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Genetically obese mice do not show increased gut permeability or faecal bile acid hydrophobicity

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Abstract

Gut barrier dysfunction may lead to metabolic endotoxaemia and low-grade inflammation. Recent publications have demonstrated gut barrier dysfunction in obesity induced by a diet high in fat, and a pathogenetic role for luminal bile acids has been proposed. We aimed to investigate whether genetically obese mice develop increased gut permeability and alterations in luminal bile acids on a diet with a regular fat content. We used seven obese male *ob/ob* mice of C57BL/6J background and ten male wild-type (WT) mice of the same strain. Faeces were collected for bile acid analysis. Intestinal permeability was measured in an Ussing chamber upon euthanasia, using 4 kDa fluorescein isothiocyanate dextran, as per mille (‰, 1/1000) of translocated dextran. We analysed the liver expression of lipopolysaccharide-binding protein (LBP), as well as serum LBP (ELISA). Intestinal permeability was not affected by genetic obesity (jejunum: 0·234 (SEM 0·04) ‰ for obese *v.* 0·225 (SEM 0·03) ‰ for WT, $P=0·93$; colon: 0·222 (SEM 0·06) ‰ for obese *v.* 0·184 (SEM 0·03) ‰ for WT, $P=0·86$), nor was liver LBP expression (relative expression: 0·55 (SEM 0·08) for obese *v.* 0·55 (SEM 0·13) for WT, $P=0·70$). Serum LBP was 2·5-fold higher in obese than in WT mice ($P=0·001$). Obese mice had increased daily excretion of total bile acids, but their faecal bile acid hydrophobicity was unchanged. In conclusion, genetic obesity did not impair gut barrier function in mice on a regular chow diet, nor was faecal bile acid hydrophobicity affected.

Key words: Bile acid: Intestinal permeability: Lipopolysaccharide-binding protein: Obesity

Certain metabolic diseases such as fatty liver disease, steatohepatitis and diabetes are related to a disruption of gut barrier function[1–3]. This barrier prevents gut luminal contents from translocating into the circulation. Upon disruption of the gut barrier, intestinal permeability to bacterial endotoxins, i.e. lipopolysaccharides (LPS), may increase and lead to systemic inflammation[4,5].

Brun *et al.*[6] reported in 2007 that the gut barrier is impaired in genetically obese *ob/ob* and diabetic *db/db* mice under a standard diet. Another pioneering group has shown decreased tight-junction protein levels[4] and increased serum endotoxins[7] in *ob/ob* mice fed with a standard chow. However, there are also contradicting data demonstrating that barrier dysfunction is not a result of genetic obesity, but is solely due to the amount of dietary fat[8]. A diet high in fat, mostly saturated, induces gut barrier dysfunction[7–10] and elevates blood endotoxins[7,9,11,12]. In human subjects, waist circumference has been suggested to be correlated with intestinal permeability[13], and elevated serum endotoxin levels have been reported after a fatty Western-type diet[14]. However, endotoxaemia does not necessarily reflect gut barrier function[15]. Only two studies have attempted to distinguish between dietary fat and obesity as a cause for increased permeability[6,8]. Using direct measures of permeability, these two studies came to contrasting conclusions. Thus, current data from animal and human studies do not allow us to draw conclusions on whether gut permeability is increased by obesity without a diet high in fat.

Mechanisms of barrier dysfunction have to a large extent remained unelucidated. We have proposed that a diet high in saturated fat impairs barrier function via alterations in luminal bile acid profile in mice[10,16]. Our diet-induced obese mice displayed increased gut permeability and a decreased faecal proportion of hydrophilic ursodeoxycholic acid, which was negatively correlated with gut permeability. Similar studies on faecal bile acids and intestinal permeability have

Abbreviations: FXR, farnesoid X receptor; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide.

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not been conducted in genetically obese mice and without a high-fat diet.

The aim of the present study was to investigate gut barrier function in the genetically obese, leptin-deficient *ob/ob* mouse strain under a standard diet. To study whether luminal bile acids play a role in barrier dysfunction irrespective of dietary fat, we also analysed faecal bile acids and a regulator of bile acid synthesis, liver farnesoid X receptor (FXR), in these mice. LPS-binding protein (LBP) was analysed from both liver and serum to see whether they reflect intestinal permeability or not.

Materials and methods

Animals

Wild-type male C57BL/6J mice (n 10) were obtained from Charles River (Sulzfield, Germany) and male *ob/ob* mice (n 7) of the same background from Charles River (Calco, Italy). Mice were housed together starting from 8 weeks of age in standard animal laboratory conditions with a 12 h dark–12 h light cycle. Mice had access to a standard chow (CRM(E); SDS Essex) and water *ad libitum*. Mice were characterised using body weight, blood glucose and serum cholesterol. All animal experiments were approved by the Animal Experiment Board in Finland.

Sample collection

At 13 weeks of age, mice were individually housed in metabolism cages for 72 h with food and water *ad libitum*. Food and water intake, as well as urinary and faecal excretion, were measured. Faeces were carefully separated and frozen at −20°C for bile acid analysis. At 15 weeks of age, mice were euthanised using CO_2 (70 % CO_2/30 % O_2; AGA). Blood was collected immediately by decapitation for blood glucose measurements. Serum was separated and frozen at −20°C for further analyses. Liver samples were snap-frozen in liquid N_2 and frozen at −80°C for quantitative RT-PCR.

Intestinal permeability measurements

Fresh segments of jejunum and colon were dissected in duplicate, opened along the mesenteric border and pinned onto sliders into an EasyMount Ussing chamber (Physiologic Instruments). Chamber halves were filled with 5 ml Ringer's solution on each side (120 mM-NaCl, 5 mM-KCl, 25 mM-$NaHCO_3$, 1·8 mM-Na_2HPO_4, 0·2 mM-NaH_2PO_4, 1·25 mM-$CaCl_2$, 1 mM-$MgSO_4$ and 10 mM-glucose). The system was water-jacketed to 37°C and carbonated with carbogen (95 % O_2, 5 % CO_2; AGA) gas flow. After an equilibration period of 10 min, solutions were replaced with fresh Ringer's solution, and 4 kDa fluorescein isothiocyanate dextran (TdB Cons) was added to the luminal side to a final concentration of 2·2 mg/ml. Serosal fluorescence was detected at 45 min with a Wallac Victor^2 1420 multilabel counter (Perkin-Elmer). Intestinal permeability was determined by comparing serosal fluorescence with luminal fluorescence as per mille (‰, 1/1000) of translocated dextran. The CV between technical replicates was 20 % for jejunum and 23 % for colon. Gut electrophysiology was monitored throughout the experiments.

Faecal bile acid analysis

Faecal samples were dried overnight with N_2 gas flow and pulverised. Bile acids were extracted and analysed from 200 mg of dried faeces by GLC according to a previously described method[17]. Internal standards were run for isolithocholic acid, lithocholic acid, epideoxycholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid and β-muricholic acid. Daily excretion was calculated from the bile acid content in dry faeces by taking into account faecal water content and daily faecal excretion, as weighed during metabolism caging. An index for faecal bile acid hydrophobicity was calculated as a percentage-weighted mean of the hydrophobicities of six bile acids – lithocholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid and β-muricholic acid – according to a previous report[18], using estimated values for lithocholic acid (1·13) and muricholic acid (−0·65).

Gene expression assays

RNA was extracted from frozen liver samples using TRIzol reagent (RT111; Molecular Research Center), as described previously[10]. RNA concentration was measured with NanoDrop 8000 (Thermo Scientific), and strands were converted to complementary DNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Reactions for quantitative PCR were run using TaqMan chemistry (Applied Biosystems) for FXR (Mm0043,6420_m1) and LBP (Mm0049,3139_m1). Gene expressions were normalised to β-actin. Reactions were run on a CFX96 real-time PCR detection system (Bio-Rad) in triplicate. Gene expression was calculated with Bio-Rad CFX Manager software using the normalised expression $\Delta\Delta C$(t) method.

Serological analyses

Serum LBP was analysed using a commercial ELISA for mouse LBP (HK205; Hycult Biotech). The serum cholesterol precursors desmosterol and lathosterol, which reflect whole-body cholesterol synthesis, and cholestanol and campesterol, which reflect cholesterol absorption efficiency also in animal studies[19], were quantified with GLC on a 50 m-long capillary column (Ultra-2; Agilent Technologies) using 5α-cholestane as the internal standard[20]. Values are expressed as ratios to cholesterol of the same run (10^2 μg/mg cholesterol).

Statistical analysis

Differences between the groups were analysed with the non-parametric Mann–Whitney U test using PASW Statistics software version 18.0.2 (IBM). Correlations were calculated as Spearman's correlation coefficient. A P value below 0·05 was considered to be statistically significant. All data are expressed

Table 1. Characterisation of mice
(Mean values with their standard errors)

	Wild type (*n* 10)		Obese (*n* 7)		
	Mean	SEM	Mean	SEM	*P*
Body weight (g)	26·2	0·71	53·6	0·70	<0·001
Blood glucose (mmol/l)	8·7	0·69	14·3	1·71	<0·01
Serum cholestanol (100 μg/mg cholesterol)	580·5	12·3	1027·0	12·0	<0·01
Serum desmosterol (100 μg/mg cholesterol)	170·0	14·2	58·1	2·5	<0·01

as means with their standard errors, and all significances are expressed as two-sided.

Results

Characterisation of mouse strains

The *ob/ob* mice were more obese ($P<0·001$; Table 1) and had higher blood glucose levels than the wild-type mice ($P<0·01$; Table 1). The ratios of serum cholesterol synthesis markers were lower ($P<0·01$) and those of absorption markers higher ($P<0·01$) in *ob/ob* mice compared with wild-type mice (shown for desmosterol and cholestanol to cholesterol in Table 1). The synthesis markers tended to be inversely correlated with the absorption markers in wild-type mice but not in obese mice (Spearman's correlation coefficient: r −0·61, $P=0·06$ in wild type and r 0·21, $P=0·65$ in *ob/ob* for desmosterol to cholestanol), which suggests impaired cholesterol homeostasis in obese mice. In metabolism cages, obese mice were hyperphagic, which was reflected as a larger daily faecal output (Table 2). Obese mice also had a higher urinary excretion rate and consumed more water (Table 2).

Intestinal permeability and lipopolysaccharide-binding protein

There were no signs of altered permeability to fluorescein isothiocyanate dextran in either jejunum ($P=0·93$; Fig. 1(a)) or colon ($P=0·86$; Fig. 1(b)) or tissue electrical resistance (Table 3), as measured in the Ussing chamber. Moreover, there was no difference in liver LBP expression, which was measured as a marker of portal endotoxin ($P=0·70$; Fig. 2(a)), nor in jejunal tissue weight ($P=0·41$; Fig. 1(c)). Jejunum weight was well correlated with jejunal permeability (r 0·66, $P<0·01$). Serum LBP was increased 2·5-fold in obese mice compared with wild-type mice ($P=0·001$; Fig. 2(b)).

Faecal bile acids and liver farnesoid X receptor

Obese mice had a 38% higher excretion of bile acids per d compared with that of wild-type mice ($P<0·01$; Fig. 3(a)), although the concentration of total faecal bile acids was smaller compared with wild-type mice (2526 (SEM 206) μg/g dry faeces *v.* 3453 (SEM 192) μg/g dry faeces, $P<0·01$) as explained by high faecal mass (Table 2). There was no difference in daily faecal bile acid excretion per daily food intake (835 (SEM 31·5) μg/g ingested food *v.* 822 (SEM 28·0) μg/g ingested food, $P=0·70$), which suggests normal bile excretion for food intake. Serum cholesterol levels were higher in obese mice compared with wild-type mice ($P<0·001$; Fig. 3(b)) The increased daily excretion of bile acids was not reflected in liver FXR expression, which was unchanged ($P=0·42$; Fig. 3(c)). To evaluate potential cytotoxicity, we calculated the hydrophobicity index of faecal bile acids. There was no difference in the calculated hydrophobicity of faecal bile acids between the obese and wild-type mice ($P=0·21$; Fig. 3(d)), despite moderate changes in the proportions of individual bile acids. The proportions of cholic acid and β-muricholic acid were approximately 40·2 and 59·4% lower in obese mice than in wild-type mice ($P<0·01$ for both; Fig. 4(a)), whereas the proportion of isolithocholic acid was higher in obese mice than in wild-type mice ($P<0·01$). The proportion of β-muricholic acid was inversely correlated with blood glucose concentration (Spearman's correlation coefficient −0·56, $P=0·019$). The daily excretion of nearly all the identified bile acids was increased in obese mice ($P<0·01$; Fig. 4(b)). On the contrary, the excretion of β-muricholic acid was decreased in obese mice ($P<0·01$). The excretion of cholic acid was unaffected by obesity ($P=1·0$).

Discussion

The purpose of the present study was to investigate whether intestinal permeability is increased in a genetically obese *ob/ob* mouse on a diet with a normal fat content. Animal studies of obesity often utilise the so-called diet-induced obesity model in which animals gain weight by the consumption of an energy-dense fatty diet. Studies on intestinal permeability in the diet-induced model cannot conclude on whether intestinal permeability was increased by the diet or the resulting adipose tissue expansion. In the present study, we used

Table 2. Metabolism caging at 13 weeks of age
(Mean values with their standard errors)

	Wild type		Obese		
	Mean	SEM	Mean	SEM	*P*
Food consumption (g/d)	4·1	0·16	5·9	0·12	<0·01
Faecal output (g/d)	0·98	0·05	2·0	0·17	<0·01
Water consumption (g/d)	3·2	0·10	9·1	0·71	<0·001
Urinary excretion (g/d)	1·1	0·09	7·3	0·51	<0·001

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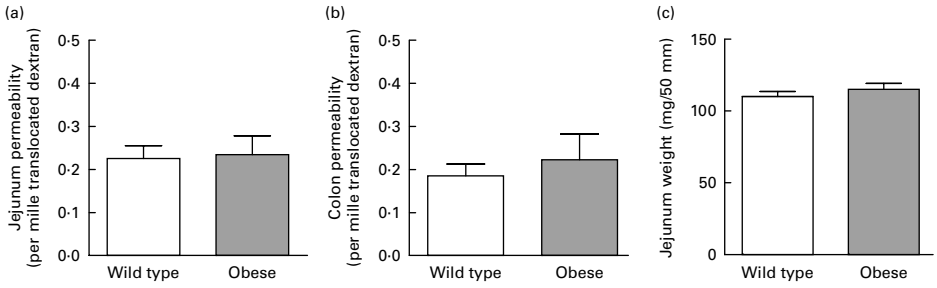

Fig. 1. Permeability of (a) jejunum, (b) colon and (c) weight of jejunum of wild-type and obese mice. Intestinal permeability was measured as the translocation of fluorescein isothiocyanate dextran (4 kDa) in the Ussing chamber system. Values are means, with their standard errors represented by vertical bars. There were no differences between the groups.

genetically obese, hyperphagic *ob/ob* mice, which became obese by eating normal chow.

In the present study, obese mice demonstrated no signs of altered barrier function when they had eaten a regular rodent chow with a normal fat content. Although the present data are supported by a rat study demonstrating that gut barrier is impaired solely by a high-fat diet, not genetic obesity[8], the present findings are in contrast with some previous studies on the *ob/ob* mouse strain[4,6,7,21]. These studies have reported increased gut permeability[6,7] and elevated portal or plasma endotoxin levels[4,6,7,21] in mice 1–3 weeks younger than those in the present study, but obtained from different suppliers. This raises the question of whether impaired barrier function is a feature specific to some distinct strain of *ob/ob* mice, but would not be related to obesity itself.

Because the term 'barrier dysfunction' includes different aspects of barrier function ranging from paracellular or transcellular translocation to submucosal or circulatory endotoxins, it is difficult to compare reports with varying methodology for barrier function. We have measured permeability in Ussing chambers as tissue resistance and the translocation of 4 kDa fluorescein isothiocyanate dextran. In previous studies, 44 kDa horseradish peroxidase has been used[6], which may translocate through a different pathway, or 4 kDa dextran has been orally administered *in vivo*[7], which could be affected by gastrointestinal transit. However, the present results on tissue resistance contradict with previous findings[6], which implies that there are true differences in permeability between these studies.

We measured the LBP from serum and assessed its hepatic expression. LBP is an acute-phase protein produced by the liver, and has an important role in the immune response to endotoxins[22]. Due to several well-known difficulties related to quantifying serum LPS levels, we measured liver LBP expression as an indirect marker of portal endotoxins and increased intestinal permeability. In concordance with our Ussing chamber measurements, liver LBP expression was unaffected by obesity. On the contrary, serum LBP was markedly increased in obese animals. An epidemiological study on Chinese men has reported a positive correlation between obesity and serum LBP, which they claimed to be a marker of endotoxaemia[23]. However, there are no studies showing that serum LBP reflects serum LPS levels in obese individuals. It has only been proven to associate with decreased fat mass after weight loss in human subjects[24]. Furthermore, adipose tissue expression of LBP correlates with adipocyte size (Robert Caesar, personal communication). Since liver LBP expression did not reflect elevated serum LBP levels in the present study, the present results do not support the use of serum LBP as a marker of endotoxaemia.

Rodent studies have consistently demonstrated increased intestinal permeability following a high-fat diet[7–12]. We have reported evidence suggesting that a diet high in fat impairs gut barrier function via a mechanism related to alterations in luminal bile acid profile[10]. In mice on a high-fat diet, the faecal hydrophobicity index was 0·214 (SEM 0·019), whereas in control mice it was 0·086 (SEM 0·006), showing a 2·5-fold increase in faecal hydrophobicity on a high-fat diet (LK Stenman, unpublished results). In the present study, in addition to a lack of impaired barrier function, *ob/ob* mice showed no change in luminal bile acid hydrophobicity. Thus, these alterations appear to be exclusive to a fatty diet and are not attributable to obesity.

Interestingly, in the present study, the excretion of muricholic acid was drastically decreased in obese mice. A previous paper reported a similarly decreased proportion of β-muricholic acid in the bile of alloxan-induced diabetic mice[25]. In a study on isolated rat livers, the 6β-hydroxylation of chenodeoxycholic acid to α-muricholic acid and further epimerisation to β-muricholic acid were approximately halved in the liver of streptozotocin-induced diabetic rats[26]. This effect was counteracted by insulin treatment, suggesting that the lack of insulin signalling leads to decreased synthesis

Table 3. Gut electrophysiology in the Ussing chamber
(Mean values with their standard errors)

	Wild-type		Obese		
	Mean	SEM	Mean	SEM	*P*
Jejunum					
Short-circuit current (μA/cm^2)	148·4	16·8	191·2	30·9	0·24
Resistance (Ω/cm^2)	31·4	2·0	32·9	3·2	0·92
Colon					
Short-circuit current (μA/cm^2)	46·6	5·2	42·3	4·9	0·63
Resistance (Ω/cm^2)	47·9	1·7	50·2	1·6	0·56

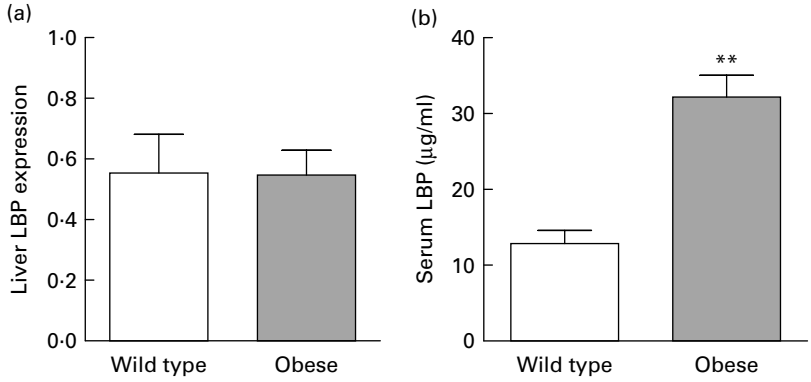

Fig. 2. (a) Liver expression and (b) serum concentration of lipopolysaccharide-binding protein (LBP) in wild-type and obese *ob/ob* mice. Liver expressions were normalised to β-actin. Values are means, with their standard errors represented by vertical bars. ** Mean value was significantly higher compared with wild-type mice ($P<0·01$).

of β-muricholic acid. In the present study, blood glucose was inversely correlated with the proportion of faecal β-muricholic acid. Thus, the present results suggest that the lack of insulin action may also impair the synthesis of β-muricholic acid.

Obese mice in the present study did not exhibit any difference in liver FXR expression despite an elevated bile acid excretion rate. FXR is a key regulator of liver bile acid synthesis as an inhibitor of the bile acid-synthesising enzyme CYP7A1[27]. We are aware of only one study on liver FXR in *ob/ob* mice stating that FXR was overexpressed[28], which was reflected as dramatically decreased CYP7A1 expression, suggesting decreased bile acid excretion. However, other results on this subject in *ob/ob* mice are conflicting[29,30]. It should also be noted that FXR influences lipid and glucose metabolism[31,32], which

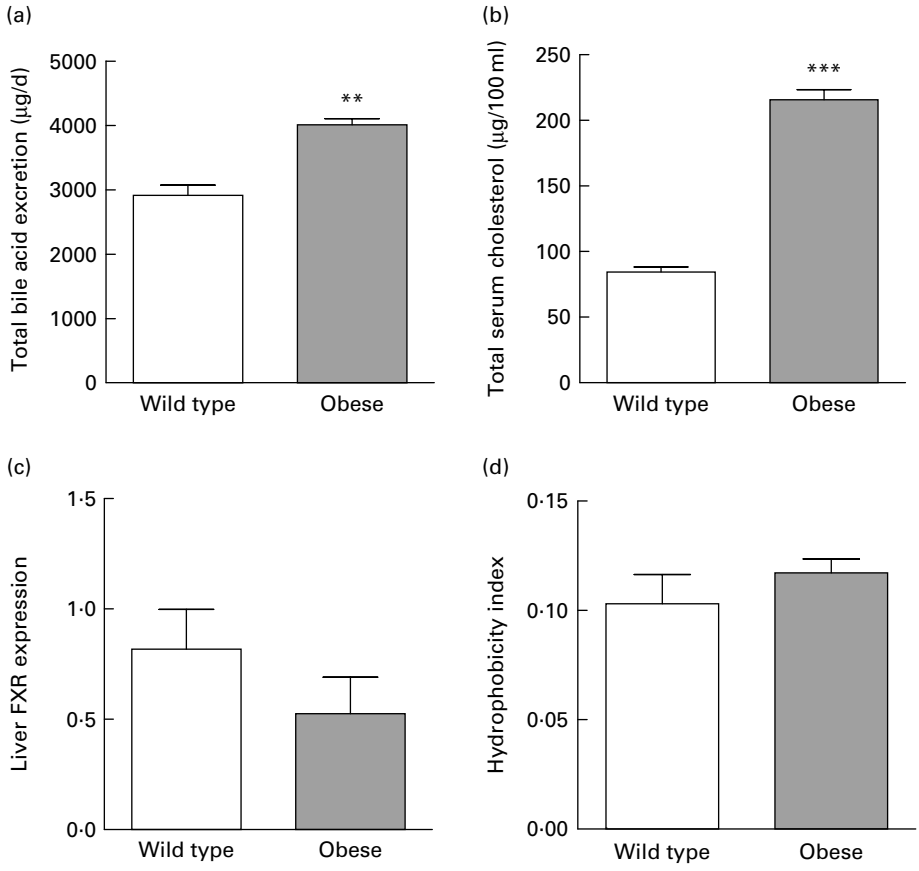

Fig. 3. (a) Total faecal bile acid excretion, (b) total serum cholesterol, (c) liver farnesoid X receptor (FXR) expression and (d) faecal bile acid hydrophobicity index in wild-type and obese mice. Liver expressions were normalised to β-actin. An index for faecal hydrophobicity was calculated as a percentage-weighted mean of hydrophobicities of individual bile acids. Values are means, with their standard errors represented by vertical bars. Mean value was significantly higher compared with wild-type mice: ** $P < 0·01$, *** $P < 0·001$.

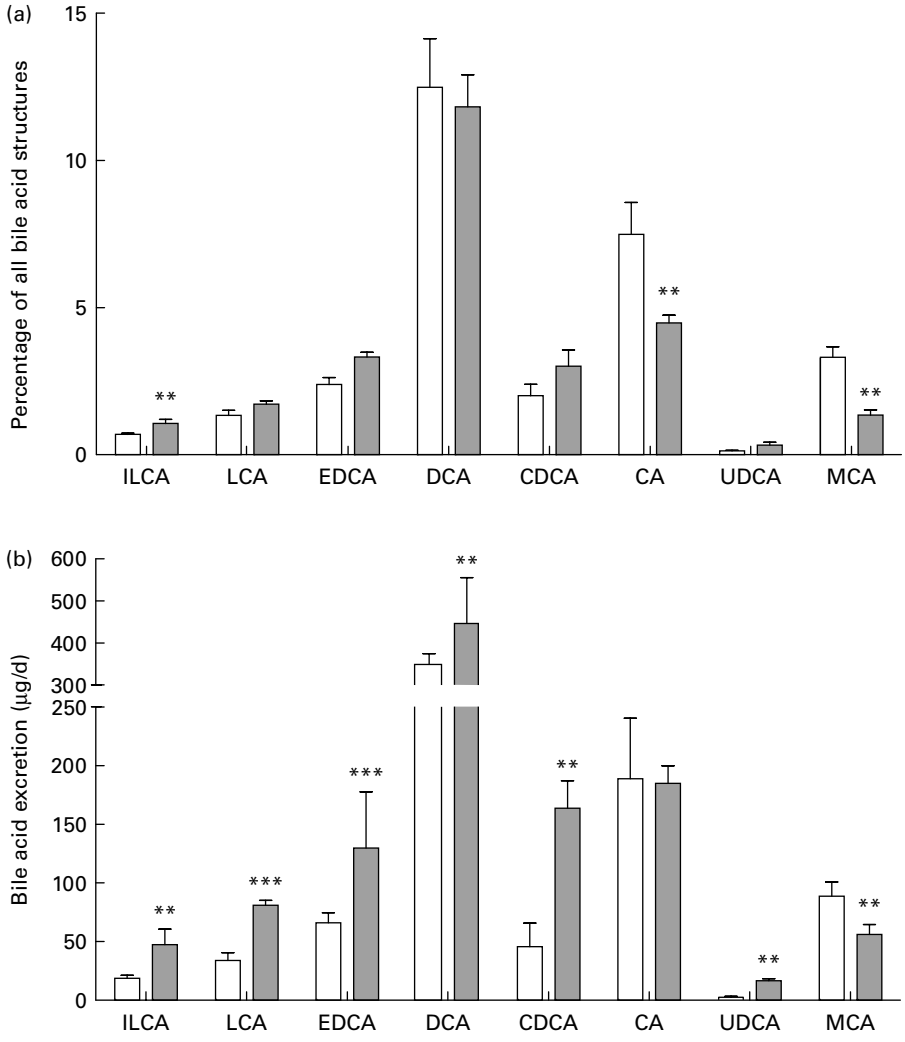

Fig. 4. Relative proportions of (a) faecal bile acids and (b) their daily excretion in wild-type and obese mice. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different compared with wild-type mice: ** $P<0{\cdot}01$; *** $P<0{\cdot}001$. ILCA, isolithocholic acid; LCA, lithocholic acid; EDCA, epideoxycholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; MCA, β-muricholic acid.

complicates the interpretation of liver FXR data in a mouse model with impaired lipid and glucose homeostasis.

As we needed a mouse model that becomes obese on a normal chow diet, we used leptin-deficient *ob/ob* mice for these experiments. Therefore, we cannot rule out the possibility that the results in the present study are partly attributable to leptin deficiency, not obesity *per se*. Leptin-deficient *ob/ob* mice are resistant to colitis induced by the exogenous stimulus dextran sodium sulphate[33], although leptin seems not to play a role in spontaneously developing colitis in IL-10 double knockout mice of the same mouse strain[34]. It cannot thus be entirely ruled out that leptin deficiency had an effect on barrier function in *ob/ob* mice.

Several studies have addressed the role of leptin in cholesterol metabolism and gut function by the re-administration of leptin into *ob/ob* mice. As these mice tend to lose weight, the effect may not be completely differentiated from obesity. However, it is possible that leptin decreases cholesterol absorption[35,36], as well as bile acid pool size and faecal bile acid excretion[36]. It was evident that in the present study, cholesterol homeostasis was disrupted in obese mice and cholesterol absorption was high, which is in line with previous results observed in leptin-deficient mice. Leptin administration has also been reported to increase the concentration of biliary muricholic acid[36,37], but it is uncertain whether this is only a secondary effect of weight loss and regained insulin function. We cannot then ascertain that all differences seen between the groups are solely obesity induced.

In conclusion, using the genetically obese *ob/ob* mouse model, we demonstrate that intestinal permeability is not altered when the animals are on a diet with a normal fat content. Faecal bile acid hydrophobicity, which has been proposed to affect barrier function, was not altered in this model. The present data also suggest that LBP levels in serum are not a marker of barrier dysfunction.

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