultured on matrix-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel), allowing the cells to attach to the dishes and spread [17]. Human islets were cultured in CMRL 1066 medium containing 5.5 mmol/l glucose, and mouse islets in RPMI 1640 medium containing 11.1 mmol/l glucose, both supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (Invitrogen, Carlsbad, CA, USA).

Insulin release and content measurements Chronic insulin release over 48 h was evaluated in the culture medium collected before the termination of the experiment. For acute insulin release in response to glucose, mouse islets or transfected human islets were washed and pre-incubated for 30 min in Krebs Ringer bicarbonate buffer (KRB) containing 2.8 mmol/l glucose and 0.5% BSA. KRB was then replaced with KRB containing 2.8 mmol/l glucose for 60 min (basal), followed by an additional 60 min in KRB with 16.7 mmol/l glucose (stimulated). Thereafter, islets were incubated in 1 ml 0.18 mol/l HCl in 70% ethanol and left overnight at 4°C. Insulin was determined using a mouse insulin ELISA kit (ALPCO Diagnostics, Salem, NH, USA). The stimulatory index was calculated by dividing stimulated insulin secretion levels by basal insulin secretion. Beta cell area of the stimulated islets was calculated under the microscope using Image-Pro Plus software. All experiments were performed in three independent rounds using islets isolated from three litters of 12 week old male *Alox5*^{-/-} and WT mice (*n*=10 for each strain), respectively, with three dishes for each genotype group and 20 islets per dish.

RNA interference experiments Short interfering RNAs (siRNAs) designed for the specific knockdown of *ALOX5* expression or as a non-specific control were synthesised by Ambion (Austin, TX, USA). Isolated human islets were plated on extracellular matrix-coated dishes and cultured in medium containing 5.5 mmol/l glucose for 24 h. The islets were then transfected with 100 nmol/l siRNA using 2.5 µl/ml Lipofectamine 2000 in OptiMEM media (Invitrogen) according to the manufacturer's protocols. The transfection medium was replaced after 12 h with antibiotic-free culture media, and 96 h later the islets were harvested for analysis. All experiments were performed in triplicate with three dishes for each treatment group, with 20 islets per dish from five donors in five independent rounds.

RNA extraction and real-time quantification Total RNA was extracted from adipose tissue or pancreatic islets using RNeasy Mini kits (Qiagen, Valencia, CA, USA). RNA was isolated from 50 mouse islets for each

genotype group, which were obtained from ten 12 week old mice, whereas 100 islets from each treatment group were used to extract RNA for the human islet experiments. The cDNA was prepared from 1 µg of total RNA using reverse transcription kits (Life Technologies, Gaithersburg, MD, USA; Applied Biosystems [ABI], Foster City, CA, USA; or Invitrogen) according to the manufacturers' protocols. Real-time expression assays were performed either on the Light Cycler quantitative PCR system (Roche, Basel, Switzerland) or an ABI 7900HT instrument. Real-time reactions were carried out in triplicate with either SYBR Green assays, as described previously [18, 19], or pre-developed assays from ABI [20]. For the SYBR Green assays, the RNA levels in each sample were calculated using standard curves generated from serial tenfold dilutions of pooled RNA from WT mice. For the pre-developed assays, RNA levels in each sample were calculated using pooled WT mice as the reference sample. Each sample was normalised to endogenous controls, such as *GAPDH/Gapdh*, *ACTB*, or *TUBA1B/Tuba1b*, and the replicates were averaged to determine differences between *Alox5*^{-/-} and WT mice or between human islets treated with control or *ALOX5*-specific siRNAs. A list of the primer sequences used for the SYBR Green assays is provided in Electronic supplementary material Table 1.

Statistical analyses Differences in measured variables between *Alox5*^{-/-} and WT mice or the siRNA treatments in human islets were determined by Student's *t* test using Statview, version 5.0 (SAS Institute, Cary, NC, USA). Values are expressed as mean±SEM, and differences were considered statistically significant at *p*<0.05.

Results

Increased adiposity in *Alox5*^{-/-} mice Based on our earlier observations that 5-LO deficiency leads to several metabolic abnormalities [14], we first characterised *Alox5*^{-/-} mice for measures related to obesity. Beginning at 10 weeks of age and continuing to 19 weeks, *Alox5*^{-/-} mice had significantly higher body weight than age-matched WT controls (Table 1). NMR analysis revealed that this difference was primarily due to increased adiposity (Table 1), which was not restricted to one anatomical region; all four adipose tissue depots were found to have an increased mass (Fig. 1a–d). In agreement with these observations, fasting plasma leptin levels (expressed per gram of body weight) were also significantly higher in female *Alox5*^{-/-} mice at 12 weeks of age, and progressively increased up to 23 weeks (Fig. 1e), mirroring what was observed for body weight and percentage body fat. As we reported previously, these differences in adiposity did not result from behavioural changes, such as food intake or activity levels [14]. To gain insight into the mechanism by which 5-LO deficiency leads to the observed metabolic changes, we measured mRNA transcript levels of genes related to insulin and leptin metabolism as well as inflammation in adipose tissue from 12-week-old female mice using real-time quantitative PCR. These analyses revealed that *Alox5* was expressed in adipose tissue of WT mice but, as expected, not in *Alox5*^{-/-} mice (Fig. 1f). In addition, expression of the gene encoding leptin (*Lep*) was increased by approximately fivefold, consistent with the higher plasma levels of the protein, whereas expression of the genes encoding the insulin receptor (*Insr*) and insulin receptor substrate 1 (*Irs1*) was decreased by ~50% (Fig. 1f). Interestingly, expression

Table 1 Measures of body weight and adiposity in *Alox5*^{-/-} and WT mice

Age	Female mice			Male mice		
	Weight (g)	Fat (g)	Percent body fat	Weight (g)	Fat (g)	Percent body fat
10 weeks						
<i>Alox5</i> ^{-/-}	19.9±0.3	2.5±0.3	12.4±1.2	26.0±2.3*	2.8±0.2*	10.8±0.5*
WT	18.6±0.1	2.0±0.3	12.2±1.3	23.8±1.7	1.9±0.4	7.9±1.0
14 weeks						
<i>Alox5</i> ^{-/-}	24.1±0.4*	2.8±0.8*	11.7±1.4*	29.0±1.6*	3.5±0.2*	12.2±0.2*
WT	21.6±0.3	2.0±0.1	9.5±0.5	26.4±1.7	2.0±0.4	7.5±0.9
16 weeks						
<i>Alox5</i> ^{-/-}	27.5±0.2*	2.9±0.6*	10.6±0.24*	30.0±1.7*	3.9±0.4*	12.9±0.5*
WT	22.8±0.1	2.0±0.01	9.3±0.2	26.4±1.5	2.0±0.1	7.6±0.2
19 weeks						
<i>Alox5</i> ^{-/-}	28.7±0.1*	3.4±0.4*	11.9±1.3*	31.2±1.9*	3.9±0.5*	12.4±1.2*
WT	23.4±0.2	2.0±0.2	8.8±0.9	27.5±1.5	2.0±0.2	7.3±0.7

Five mice of each genotype group were weighed at the indicated ages and their adiposity determined by NMR. The data are shown as mean±SEM, and statistical comparisons were only made within each sex group

**p*<0.05 vs WT mice

of inflammatory genes, such as *Tlr4*, *Il1b*, *Il-6*, and *Tnfa*, was decreased in *Alox5^{-/-}* compared with WT mice (Fig. 1g).

Impaired glucose and insulin metabolism in *Alox5^{-/-}* mice We next investigated whether the increased adiposity of *Alox5^{-/-}* mice was associated with other metabolic abnormalities, using a series of time course experiments with mice at different ages. As shown in Fig. 2a, male and female *Alox5^{-/-}* mice had fasting plasma glucose levels similar to those in WT mice at 8 weeks of age but had significantly elevated levels at 12 weeks of age, which remained higher up to 23 weeks of age. By contrast, fed plasma glucose levels became elevated at 18 weeks (Fig. 2b), whereas lower fasting insulin levels were observed starting at 12 weeks of age (Fig. 2c). These results suggest a progressive impairment of glucose homeostasis in *Alox5^{-/-}* mice.

To explore these differences further, we first assessed insulin sensitivity by IPGTT. As shown in Fig. 2d, 12-week-old male and female *Alox5^{-/-}* mice exhibited significantly higher plasma glucose levels than WT mice at all time points following a bolus glucose injection. This was accompanied by significantly lower insulin levels at the 0, 15, and 30 min time points (Fig. 2e). These experiments were repeated at 18 weeks of age, where again we observed significantly higher fasting and fed plasma glucose (Fig. 2f) and lower fasting insulin levels (Fig. 2g) in *Alox5^{-/-}* mice. An IPGTT at 18 weeks of age also demonstrated signifi-

cantly elevated plasma glucose levels in *Alox5^{-/-}* mice at all time points post-injection (Fig. 2h). Based on these observations, we next carried out IPITTs to assess insulin sensitivity. Interestingly, although *Alox5^{-/-}* mice had elevated plasma glucose levels at all time points following insulin injection, including at baseline (Fig. 2i), the rate of glucose disposal was the same as that for WT mice, since the difference between plasma glucose levels at 60 min and those at 0 min were not significantly different between the two strains (Fig. 2j). These results suggest that 5-LO deficiency does not alter insulin sensitivity noticeably, despite an increase in adiposity.

Another series of IPGTTs was carried out on mice at 23 weeks of age, to gain a better understanding of glucose and insulin dynamics. Similar to the results in younger mice, 23-week-old *Alox5^{-/-}* mice exhibited higher plasma glucose levels than the WT mice at baseline and throughout the duration of the experiment (Fig. 2k). Plasma insulin levels were significantly lower in the *Alox5^{-/-}* mice at baseline and at the 10, 15 and 30 min time points (Fig. 2l), thus suggesting an impairment of insulin secretion. We also used the IPGTT data to calculate the mean AUC, which is increased for glucose and decreased for insulin levels in *Alox5^{-/-}* mice compared with WT mice (Fig. 2k,l).

Impaired beta cell function in *Alox5^{-/-}* mice To assess whether the decreased insulin response in *Alox5^{-/-}* mice was mediated by altered beta cell mass and function, we

Fig. 1 *Alox5^{-/-}* mice have increased adiposity and altered gene expression in adipose tissue. The mass of epididymal (a), omental (b), retroperitoneal (c) and subcutaneous (d) fat depots are all increased in 23-week-old *Alox5^{-/-}* mice compared with WT mice. Each fat pad was dissected out and weighed individually. e Beginning at 12 weeks of age, *Alox5^{-/-}* mice (black bars) have significantly higher fasting plasma leptin levels (expressed per unit of body weight) than WT mice (white bars), and this difference becomes progressively more pronounced by 23 weeks of age. Gonadal adipose tissue expression of

Lep is approximately fivefold higher in 12-week-old *Alox5^{-/-}* mice than in age-matched WT mice, whereas the expression of *Insr* and *Irs1* is decreased by ~50%. The expression of *Irs2* and *Irs3* is not significantly different. f, g The expression of inflammatory genes, such as *Tlr4*, *Il1b*, *Il6* and *Tnfa*, is also decreased in adipose tissue from *Alox5^{-/-}* mice. Gene expression was quantified in triplicate, normalised to an endogenous control, and expressed as arbitrary units (AU). All data are from female mice ($n=7-12$ per group) and expressed as mean+SEM. * $p<0.05$ vs WT mice

Fig. 2 Impaired glucose tolerance and insulin secretion in *Alox5*^{-/-} mice. Fasting plasma glucose levels are significantly higher from 12 weeks of age (**a**), fed plasma glucose levels become significantly elevated from 18 weeks (**b**), and fasting insulin levels are significantly lower from 12 weeks (**c**) in male and female *Alox5*^{-/-} mice (circles) compared with WT mice (squares). Post-glucose injection during an IPGTT, plasma glucose levels are significantly higher at all time points (**d**), whereas plasma insulin levels are lower at the 0, 15 and 30 min time points (**e**) in 12-week-old male *Alox5*^{-/-} mice (circles or black bars) compared with WT mice (squares or white bars). At 18 weeks, fasting and fed plasma glucose levels are higher (**f**) and fasting insulin levels are lower (**g**) in male and female *Alox5*^{-/-} (black bars) compared with WT (white bars) mice. **h** At this age, *Alox5*^{-/-} male (circles, dashed line) and female (circles, solid line) mice exhibit significantly higher glucose levels than male (squares, dashed line) and female (squares, solid line) WT mice at all time points following a bolus glucose injection during an IPGTT. **i** An IPITT demonstrates

that although 18-week-old male (circles, dashed line) and female (circles, solid line) *Alox5*^{-/-} mice have higher plasma glucose levels than age-matched male (squares, dashed line) and female (squares, solid line) WT mice at baseline, the rate of decrease after administration of an insulin bolus is the same. **j** This is further shown by the comparable changes in glucose levels from baseline to 60 min post-injection in *Alox5*^{-/-} (black bars) and WT (white bars) mice. An IPGTT performed at 23 weeks of age demonstrates that glucose clearance is decreased in female *Alox5*^{-/-} mice (circles) compared with sex-matched WT mice (squares; **k**), which results, at least in part, from impaired insulin secretion at the baseline, 10, 15 and 30 min time points (**l**). Please note that the scales shown on x-axes of the graphs shown in **k** and **l** are non-linear. All data are from ten to 15 mice of each genotype at the indicated ages. IPITT and IPGTT experiments were performed as described in the “Methods” section. Data are shown as mean+SEM. **p*<0.05 for *Alox5*^{-/-} vs WT mice. AU, arbitrary units

analysed the morphology, histology and function of pancreatic islets isolated from 12-week-old mice in vitro. When stained for insulin, glucagon, somatostatin and pancreatic polypeptide (PP), islets from both *Alox5*^{-/-} and WT mice contained the appropriate ratio of alpha-, beta-, delta- and PP-staining cells (Fig. 3a,b). However, beta cell volume of *Alox5*^{-/-} islets was increased 1.7-fold compared with that of WT islets (Fig. 3c). Since pancreatic weight was decreased in *Alox5*^{-/-} compared with WT mice (Fig. 3d), beta cell mass was not significantly different, despite the larger islets (Fig. 3e). We next analysed the islets for the presence of hypertrophy and/or hyperplasia. As shown in Fig. 3f, *Alox5*^{-/-} islets have 1.4-fold fewer beta cells per mm² of islet, suggesting the presence of larger beta cells and hypertrophy. This is supported by measurements of nuclear

size and nuclear distance between neighbouring beta cells, which were both 1.3-fold larger in *Alox5*^{-/-} compared with WT islets (Fig. 3g,h). To further analyse islet size, we measured mean islet area, and this was found to be 5.7-fold larger in *Alox5*^{-/-} mice (Fig. 3i), and, accordingly, leads to a fourfold increase in the number of beta cells per islet (Fig. 3j). Taken together, these data show that *Alox5*^{-/-} islets exhibit both hyperplasia and hypertrophy, with hyperplasia being more prominent, since beta cells from *Alox5*^{-/-} mice are 1.3-fold larger but are fourfold more frequent per islet.

A series of in vitro experiments was conducted to analyse insulin secretion in isolated islets from *Alox5*^{-/-} and WT mice. During a 48 h culture, chronic insulin release, defined as the amount of insulin released into the medium, was significantly decreased by 2.9-fold in *Alox5*^{-/-}

Fig. 3 Morphology and histology of the pancreas in $Alox5^{-/-}$ and WT mice. Double immunostaining of representative pancreatic sections (magnification: $\times 250$) for insulin, glucagon, somatostatin and PP shows normal islet morphology and cellularity in both $Alox5^{-/-}$ (a) and WT (b) mice. The right-hand panel for each genotype group shows overlaid immunostaining images. $Alox5^{-/-}$ mice have an increased beta cell area as a percentage of total pancreatic area (c), but a decreased pancreatic weight (d). e Beta cell mass is not significantly different between the two groups of mice. The number of beta cells counted per mm² islet is decreased in $Alox5^{-/-}$ mice (f),

suggesting the presence of hypertrophic cells. This is supported by the increased nuclear size (g) and distance between the nuclei of adjacent beta cells (h) in $Alox5^{-/-}$ islets. Mean islet area is increased 5.7-fold (i) and there are fourfold more beta cells per islet (j) in $Alox5^{-/-}$ mice compared with WT mice. Ten representative 20 μ m sections from each pancreas (spanning the width of the pancreas) were used in these analyses, and the results are from ten male mice of each genotype from three litters at 12 weeks of age. Data are shown as mean $\pm$ SEM. * $p < 0.05$ vs WT mice

islets (Fig. 4a). Similarly, insulin secretion was significantly lower in $Alox5^{-/-}$ islets over a 60 min pre-culture under basal conditions with 2.8 mmol/l glucose (Fig. 4b), as well as during a 60 min time course experiment under stimulated conditions with 16.7 mmol/l glucose (Fig. 4b). Such an impairment of insulin secretion by $Alox5^{-/-}$ islets is further illustrated by the decreased stimulatory index shown in Fig. 4c. Since $Alox5^{-/-}$ islets were larger, we also analysed their insulin content (expressed per beta cell area), and this was found to be significantly lower than that in WT islets (Fig. 4d). To help understand the molecular mechanisms underlying these histological and functional differences, we quantified the levels of the insulin (*Ins2*) and *Pdx1* mRNAs. Pancreatic duodenal homeobox 1 (PDX1) is involved in pancreatic development and differentiation, and transcriptionally activates many beta cell genes encoding proteins

involved in glucose-stimulated insulin secretion, including glucokinase, glucose transporter 2, as well as insulin itself [21]. As shown in Fig. 4e, these analyses revealed that insulin and *Pdx1* gene expression were five- and threefold lower in $Alox5^{-/-}$ islets than in WT islets, respectively, consistent with the decreased insulin secretion and content.

Direct effect of 5-LO on INS expression and secretion in human islets To determine whether impaired insulin secretion as a result of 5-LO deficiency is due to a direct effect in the pancreas or secondary to increased adiposity, we carried out siRNA knockdown experiments in isolated human islets. For these experiments, siRNAs were designed to be either specific for *ALOX5* or a non-specific control. Transfection of control siRNA (siScramble) into islets had no effect on *ALOX5*, *INS* or *PDX1* expression (Fig. 5a). By

Fig. 4 Impaired beta cell function and altered gene expression in isolated islets from *Alox5*^{-/-} mice. **a** Chronic insulin secretion during a 48 h culture is significantly decreased in isolated islets from *Alox5*^{-/-} mice compared with WT mice. Insulin secretion is defined as the amount secreted into the medium and is expressed as a ratio of secreted insulin to insulin content. **b** Glucose-stimulated insulin secretion is impaired in *Alox5*^{-/-} (black bars) vs WT (white bars) islets. Basal insulin secretion was measured after a 60 min incubation in KRB containing 2.8 mmol/l glucose, and stimulated insulin secretion was measured at 5, 10, 15, 30 and 60 min after changing the medium to KRB containing 16.7 mmol/l glucose. **c** Compared with WT islets, *Alox5*^{-/-} islets have a reduced stimulatory index, which was calculated by dividing stimulated insulin release by basal insulin release. **d** Despite having larger islets, the insulin content of

Alox5^{-/-} islets, expressed per beta cell area, is lower than that of WT islets. **e** Expression levels of the *Ins2* (which encodes insulin; black bars) and *Pdx1* (white bars) genes are lower in isolated islets from *Alox5*^{-/-} mice than in those from WT mice, as assessed by real-time quantitative PCR. The mRNA levels of each gene were measured in triplicate, normalised to an endogenous control, and are expressed in arbitrary units (AU). All data are from three independent experiments from three litters of 12-week-old male *Alox5*^{-/-} and WT mice ($n=10$ for each genotype). Cell culture results are from three independent experiments from three dishes for each genotype group, with 20 islets per dish. Total RNA was isolated from 50 islets of each genotype, with ten mice in each group. Data are shown as mean+SEM. * $p<0.05$ vs WT mice

contrast, two different siRNAs specific for *ALOX5* (si5-LO1 and si5-LO2) each significantly decreased *ALOX5* expression by over 50% and concomitantly decreased insulin and *PDX1* mRNA levels (Fig. 5a). Although insulin secretion under basal conditions (2.8 mmol/l glucose) was not affected by an *ALOX5*-specific siRNA, stimulated insulin secretion (secretion after 60 min with 16.7 mmol/l glucose) was significantly attenuated (Fig. 5b), thus leading to a decreased stimulatory index (Fig. 5c). As shown in Fig. 5d, the insulin content of *ALOX5* siRNA-treated islets was also reduced relative to that of islets treated with the control siRNA. Importantly, these results are consistent with the in

vivo and in vitro experiments described above for *Alox5*^{-/-} mice, and demonstrate that 5-LO directly regulates insulin gene expression and protein secretion at the level of pancreatic islets.

Discussion

With the exception of the genes known to cause maturity-onset diabetes of the young, the genetic factors controlling insulin secretion are not well known. Notably, recent genetic studies in humans have provided evidence that

Fig. 5 Inhibition of 5-LO expression in human islets by siRNA decreases *INS* expression and insulin secretion. Human islets were transfected with either one of two *ALOX5*-specific siRNAs (si5-LO1 or si5-LO2) or a control siRNA (siScramble) for 96 h. **a** *ALOX5*-specific siRNA significantly decreases *ALOX5* (grey bars), *INS* (black bars) and *PDX1* (white bars) expression. Total RNA was extracted from 100 islets for each treatment group, and gene expression was carried out with real-time quantitative PCR. **b** Glucose-stimulated insulin secretion is decreased in human islets treated with an *ALOX5*-specific siRNA (si5-LO1). Cultured islets were incubated under basal conditions in KRB containing 2.8 mmol/l glucose (black bars) for

60 min followed by a successive incubation for 60 min under stimulated conditions with KRB containing 16.7 mmol/l glucose (white bars). Treatment with an *ALOX5*-specific siRNA decreased the stimulatory index (**c**), defined as the ratio between stimulated and basal values of insulin secretion, as well as the insulin content of human islets (**d**). No differences were observed with a non-specific control siRNA (siScramble). Results are for five independent experiments from five donors in triplicate, with three dishes for each treatment group, with 20 islets per dish. Data are expressed as mean+SEM. * $p<0.05$ si5-LO1 and/or si5-LO2 vs scramble siRNA

genes controlling beta cell function also play an important role in adult-onset type 2 diabetes, suggesting that insulin secretion-related mechanisms may make more significant genetic contributions to common forms of type 2 diabetes than previously recognised [22–26]. Thus, identification of novel pathways regulating beta cell function and/or insulin secretion may lead to a better understanding of type 2 diabetes and its underlying aetiology. We now provide evidence that the 5-LO/LT pathway is another mechanism by which such processes are regulated. Importantly, our observations are in agreement with a series of earlier studies carried out by Metz et al. demonstrating that lipoxygenase inhibitors decrease glucose-stimulated insulin secretion in a dose-dependent manner [27, 28] and that arachidonic acid metabolites play a role as ‘third messengers’ for hormone release [29, 30]. More recently, Prasad and colleagues have shown that 12/15-LO, a related enzyme that also metabolises arachidonic acid, influences beta cell function and insulin secretion as well [31].

In this study, we demonstrate that 5-LO deficiency in mice impairs glucose/insulin homeostasis from an early age. For example, in vitro and in vivo experiments showed that the insulin response is decreased in isolated islets from 12-week-old *Alox5*^{−/−} mice. These observations suggest that 5-LO deficiency alters beta cell function relatively early in life and prior to the onset of more pronounced obesity. Such abnormalities continue as the mice age, since decreased fasting and glucose-stimulated plasma insulin levels are also observed at 18 and 23 weeks of age. It is possible that overall pancreatic function is also compromised given the significantly reduced weight of the pancreas and insulin content of *Alox5*^{−/−} islets. Interestingly, our histological analyses suggest that *Alox5*^{−/−} islets are larger and contain beta cells that have undergone both hyperplasia and hypertrophy. These morphological changes may indicate compensatory mechanisms for the reduced insulin secretory capacity of *Alox5*^{−/−} islets and/or that LTs are required for normal pancreatic development. Furthermore, siRNA knockdown experiments in human islets decreased gene expression and insulin secretion, consistent with the observations in isolated *Alox5*^{−/−} islets. Taken together, our results indicate a direct role for 5-LO in the regulation of insulin production and secretion, both in an acute setting and over the long term.

Obesity is now widely recognised to be associated with a heightened inflammatory state involving elevations in the circulating levels and production of inflammatory cytokines such as C-reactive protein, IL-6 and TNF- α [32]. Moreover, two separate reports have shown that adipose tissue is characterised by macrophage infiltration, which could affect its normal function and inflammatory properties [33, 34]. In this regard, we show that although adiposity is increased in *Alox5*^{−/−} mice from as early as 12 weeks of age, the expression of inflammatory genes in adipose tissue is lower.

Thus, the mechanism by which 5-LO and LT deficiency leads to abnormal adipose biology and increased fat mass is not entirely clear. It should be noted, however, that 5-LO could presumably play a direct mechanistic role given that it is produced in both macrophages and adipose tissue.

Leptin is secreted by adipose tissue to regulate energy expenditure and food consumption. Interestingly, *Alox5*^{−/−} mice exhibit hyperleptinaemia but do not have increased food intake [14], suggesting that leptin signalling in the hypothalamus is either not affected by 5-LO deficiency or that there are other compensatory mechanisms. In addition, elevated plasma leptin levels in *Alox5*^{−/−} mice appear to result from transcriptional upregulation of the gene, although it is possible that other mechanisms such as decreased clearance and/or leptin resistance may be involved as well. The effect of elevated leptin levels on the so-called ‘adipose-insular axis’ may also provide another potential mechanism for the impaired insulin secretion in *Alox5*^{−/−} mice. For example, since leptin has been shown to inhibit insulin biosynthesis and secretion [35, 36], the increased plasma leptin in *Alox5*^{−/−} mice could also decrease insulin production and secretion from beta cells, in addition to what is directly affected by 5-LO itself. This could be particularly relevant in older mice in which adiposity has substantially increased.

In previous studies, we reported that 5-LO deficiency dramatically decreases aortic lesion development in two mouse models [3, 4] and that humans carrying certain *ALOX5* gene variants have increased carotid intima-media thickness [5]. Moreover, recent studies in humans and mice have demonstrated that various pharmacological modifiers of the 5-LO pathway decrease inflammatory biomarkers and aortic lesion formation, respectively [9, 13, 37], raising the possibility of developing 5-LO pathway inhibitors for the treatment of CVD. Our results suggest that it would be important for such therapeutic strategies to carefully consider potentially undesirable metabolic effects. While complete inactivation of 5-LO may result in developmental changes altering adipose tissue metabolism and/or pancreatic function that would otherwise not occur if the 5-LO pathway is inhibited in adulthood, our siRNA experiments, designed to directly perturb *ALOX5* in human islets, suggest that decreased 5-LO levels and/or activity can have an acute effect on islet function and insulin secretion. In addition, the LT pathway bifurcates to the LTB₄ and cysteinyl LT branches after the generation of LTA₄ by 5-LO, raising the possibility that a deficiency of both types of LTs is required for the development of metabolic abnormalities. For example, montelukast, which only blocks cysteinyl LT signalling, is widely used for the treatment of asthma but has not been reported to be associated with metabolic disturbances. Finally, since arachidonic acid can also be converted into other fatty acid derivatives by the cyclooxygenase and 12/15-LO enzymes, it is possible that the

metabolic phenotypes observed for the *Alox5*^{-/-} mice result from the increased production of other eicosanoids owing to shunting of arachidonic acid to these alternative pathways. For example, Goulet et al. reported that production of prostaglandins and thromboxanes is increased in macrophages from *Alox5*^{-/-} mice [38], but it is not known whether the metabolic consequences we observe with 5-LO deficiency are due directly to the lack of LTs and/or indirectly to the increased production of other eicosanoids. Thus, additional studies will be required to determine whether pharmacological antagonism of the 5-LO pathway will provide beneficial CVD effects in humans without compromising adiposity and pancreatic function.

In conclusion, we provide strong evidence that the inflammatory 5-LO/LT pathway has pleiotropic effects on adiposity and type 2 diabetes-related traits. Specifically, decreased 5-LO production leads to generalised obesity and pancreatic dysfunction in mice, and impaired insulin secretion in human islets. These results provide a new mechanism by which metabolic abnormalities associated with obesity and type 2 diabetes can arise.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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