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Cholesterol mobilization from hepatic lipid droplets during endotoxemia is altered in obese ob/ob mice

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The innate immune response to pathogens during the acute phase response includes lipid metabolism adaptations. Hepatic triacylglycerol (TG) and cholesteryl ester (CE) storage in and mobilization from lipid droplets (LDs) respond to metabolic changes under the control of liver X receptor (LXR) transactivation and cytokine transduction. To evaluate whether alterations of these mechanisms have an impact in the adaptive response to endotoxemia, we analysed liver metabolism changes in lipopolysaccharide (LPS)-treated ob/ob mice, which show altered metabolic and innate responses and a higher sensitivity to sepsis. Lipid composition of serum lipoproteins and hepatic LDs was determined in wild type and ob/ob mice 24 h after LPS treatment. Liver metabolic profiling was done by measuring enzyme activities and mRNA levels. Increased CE hydrolase activity in LDs from endotoxemic mice was accompanied by a lower content of CE and low or no induction of LXR-mediated expression of genes involved in HDL secretion. The attenuated response in liver lipid mobilization accompanied by the strain-specific cholesterol enrichment of secreted VLDL might lead to accumulation of LDL cholesterol. According to our findings, obese leptin-deficient mice present an altered control of hepatic lipid metabolism responses to LPS, which might be, in part at least, a consequence of impaired LXR.

Keywords: lipid/lipopolysaccharide/lipoprotein/liver/mouse.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGH, diacylglycerol hydrolase; FC, free cholesterol; LD, lipid droplet; LXR, liver X receptor; PNSN, postnuclear supernatant; TG, triacylglycerol; TGH, triacylglycerol hydrolase; WT, wild type.

Formation and utilization of lipid droplets (LDs) are essential processes in cellular lipid metabolism. In hepatocytes LDs accumulate and mobilize lipids according to cellular needs, channelling them to lipoprotein secretion, energy obtaining or biosynthesis pathways (*1*). LD biology has many other implications in cell and tissue biology; among others, it is known to be linked to the inflammatory response.

Liver responses to lipopolysaccharide (LPS) treatment in mice include specific modifications in LD metabolism mediated by proinflammatory cytokines (tumour necrosis factor (TNF)α, interleukin (IL)-1β and IL-6) and liver X receptor (LXR). Cytokine-mediated control mechanisms include modulation of acylglyceride hydrolytic activities leading to decreased cellular triacylglycerol (TG) stored in LDs (*2*) while LXR reduces LD-associated cholesteryl ester (CE) levels. It has been described that TNFα administration also induces an increase in lipolysis rate on LDs in adipocytes (*3*, *4*). Other works show that in clinical and experimental sepsis LDs accumulate in immune cells and associate with proteins involved in the inflammatory response (*5*, *6*).

Specific adaptations of liver lipid metabolism contribute to the so-called lipemia of sepsis, one of the best known clinical features related to lipid metabolism in the acute phase response to infections. Lipoproteins have the ability to bind and accelerate the clearance of endotoxins, and by that they help in the control of the inflammatory response, limiting the potential tissue injury associated to it (*7*). Proinflammatory agents induce liver lipoprotein secretion by modulating many steps of VLDL or HDL particle formation, including lipid supply from LDs. We have previously described that in the liver of LPS-treated wild type (WT) mice hepatocytes channel LD-associated acylglycerides to VLDL particle formation, while cholesterol stored as CE in this organelle is directed to nascent HDL particles (*2*).

Those observations have led us to wonder whether the hepatic metabolic response to LPS treatment is altered in ob/ob mice. These mutant animals lack leptin, a hormone secreted by adipose tissue which operates in the hypothalamus and induces satiety. Therefore, ob/ob mice are hyperphagic and develop obesity, insulin resistance and hepatic steatosis, and have an increased sensitivity and mortality during endotoxemia (*8*). We hypothesized that the alteration in the hepatic lipid metabolism of ob/ob mice might lead to an altered hepatic adaptation to endotoxemia, and this would explain the higher mortality of these animals. Indeed, in this work we show that the mechanisms that control the response of hepatic

cholesterol mobilization during endotoxemia are altered in ob/ob mice, leading to an adverse lipoprotein profile.

Materials and Methods

Chemicals and reagents

Free cholesterol (FC), cholesteryl oleate, TG, diacylglycerol (DG), sodium taurocholate, oleoyl coenzyme A and LPS (*Escherichia coli* serotype O111:B4) were from Sigma (MO). DG used as a standard for lipid quantification by TLC was from Avanti Polar Lipids, Inc. (AL). Lecithin for enzymatic assays was from Lipid Products (UK). Silica plates for TLC were from Macherey-Nagel (Germany). Organic solvents for TLC were from Scharlau (Spain). Glycerol tri[1-^{14}C]oleate, cholesteryl[1-^{14}C]oleate and [1-^{14}C]oleoyl CoA were from GE Healthcare (UK). 1,2-dioleoyl rac-[^{14}C(U)]glycerol was from American Radiolabeled Chemicals (MO). All other compounds were of the necessary quality for our purposes.

Animals and LPS treatment

8-week-old C57BL/6J (WT) and B6.V-Lepob/J (ob/ob) female mice (Jackson Laboratory, Maine) were housed in a temperature-controlled room with a 12–h-light/dark cycle and *ad libitum* access to water and food. Animals were intraperitoneally treated with 1 mg/kg body weight of LPS in saline or saline alone and simultaneously food-deprived. Mice were euthanized 24 h later to obtain serum and the liver; this time point was selected because it has been described that leptin secretion is suppressed after 18 h of fasting (*9*) and so the differences between WT and ob/ob mice observed 24 h after LPS treatment are mainly consequence of obesity and not leptin deficiency.

To confirm acute phase response, serum biochemical markers were determined using an automatic Cobas clinic analyser (Laboratorio Lafita, Spain) and amyloid A by ELISA (Tridelta Development Ltd., Ireland) (Table I).

In order to assess the *in vivo* hepatic lipid secretion, lipoprotein lipase inhibitor Poloxamer (P-407; Life Technologies, Spain) was used as previously described (*10*). Poloxamer was administered intraperitoneally 20 h after vehicle or LPS injection and 4 h later serum VLDL were purified by gel filtration and lipid characterization was performed as described below.

All the procedures that involved animal handling were approved by the Ethics Committee for Animal Welfare of the University of the Basque Country UPV/EHU.

Serum lipoprotein profiling

Serum lipoproteins were separated and analysed as we previously described (*2*) using an ÄKTA-FPLC (fast protein liquid chromatography) system equipped with a Superose 6 10/300 GL column connected in tandem to a Superdex 200 10/300 GL column (GE Healthcare Europe GmbH, Germany).

Table I. Liver and serum parameters of ob/ob mice treated with endotoxin

	Control	LPS
Hepatic index (%)	6.44 ± 0.26	5.98 ± 0.27
Glucose (mg/dl)	397 ± 42	63.4 ± 7.7***
GOT (U/l)	300 ± 60	632 ± 285
GPT U/l)	195 ± 46	132 ± 43
Albumin (g/l)	45.0 ± 0.9	36.1 ± 1.2***
Total protein (g/l)	56.8 ± 0.8	49.9 ± 0.7***
Serum amyloid A (μg/ml)	71.7 ± 23.9	755 ± 57**

LPS, lipopolysaccharide; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase. Mice were administered saline or 1 mg/kg LPS intraperitoneally and food-deprived. Blood and liver were obtained after 24 h. Serum GOT and GPT activities and albumin concentration were quantified by standard biochemical methods. Serum amyloid A concentration was measured by ELISA. These results were obtained in parallel to those presented in a previous work (*2*) on WT mice. Data are expressed as the mean ± SEM of at least five animals in each group. Student's *t*-test: $^{**}P<0.01$, $^{***}P<0.001$.

Isolation of subcellular fractions and determination of enzymatic activities

Postnuclear supernatant (PNSN) and microsomal, cytosolic and lysosomal fractions were isolated from liver homogenates by differential centrifugation as described elsewhere (*11*). LD subpopulations were purified by sucrose density gradient ultracentrifugation as described by Arisqueta *et al.* (*2*). Enzymatic activities were assayed as described by Arisqueta *et al.* (*2*).

Lipid extraction and quantification

Lipids were exhaustively extracted following the method described by Bligh and Dyer (*12*). Lipid quantification was performed by TLC and image analysis as described elsewhere (*13*).

Relative mRNA quantification by real time RT-PCR

Total RNA was extracted using Trizol Reagent (Life Technologies, Spain) and cDNAs were obtained by retrotranscription (SuperScript III RT; Life Technologies, Spain) following the manufacturers' instructions. Quantification of mRNA by real time PCR was performed using the SYBR Green detection system (Applied Biosystems, CA) in an Applied Biosystems 7000 RT-PCR System. Samples were analysed in triplicate and 18 S rRNA was used for normalization.

All primers used have been previously described (*2*) except for the following: Abcg5 Fw: tgtgattgtcaccatccaccag, Rv: ccacagaacaccaactctccgt; Abcg8 Fw: ttcacagcccacaatctggtg, Rv: atgtcagagcgaggctggtg; Abcb4 Fw: cgtcaggtgtctaagggaaatcat, Rv: tagcggata ttttcagcgatcgt; Ch25h Fw: ctcttcgacaccgagatcttcg, Rv: ttctggtgatgcaccttgtgg; Hsl Fw: ccagttcacacctgccatcc, Rv: tgtctcgttgcgtttgta; Pnpla2 Fw: ctgcctgctgattgccatg, Rv: gatgacgttctctccgctgtgaaac; Pnpla3 Fw: aggagaggccaaagctgagg, Rv: ccacccatcatctagacactgttg.

Protein measurement and Western blotting

Protein concentration was routinely determined by the bicinchoninic acid method (Thermo Fisher Scientific, Spain) including 2% SDS in all samples to avoid erroneous measures due to the presence of lipid, except for the F1 LD subpopulation samples, in which due to the high amount of lipid the Lowry protein assay modified by Peterson (*14*) was used as previously described (*2*).

For Western blotting analysis, protein fractionation by SDS-PAGE and immunodetection were performed as described elsewhere (*15*). Guinea pig anti-mouse perilipin-2 (Progen, Germany) was used as primary antibody and anti-guinea pig IgG (Sigma, MO) as secondary antibody.

Statistics

Statistical analysis was performed using GraphPad Prism version 5.02 (GraphPad Software, CA). Comparisons between two groups were performed by the unpaired Student's *t*-test. Two-way ANOVA and Bonferroni test were performed to assess the effect of LPS treatment in the three LD subpopulations and the differences between them. A $P < 0.05$ was considered statistically significant.

Results

Responses of hepatic TG and CE metabolism to LPS

Aiming to evaluate the importance of the control mechanisms that modulate the hepatic LD metabolism in the response to endotoxemia in ob/ob mice, we first analysed the neutral lipid, *i.e.* TG and CE, mobilization and storage capacity in crude liver extracts and relevant subcellular fractions. As shown in Fig. 1A, LPS treatment did not induce any significant changes in TG, DG or CE hydrolytic activities (TGH, DGH and CEH, rspectively) in liver PNSN. Regarding subcellular fractions, endotoxemia induced a slight but general drop in lysosomal hydrolytic activities which was significant in the case of DGH (Fig. 1A). Comparison with data from WT mice (below each

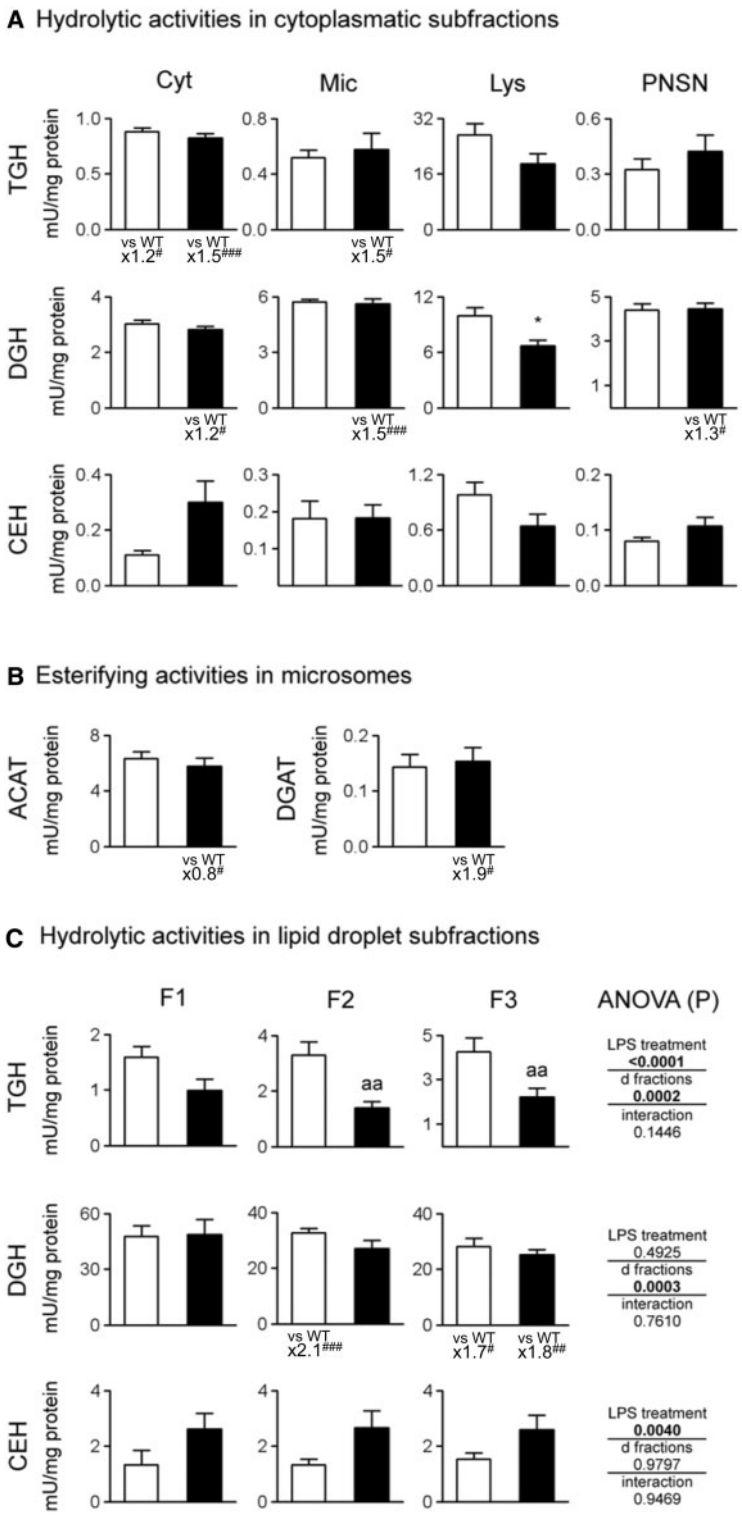

Fig. 1 Effect of LPS on relevant activities of lipid hydrolysis and esterification in hepatic subcellular fractions from ob/ob mice. (A) PNSN and cytosolic (Cyt), microsomal (Mic) and lysosomal (Lys) fractions were isolated from liver homogenates of LPS-treated ob/ob mice (1 mg/kg body weight in saline) (filled bars) and control ob/ob mice (saline) (open bars) after 24 h of fasting and hydrolytic activities (triacylglycerol hydrolase, TGH; diacylglycerol hydrolase, DGH; cholesteryl ester hydrolase, CEH) were measured. **(B)** Esterifying activities (acyl CoA:cholesterol acyltransferase, ACAT; diacylglycerol acyltransferase, DGAT) were also measured in the microsomal fraction. **(C)** Three LD subpopulations (F1–F3) were isolated from PNSN according to their density and hydrolytic activities were measured. Activity units (U) are defined as μmol of released product (fatty acids for TGH and CEH and glycerol for DGH) per min for hydrolytic activities and μmol of fatty acid incorporated per min for esterifying activities. These results were obtained in parallel to those presented in a previous work (*2*) on WT mice; when significant differences have been observed with respect to WT animals, fold-changes are expressed under the graphs. Data are expressed as the mean ± SEM ($n = 5$). Student's *t*-test was performed to assess the effect of LPS treatment on subcellular fractions: $^{*}P < 0.05$. The same test was performed to express significant differences with respect to WT animals: $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$. Two-way ANOVA was performed to assess the effect of LPS treatment on all the three density (d) subpopulations of LDs and the differences between subpopulations; *P* values for both and their interaction are separately expressed in the ANOVA column. Bonferroni post-test was performed to compare control and LPS groups in each LD subpopulation: $^{aa}P < 0.01$.

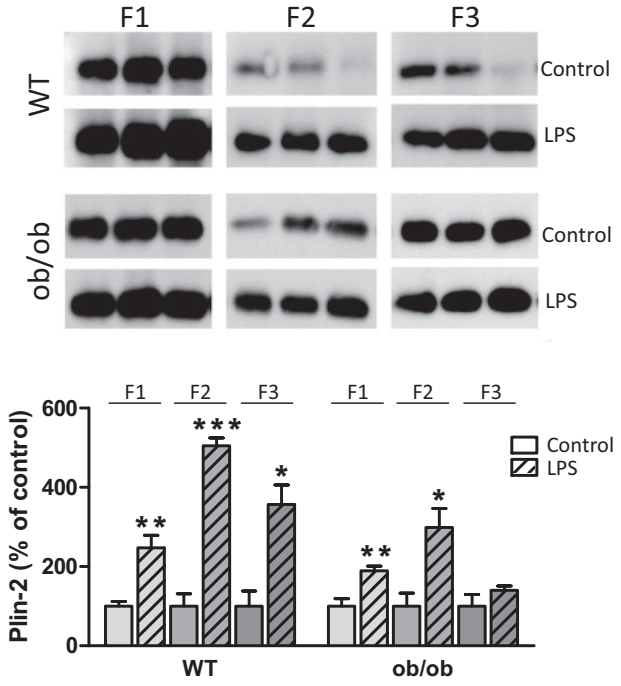

Fig. 2 Effect of LPS on perilipin-2 levels in purified LD fractions F1-F3 from WT and ob/ob mice. Equal protein quantities were loaded for each type of sample (F1: 0.25 μg, F2 and F3: 0.65 μg), and subjected to SDS-PAGE and immunoblotting. The pair of images shown for each fraction and strain corresponds to areas cut out from the same blot. Quantification was performed by optic densitometry and compared data were obtained from the same blot. Data in the graph (arbitrary units) are expressed as the mean ± SEM and each bar corresponds to LD preparations from five animals. Student's *t*-test: $*P<0.05$, $**P<0.01$. $***P<0.001$.

bar in the graphs of Fig. 1 and in (*2*)) reveals that adaptive changes in TG mobilization in neutral compartments (cytosol and microsomes) disappear in ob/ob mice. On the contrary, although no change was observed in WT mice, a 2-fold increase in cytosolic CEH activity (*t*-test: $P = 0.0525$) occurred in obese mice. Figure 1B shows that no changes occurred in the esterifying activities DG acyltransferase (DGAT) and acyl-coenzyme oA:cholesterol acyltransferase (ACAT) due to LPS treatment.

As it occurred in the cytosolic fraction, CEH activity in LDs from the liver of septic obese mice was higher than in LDs from control, fasted animals (Fig. 1C). Treatment with LPS also induced a reduction in TGH activity in LDs, particularly in the fractions with the highest density (Fig. 1C). Data corresponding to TG and DG hydrolysis were very similar to those obtained in LDs isolated from WT mice. This reduction of TG hydrolytic activity was accompanied by an increase of perilipin-2 expression in LDs, specifically in F1 and F2 subpopulations in both strains (Fig. 2). Perilipin-2 is the main structural protein in hepatic LDs and is thought to prevent lipolysis and TG turnover (*16*).

Next, we quantified the mRNA levels of several hydrolytic and biosynthetic enzymes with the aim of clarifying whether, besides perilipin-2 expression, the enzymatic activity modulation had a basis on the regulation of gene expression and which enzyme was implicated, if any, in the metabolic adaptations. As shown in Fig. 3, Pnpla2 (which codifies the major neutral liver TGH) mRNA levels did not change after LPS administration. Among the hydrolases only the endoplasmic reticulum carboxylesterase Ces1d mRNA levels diminished in both strains. The increase in CEH activity in the cytosolic fraction and LDs in LPS-treated obese mice is paralleled by the changes in Hsl expression (Fig. 3). Regarding the biosynthetic enzymes Dgat1, Dgat2 and Soat1, the main acyltransferases in the synthesis of TG and CE, they showed no significant modifications.

To determine the consequences of the altered hepatic LD metabolic profile in ob/ob mice during endotoxemia, we quantified lipid levels in liver LD subpopulations to assess compositional adaptations. One of the parameters that showed a clear tendency to change after LPS treatment was the TG/DG ratio of the F1 LD subpopulation which nearly doubled (Table II). This rise reflects the higher biosynthesis/hydrolysis ratio in endotoxemic ob/ob mice.

The second one is the CE molar fraction in the F2 LD subpopulation, which decreased significantly. This change gained significance when the three LD subpopulations were considered together showing a significant decrease of about 50% (ANOVA: $P<0.001$) (Table II). The modifications in cholesterol management were reflected in a lower CE/FC ratio value in LDs (ANOVA: $P = 0.059$).

In summary, we observed that while adaptive responses to LPS observed in acylglyceride metabolism of WT mice do not operate in ob/ob mice, which make the TG/DG ratio increase (it decreased in WT mice), cholesterol metabolism is deeply affected. We observed a higher CEH activity in LDs which led to a decrease of the CE molar fraction in that organelle.

Serum cholesterol distribution

Next we wondered whether these LD-associated metabolic adaptations in the ob/ob mice would be reflected in the lipoprotein profile as it occurs in septic WT mice (*2*). We determined whole serum TG, total cholesterol and HDL-cholesterol using an automatic Cobas clinical analyser (Roche Diagnostics) (Fig. 4A). As it occurred in WT mice, there was not a significant increase in serum TG content in LPS-treated obese mice. Serum total cholesterol increased almost 50% even though HDL-cholesterol underwent a 50% decrease, which indicates a big increase in cholesterol levels of apoB-containing lipoproteins, *i.e.* LDL. To clarify this question, then we fractionated serum lipoproteins from treated and control WT and ob/ob mice by gel-permeation using a double column FPLC system (described in 'Material and Methods' section) and measured protein (Fig. 4B), total cholesterol and phospholipid (PL) (Fig. 4C). The cholesterol distribution profile of ob/ob mouse serum shows a rise in the range of LDL (quantified in Table III) due to LPS treatment while in WT mice the main lipoprotein class that increased in endotoxemia was HDL. Moreover, the PL peak in

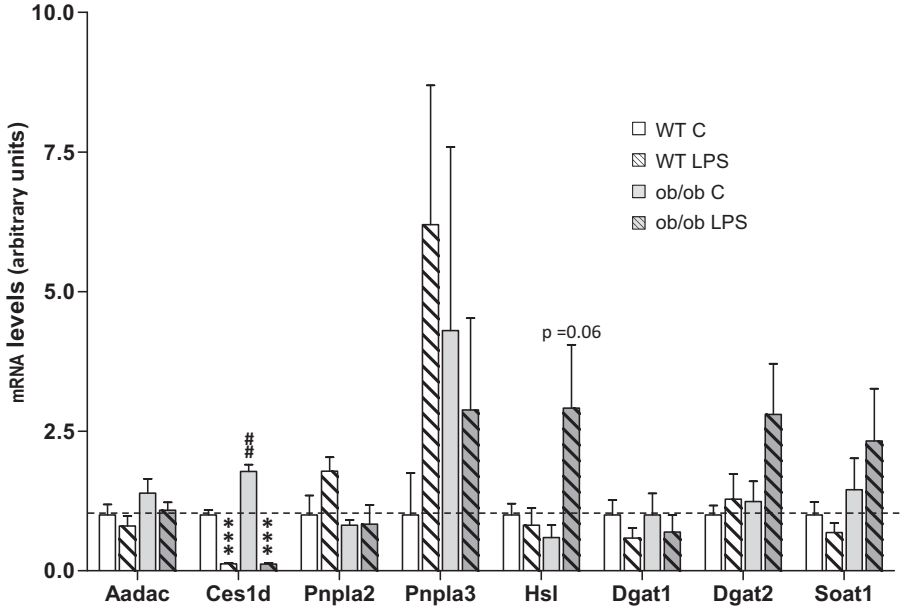

Fig. 3 Effect of LPS on mRNA levels of enzymes that catalyze lipid carboxyl ester hydrolysis and esterification reactions in the liver. Total RNA was extracted from livers of LPS-treated WT and ob/ob mice (1 mg/kg body weight in saline) and control (C) mice (saline) after 24 h of fasting. After retrotranscription relative measurement of the mRNA was performed by quantitative PCR as described in Materials and Methods. All the results were normalized with 18 S rRNA expression levels. Results correspond to the mean ± SEM of 4 animals. Student's *t*-test: ***$P<0.005$ when comparing LPS-treated animals with their controls; ##$P<0.01$ when comparing the two genotypes in the same condition.

Table II. Effect of LPS on lipid composition of hepatic lipid droplets from ob/ob mice

	F1		F2		F3		ANOVA (*P*)		
	Control	LPS	Control	LPS	Control	LPS	Treatment	Fractions	Interaction
Total protein (mg/liver g)	2.3 ± 0.3	2.3 ± 0.5	0.34 ± 0.02	0.4 ± 0.03	0.4 ± 0.1	0.4 ± 0.03	0.960	**<0.001**	0.993
Total lipid (μmol/liver g)	76 ± 4	106 ± 8***	0.85 ± 0.09	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	**0.003**	**<0.001**	**<0.001**
TG (%)	91 ± 1	94 ± 1	61 ± 4	59 ± 3	59 ± 5	60 ± 4	0.909	**<0.001**	0.803
DG (%)	3.2 ± 0.3	2.0 ± 0.3	2.6 ± 0.7	2.4 ± 0.5	5.6 ± 1.5	4.9 ± 1.5	0.364	**0.010**	0.847
CE (%)	2.5 ± 0.4	1.5 ± 0.3	3.4 ± 0.7	1.7 ± 0.4*	3.0 ± 0.3	1.5 ± 0.2	**<0.001**	0.465	0.745
FC (%)	0.9 ± 0.2	0.7 ± 0.2	4.2 ± 0.2	4.4 ± 1.0	6.9 ± 1.4	8.7 ± 2.2	0.528	**<0.001**	0.627
TG/DG	29 ± 3	50 ± 7*	28 ± 5	28 ± 5	13 ± 2	16 ± 4	0.052	**<0.001**	0.075
CE/FC	2.8 ± 0.4	2.4 ± 0.3	0.8 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.03	0.059	**<0.001**	0.971

TG, triacylglycerol; DG, diacylglycerol; CE, cholesteryl ester; FC, free cholesterol. LD density subpopulations (F1–F3) were isolated from liver homogenates of LPS-treated (1 mg/kg body weight in saline) and control (saline) ob/ob mice after 24 h of fasting as described in 'Materials and Methods' section. Lipids were extracted and the different species were separated by TLC and measured by optic densitometry. Total lipid quantities express the summation of all the measured species, which are then expressed as the percentage of the summation. These results were obtained in parallel to those presented in a previous work (*2*) on WT mice. Data are expressed as the mean ± SEM of five animals per group. Two-way ANOVA was performed to assess the effect of LPS treatment on all the three subpopulations and the differences between subpopulations; *P* values for both and the interaction are separately expressed in the ANOVA column and highlighted in bold when <0.05. Bonferroni post-test was performed to compare control and LPS groups in each LD subpopulation: *$P<0.05$, ***$P<0.001$.

the 10-nm diameter particles trended inversely in the two genotypes, resulting in the disappearance of the differences of the control condition (Table III).

Aiming to analyse whether the rise in plasma LDL cholesterol could be linked to the higher capacity of cholesterol mobilization in hepatic LDs after LPS treatment (Fig. 1C), control and endotoxemic mice were treated with Poloxamer, a lipoprotein lipase inhibitor that allows to analyse newly secreted VLDL. On the one hand we separately measured FC and CE in serum lipid extracts of LPS-treated WT and ob/ob mice with and without Poloxamer administration 4 h before euthanization; we also quantified total cholesterol and TG in fractionated, newly secreted VLDL (diameter > 65 nm). Figure 4D shows that newly secreted FC of LPS-treated obese mouse serum increases at the expense of CE (upper panel) without significant changes in the enrichment of total cholesterol in newly secreted VLDL (lower panel). LPS treatment did not modify FC secretion in WT mice and CE secretion was slightly but significantly lower. Total cholesterol in newly secreted VLDL in the obese strain is identical in both LPS-treated and control animals but significantly higher than in WT mice. These data indicate that newly secreted VLDL is cholesterol-enriched in ob/ob mice with higher FC content due to

Table III. Effect of LPS on the lipid levels in fractionated serum lipoproteins from WT and ob/ob mice

Fraction lipid (μg/ml)		WT C	WT LPS	ob/ob C	ob/ob LPS
>65 nm (VLDL)	Cho	3 ± 1	1 ± 2	6 ± 1	39 ± 13
	PL	7 ± 2	26 ± 6*	15 ± 2	54 ± 12*
25–40 nm (IDL)	Cho	13 ± 3	48 ± 5**	37 ± 9$^{\#}$	68 ± 9
	PL	12 ± 3	36 ± 3**	31 ± 4$^{\#}$	51 ± 6
18–25 nm (LDL)	Cho	44 ± 2	97 ± 10**	269 ± 21$^{\#\#\#}$	402 ± 3* $^{\#\#\#}$
	PL	56 ± 8	91 ± 10*	223 ± 17$^{\#\#\#}$	282 ± 12* $^{\#\#\#}$
13–18 nm (HDL)	Cho	120 ± 5	200 ± 11***	171 ± 21$^{\#}$	177 ± 22
	PL	126 ± 10	127 ± 6	144 ± 14	157 ± 14
10–11 nm (discoidal HDL)	Cho	5 ± 1	3 ± 1	6 ± 0.2	5 ± 1
	PL	54 ± 6	61 ± 5	105 ± 20$^{\#}$	79 ± 15

WT, wild type; C, control; LPS, lipopolysaccharide; Cho, total cholesterol; PL, phospholipids. These data represent the summation of lipids in the fractions corresponding to the ranges of lipoproteins indicated in Fig. 4B whose Cho and PL levels are represented in Fig. 4C. Data are expressed as the mean ± SEM of four animals per group. Student's *t*-test: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ when comparing LPS-treated animals with their controls; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ when comparing the two genotypes in the same condition.

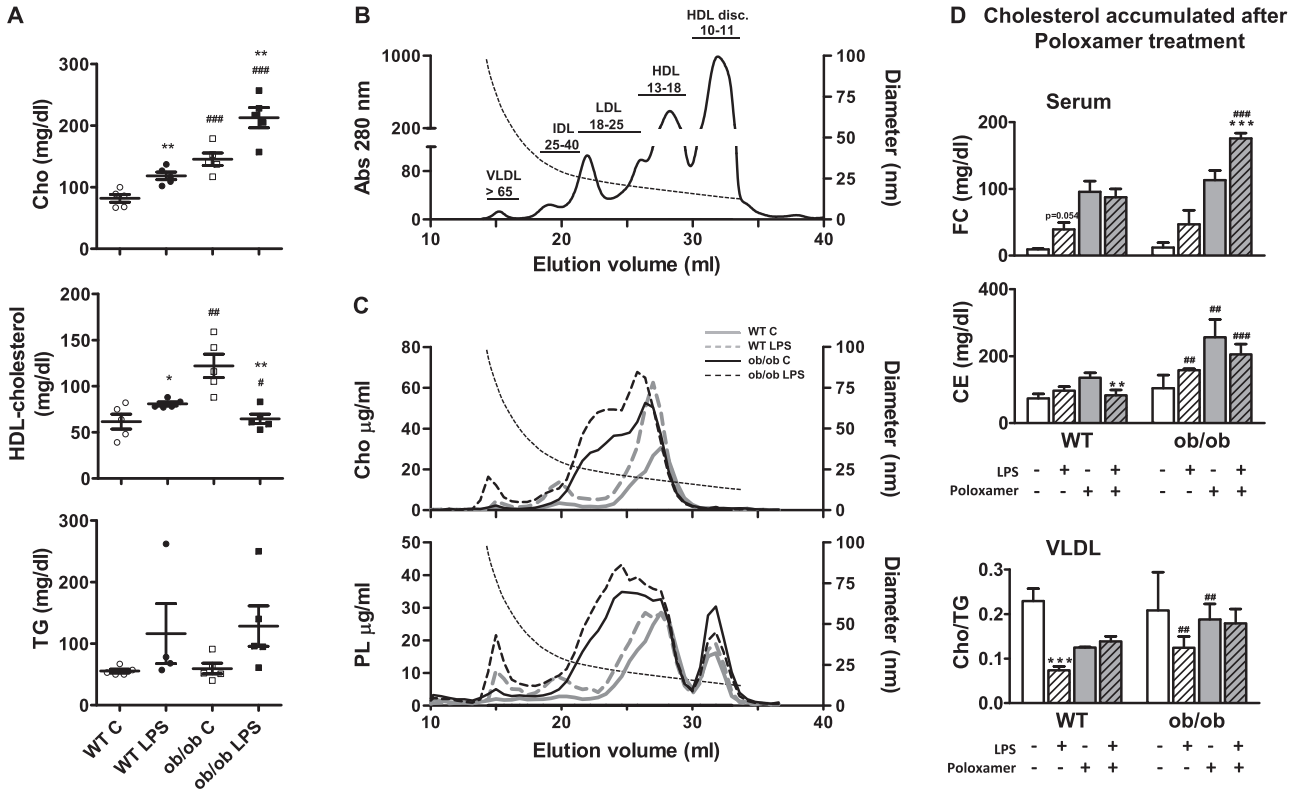

Fig. 4 Effect of LPS on the lipoprotein distribution of serum cholesterol. (A) Whole serum total cholesterol (Cho), HDL-cholesterol and TG (determined using an automatic analyser as described in 'Materials and Methods' section) of control (C) and LPS-treated (1 mg/kg body weight in saline) WT and ob/ob mice. **(B)** Sera were fractionated by gel filtration FPLC. A representative proteinogram (absorbance at 280 nm; arbitrary units) is shown. Size ranges (diameter in nm) used to define lipoprotein classes are indicated above the proteinogram. The dotted line represents particle size. **(C)** Cho and PL were enzymatically determined in fractionated sera. Each of the 40 points of the chromatographic profiles corresponds to the mean of four animals. **(D)** Poloxamer was administered intraperitoneally 20 h after vehicle or LPS injection and 4 h later serum was obtained and VLDL were purified by gel filtration. Whole serum lipids were extracted, separated by TLC and FC and CE were quantified. Cho and TG in fractionated VLDL were enzymatically quantified. All values correspond to the mean ± SEM of four animals. Student's *t*-test: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ when comparing LPS-treated animals with their controls; $\#P<0.05$, $\#\#P<0.01$ and $\#\#\#P<0.001$ when comparing the two genotypes in the same condition.

LPS treatment. Regarding the data sets corresponding to mice non-treated with Poloxamer, net values and differences between control and endotoxemic conditions are similar in WT and ob/ob mice, which indicates that plasmatic metabolization of VLDL-cholesterol was similar in both strains.

Attenuated transduction of LPS signal

In a previous work, we showed that the hepatic lipid metabolism adaptations during endotoxemia involve several specific changes in response to LXR transduction and inflammatory cytokines (*2*). To assess the contribution of LXR-mediated mechanisms in the

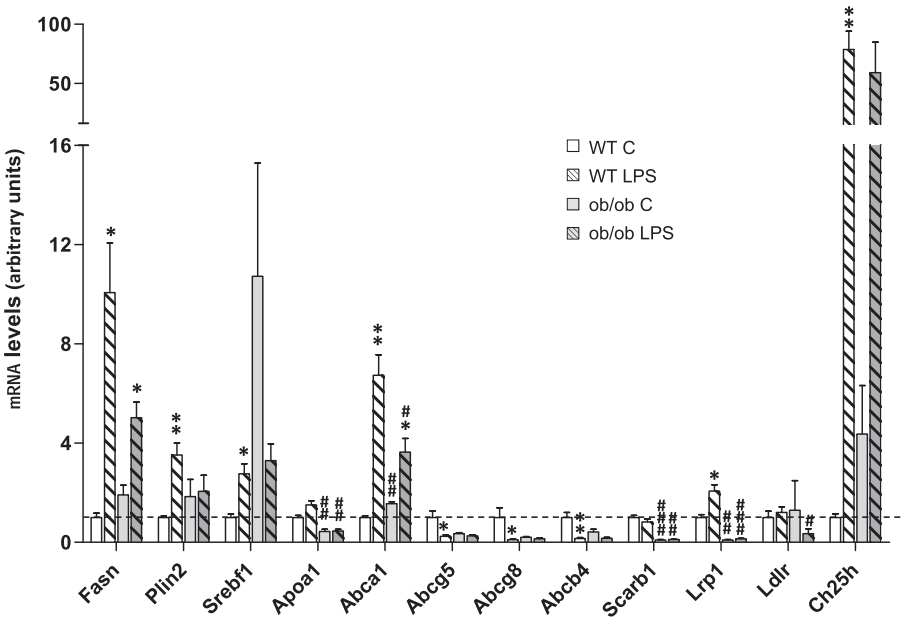

Fig. 5 Effect of LPS on the hepatic expression of genes related to lipid transport or regulatory processes. Student's *t*-test: $*P<0.05$, $**P<0.01$ when comparing LPS-treated animals with their controls; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ when comparing the two genotypes in the same condition. See the legend of Fig. 3 for further information.

alterations of cholesterol mobilization in obese mice, mRNA levels of relevant target genes were quantified. Additionally, expression of genes responsible for cholesterol management was also determined. Data in Fig. 5 suggest that obese mice have an attenuated response to LPS in LXR transactivation compared with WT mice: Abca1 and Fasn mRNA levels increased about 2.5-fold (6.7- and 10.1-fold, respectively, in WT mice) and no changes in Srebf1 and Plin2 expression were detected. Moreover, LPS induced a 78-fold increase in Ch25h expression in WT mice while the overexpression was much lower (13-fold) and not statistically significant in leptin-deficient mice; this gene encodes the enzyme cholesterol 25-hydroxylase which produces 25-hydroxycholesterol, a natural LXR agonist, whose plasmatic levels have been reported to be enhanced as a result of the dramatic upregulation of cholesterol 25-hydroxylase after endotoxic insult (*17–19*). Notably, other genes involved in hepatic cholesterol export (basolateral: Apoa1; apical/canalicular: Abcg5, Abcg8 and Abcb4) and uptake (Scarb1, Ldlr and Lrp1) were unaltered in endotoxemic obese mice (Fig. 5) compared with control, fasted mice, while the expression of some genes related with cholesterol transport processes (Apoa1, Scarb1 and Lrp1) was dramatically lower in obese mice than in WT ones.

Discussion

Liver LDs were recently reported to play a role in the control of lipid metabolism responses during endotoxemia (*2*). It was shown that hepatic neutral lipid secretion is regulated by cytokines and by the activation of LXR both induced by LPS treatment. As part of the innate immune response, septic hyperlipidemia helps to clear endotoxins, control the inflammatory response and limit the tissue injury it could cause (*7*). It has been reported that leptin-deficient ob/ob mice show higher sensitibility and mortality after LPS treatment than WT mice (*8*). We hypothesized that alterations of the immune responses and the basal hepatic lipid management in this mouse strain might lead to altered liver metabolic adaptations to LPS treatment leading to an inefficient lipemic profile.

To examine whether an altered metabolic control in the liver underlies impaired responses of lipemia in endotoxemic ob/ob mice, we quantified hydrolyzing activities that make neutral lipids accessible for cellular processes like lipoprotein biogenesis (TGH, DGH and CEH) in LDs and other subcellular fractions relevant for neutral lipid metabolism (Fig. 1). LPS had different effects on the livers of ob/ob mice and on WT animals (*2*). We observed a stronger inhibition of TGH activity in LDs of obese mice which was not observed in the cytosolic activity and was, therefore, LD-specific. It cannot be discarded that this inhibition is a consequence of modifications in the expression of endoplasmic reticulum resident enzymes (*i.e.* carboxylesterases) but it is probably a consequence of the parallel enrichment of LDs with perilipin-2 (Fig. 2). Perilipin-2, the main structural protein in hepatic LDs, is thought to prevent lipolysis in LDs (*16*). In fact, its expression positively correlates with LD-associated lipid levels (*20–23*).

At the same time, the inhibition of neutral DGH activity observed in the liver of endotoxemic WT animals (*2*) did not happen in obese mice. Furthermore, the TG/DG ratio tended to increase in LDs (Table II) indicating that the hydrolysis-esterification cycle was directed toward TG accumulation. Gene expression analysis seems to support this hypothesis, since most of the hydrolases we have studied (Aadac, Pnpla2, Pnpla3) show no modifications in their mRNA levels while Ces1d is dramatically underexpressed in both WT and

ob/ob mice. Only Hsl is overexpressed in a nearly significant fashion; this enzyme has been previously reported to have a role in CE hydrolysis (*24*, *25*), so we can speculate that this overexpression might be related to the increase in CEH activity observed in the cytosolic fraction and LDs of LPS-treated ob/ob mice (Fig. 1A).

Moreover, neither whole serum (Fig. 4A) nor VLDL-associated (Table III) TG levels were significantly affected; there was not a significant LPS-mediated increase of triglyceridemia or reduction of hepatic steatosis (Supplementary Fig. S1). Therefore, the metabolic adaptations mediated by cytokines that differentially regulate acylglyceride hydrolysis leading to enhanced lipoprotein-associated TG levels seem not to operate in this animal model, although this issue remains an open question.

Activation of LXR in response to LPS treatment seems to promote HDL particle formation in WT mice, leading to an increase of HDL-cholesterol levels in serum and the concomitant depletion of hepatic LD-associated CE (*2*). It has been described that serum HDL-cholesterol in leptin-deficient mice is elevated due to the altered hepatic catabolism of HDL (*26*). In endotoxemic ob/ob mice hepatic gene expression of some LXR target genes (Fig. 5) was only moderately elevated (Abca1) or unchanged (Srebf1, Plin2), which indicates an attenuated response to LPS treatment in LXR transactivation. Because mRNA levels of hepatocyte transporters involved in the hepatic steps of reverse cholesterol transport and biliary excretion (Scarb1 and Abcg5/g8) were not modified, the reduced HDL-cholesterol levels (Fig. 4A) seem to be a consequence of diminished secretion and/or plasmatic maturation. Nevertheless, the hepatic ability to mobilize cholesterol accumulated in LDs is enhanced in LPS-treated obese mice (Fig. 1C). Cholesterol stored in LDs might be mobilized to be channelled to the VLDL cargo and ultimately accumulate in serum LDL instead of HDL in ob/ob mice.

To test that hypothesis, we analysed the cholesterol content of newly secreted VLDL (Fig. 4D) using the lipoprotein lipase inhibitor Poloxamer (*27*). Cholesterol levels of newly secreted VLDL were higher in obese mice than in WT mice. After Poloxamer injection, LPS treatment promoted the accumulation of serum FC in ob/ob but not in WT mice, apparently at the expense of CE. This fact correlated with the increase of CEH activity (Fig. 1) and the decrease of CE mass percentage (Table II) in hepatic LDs from obese mice after LPS treatment.

Lipoprotein profiles in Fig. 4 and Table III also show that the high levels of small PL-rich lipoprotein particles (*i.e.* nascent HDL of ~10 nm in diameter) tended to be lower after LPS treatment in obese mice. Together with the lower HDL-cholesterol levels these data indicate lowered reverse cholesterol transport in endotoxemic obese mice. Thus, the altered serum cholesterol profile in endotoxemic ob/ob mice seems to be related to both a higher FC channelling to VLDL secretion from hepatic LDs CE stores and a lower cholesterol return to the liver.

In conclusion, this work shows that hepatic metabolic adaptations that modulate TG and CE storage and mobilization in LDs after LPS treatment are altered in ob/ob mice. Control mechanisms triggered by LXR transactivation are attenuated leading to a probably detrimental serum lipoprotein profile. These alterations are likely to be consequence of obesity and not of leptin deficiency, since fasting induces a dramatic decrease of circulating leptin levels in WT mice in just 2 h and the almost complete suppression in 18 h (*9*). It would be interesting to know whether the altered serum HDL profile alone, or together with that of apoB-containing lipoproteins, are behind the increased sensitivity of ob/ob mice to LPS treatment.

Supplementary Data

Supplementary data are available at *JB* Online.

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Conflict of Interest

None declared.

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