LXR activation by GW3965 alters fat tissue distribution and adipose tissue inflammation in ob/ob female mice

Amena Archer, 1* Émilie Stolarczyk, 2† Maria Luisa Doria, 2* Luisa Helguero, 6 Rosário Domingues, 7 Jane K. Howard, † Agneta Mode, * Marion Korach-André, * and Jan-Åke Gustafsson 1*†**

Department of Biosciences and Nutrition, 1 Karolinska Institutet, Huddinge, Sweden; Division of Diabetes and Nutritional Sciences, † King’s College London, London, United Kingdom; Department of Organic Chemistry and Natural Products, 7 Universidade de Aveiro, Aveiro, Portugal; and Department of Biology and Biochemistry, 1*†** Center for Nuclear Receptors and Cell Signaling, University of Houston, Houston, TX

Abstract To investigate the role of liver X receptor (LXR) in adipose tissue metabolism during obesity, ob/ob mice were treated for 5 weeks with the synthetic LXR agonist GW3965. MRI analysis revealed that pharmacological activation of LXR modified fat distribution by decreasing visceral (VS) fat and inversely increasing subcutaneous (SC) fat storage without affecting whole body fat content. This was concordant with opposite regulation by GW3965 of the lipolytic markers hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) in the two fat depots; moreover, the expression of genes involved in lipogenesis was significantly induced in SC fat. Lipidomic analysis suggested that changes in lipid composition in response to GW3965 also varied between VS and SC fat. In both depots, the observed alteration in lipid composition indicated an overall change toward less lipotoxic lipids. Flow cytometry analysis showed decreased immune cell infiltration in adipose tissue of ob/ob mice in response to GW3965 treatment, which in VS fat mainly affected the macrophage population and in SC fat the lymphocyte population. In line with this, the expression and secretion of proinflammatory markers was decreased in both fat deposits with GW3965 treatment.—Archer, A., É. Stolarczyk, M. L. Doria, L. Helguero, R. Domingues, J. K. Howard, A. Mode, M. Korach-André, and J.-A. Gustafsson. LXR activation by GW3965 alters fat tissue distribution and adipose tissue inflammation in ob/ob female mice. J. Lipid Res. 2013. 54: 1300–1311.

Supplementary key words liver X receptor • lipidomic analysis • immune cell infiltration

Overweight and obesity are worldwide public health issues associated with a number of diseases, such as cardiovascular diseases, insulin resistance (IR), type 2 diabetes (T2D), hepatic failure, and certain forms of cancer (1, 2). The close relationship between obesity and IR may involve two nonexclusive mechanisms: lipotoxicity (3, 4) and low-grade inflammation in the white adipose tissue (WAT) (5, 6). Lipotoxicity is thought to be related to the fat mass expansion during obesity and to an imbalance between excess of energy entering the tissue and limited oxidative and storage capacity (3), leading to the formation of toxic lipid metabolites, such as reactive oxygen species, diacylglycerol (DAG), and ceramides. Low-grade inflammation in WAT is characterized by macrophage infiltration, leading to production and secretion of cytokines in the tissue. Recent studies show that prevention of macrophage accumulation in adipose tissue decreases adipose inflammation and improves insulin sensitivity in various animal models of obesity (7, 8). Macrophages can display polarized functional properties and thereby exert proinflammatory (M1 macrophages) or anti-inflammatory (M2 macrophages) actions in adipose tissue (9). In addition to macrophages, adipose tissue harbors lymphocytes and natural killer cells, which may also contribute to the adipose tissue inflammation process (10, 11).

Abbreviations: ATGL, adipose triglyceride lipase; BW, body weight; Chol, cholesterol; HSL, hormone-sensitive lipase; IL, interleukin; IR, insulin resistance; LXR, liver X receptor; MCP-1, monocyte chemoattractant protein 1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; SC, subcutaneous; SVF, stromal vascular fraction; TA, tibialis anterior; TF, total fat; TG, triglyceride; tHSL, total HSL; TNF, tumor necrosis factor; TLR, Toll-like receptor; VS, visceral; WAT, white adipose tissue.

1 To whom correspondence should be addressed. e-mail: amena.archer@ki.se; amena.archer@gmail.com
2 E. Stolarczyk and M. L. Doria contributed equally to this work.
3 The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures and three tables.

This work was supported by the Swedish Science Council, Emil och Wera Cornellis Stiftelse, the Emerging Technology Fund of Texas under Agreement 300-9-1958, and the Robert A. Welch Foundation Grant E-0004. MRI experiments were supported by the experimental MR center at Karolinska Institutet and Karolinska Experimental Research Imaging Center. E.S. and J.K.H. were supported by the Medical Research Council (MRC), United Kingdom. M.L.D., R.D., and L.H. were supported by the Portuguese Science and Technology Foundation projects Ciencia 2008, PEst-C/QUI/UI0062/2011) and RNEM (REDE/1504/REM/2005-Portuguese Mass Spectrometry Network).

Manuscript received 7 December 2012 and in revised form 6 February 2013.

Published, JLR Papers in Press, February 27, 2013 DOI 10.1194/jlr.M033977

This article is available online at http://www.jlr.org

1300 Journal of Lipid Research Volume 54, 2013
Accumulating evidence suggests important differences between subcutaneous (SC) and visceral (VS) fat. SC adipose tissue is essential for lipid homeostasis and represents an easily available metabolic reservoir for energy. Both storage capacity impairment in SC fat and triglyceride (TG) accumulation in VS fat induce circulating lipids as well as lipid accumulation in other compartments, such as liver and skeletal muscle, leading to lipotoxicity and IR (12–14). VS fat is more metabolically active than SC fat with a higher lipolytic activity and an increased sensitivity to catecholamine-induced lipolysis (15). In humans, a decreased percentage of VS fat and VS/SC fat ratio, but not a loss of SC fat or body weight, is associated with improved insulin sensitivity and glucose tolerance (16, 17). Moreover, VS fat appears to be more susceptible than SC fat to low-grade inflammation associated with obesity (18, 19).

There is an intimate relation between inflammation and lipid metabolism. In this crosstalk, the liver X receptors (LXRs) act to integrate the metabolic and inflammatory signaling (20, 21). The LXRs, LXRα and LXRβ encoded by distinct nuclear receptor genes, are transcription factors activated by oxysterols. As such the LXRs control intracellular sterol and lipid homeostasis by regulating key genes involved in reverse cholesterol transport, cholesterol disposal, lipogenesis, and glucose metabolism. Target genes include the apolipoprotein E (ApoE) (22), the ATP-binding cassette (ABC) transporters Abca1, Abcg1, Abcg5, and Abcg8 (23); the sterol regulatory element-binding protein 1c (Srebp1c), which is the master regulator of lipogenesis (24); and the glucose transporter (Glut4) (25).

LXR plays an important role in regulation of inflammation, which is a key factor in the development of atherosclerosis; LXR activation attenuates inflammation and expression of genes encoding proinflammatory factors, such as interleukin (IL)-6, IL-1β, inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein 1 (MCP-1), and metalloproteinase 9 (MMP-9) in various cell types, including macrophages (26, 27).

Much is known about the role of LXR in hepatic lipid homeostasis and the atherosclerotic process, but much less is known about the influence of LXR on lipid metabolism in WAT and low-grade inflammation associated with obesity. Both LXR paralogs are expressed in the WAT and apparently at higher levels in the SC fat than in the VS fat (28). In the present study, we used the ob/ob mouse to investigate the impact of pharmacological activation of LXRs on the WAT environment and on low-grade inflammation in VS and SC WAT.

RESEARCH DESIGN AND METHODS

Animals and experimental design

Four- to five-week-old ob/ob female mice (B6.V-Lep<sup>ob</sup>/J, stock no.000632, the Jackson Laboratory) were maintained in a 12 h light-dark cycle (21°C) with free access to water and chow diet (R34, Lantmännen Lantbruk). Mice received GW3965 (10 mg/kg) (treated) or vehicle (control) in the drinking water for 5 weeks; the concentration of vehicle components in the water was 0.5% hydroxypropyl methyl cellulose, 0.1% Tween 80, 3.6 g/l NaH<sub>2</sub>PO<sub>4</sub>, and 5.5 g/l NaHPO<sub>4</sub>. Food and drink intake was measured three times a week over a period of 3 weeks in the middle of the treatment. Two independent experiments were carried out with 7–10 animals per group. At the end of the experiments, mice were sacrificed under 4% isoflurane after 2 h of food deprivation, and blood was collected by heart puncture. Tibialis anterior (TA) muscle, perigonadal fat, representing VS fat, and SC fat pads were collected, immediately frozen in liquid nitrogen, and stored for further analysis or directly used for cell fractioning or explant cultures. All experiments were approved by the local Ethical Committee of the Swedish National Board of Animal Research.

In vivo MRI

The animals were anesthetized using 4% isoflurane in a 3:7 mixture of oxygen and air before being positioned prone in the MR-compatible animal holder. Core body temperature and respiration were monitored during scanning (SA-instruments); body temperature was maintained at 37° with a warm air thermostat system (SA-instruments). MRI experiments were conducted using a horizontal 9.4 T magnet with a bore size of 31 cm (Varian). A gradient system with a 12 cm inner diameter and a maximum gradient strength of 600 mT/m was used. A volume coil with 40 mm inner diameter and 110 mm RF-window was employed both for excitation and detection. Forty axial slices of 1 mm thickness with 0.7 mm gap, covering the mice from neck to tail, with a field of view (FOV) of 51.2 × 51.2 mm<sup>2</sup> and a matrix size of 256 × 96 were acquired. The fast spin-echo sequence was employed with etl = 8 and kzero = 8, resulting in an effective echo time of 65 ms. Preceding every excitation pulse, a 2 ms gauss pulse selective for water followed by a 1.5 ms crusher gradient of 17 G/cm was applied to saturate water, while leaving the fat signal unperturbed. Respiration gating was employed, with four blocks of data acquired during each expiration period, resulting in a total scan time of approximately 5 min per animal. Signal from fat on each slice was computed using the Fiji software (http://fiji.sc) to calculate total fat and VS fat. SC fat was calculated as the difference between the total abdominal fat signal minus VS fat.

Insulin tolerance test

Mice were fasted for 4 h prior to the test. Insulin (1 U/kg Actrapid; Penfill, Novo Nordisk) was injected intraperitoneally, and blood samples were obtained via tail nick at times 0, 15, 30, 60, and 120 min. Blood glucose level was measured with the OneTouch Ultra glucometer (Accu-Check Sensor, Roche Diagnostics).

Stroma vascular fraction purification and flow cytometry analysis

Mononuclear cells were extracted from adipose tissue as described previously (29). Briefly, VS and SC adipose tissues were digested by Dispase II (Roche), and after centrifugation, the stroma vascular faction (SVF) containing preadipocytes and mononuclear cells was collected for analysis. Extracted cells were stained with antibodies conjugated to fluorochromes, CD45 clone 30-F1, CD3 clone 145-2C11, CD4 clone GK1.5, CD8 clone 53-6.7, NKp46 clone 29A1.4, B220 clone RA3-6B2, CD11b clone M1/70, CD11c clone N418, F4/80 clone BM8, and CD206 clone 310301 (eBiosciences and R and D). Samples were acquired using an LSRII cytometer (Becton Dickinson), and data were analyzed using FlowJo software (Tree Star).
Adipose tissue explants
VS and SC adipose tissue explants, about 120 mg, were cultured in 1 ml RPMI 1640 media (Invitrogen) supplemented with penicillin (100 U/ml) and streptomycin (100 pg/ml) for 24 h at 37°C in an atmosphere of 5% CO₂. Media was collected, frozen, and kept at −70°C until analysis.

Quantitative PCR
Total RNA was extracted by using TRIzol according to the manufacturer’s instructions (Invitrogen). Expression levels of mRNA were quantified using an ABI 7500 instrument and the FAST SYBR green technology (Applied Biosystems). Relative gene expression changes were calculated with the comparative Ct method using 36B4 as an internal reference gene for fat tissue samples and TFIB for TA muscle samples.

Western blotting
Proteins were extracted from fat samples using a lysis buffer complemented with protease and phosphatase inhibitors (Roche) as described previously (30). The following primary antibodies were used: anti-ATGL (#2138, Cell Signaling); anti-HSL total (#4107, Cell Signaling); anti-p563-HSL (#4139, Cell Signaling); and anti-α-tubulin. Secondary antibodies were HRP-conjugated anti-rabbit (Cell Signaling) or anti-mouse (Amersham). α-tubulin was used as a loading control, and p563-HSL levels were normalized to total HSL (tHSL). Visualization was carried out using ECL plus (Amersham), and signal quantification was performed by ImageJ software (http://rsb.info.nih.gov/ij/).

Histology of the liver and adipose tissue
Liver, VS fat, and SC fat samples were dissected and fixed overnight in 4% (wt/vol) paraformaldehyde at 4°C and embedded in paraffin. Sections (4 µm thickness) were stained with hematoxylin and eosin according to standard histological procedures.

ELISA
IL-6, MCP-1, and adiponectin in media from adipose tissue explants and SAA-3, adiponectin, and insulin levels in serum were measured by commercial ELISA kits (IL-6 # M6000B, MCP1 #MJE00, and adiponectin #MRP300 from R and D, and SAA-3 #EZMSAA3-12K and insulin #EZRMI-13K from Millipore).

Lipidomic analysis
Lipids were extracted with chloroform:methanol:water (4:2:1) and mechanical disruption, dried under N₂ flow, and solubilized in chloroform. The total lipid extract was fractionated into triglyceride (TG), cholesterol (Chol), and phospholipid (PL) fractions using solid phase extraction with 500 mg aminopropyl minicolumns (Supelco) (31). TG and Chol contents were quantified using the colorimetric kits Liquick Cor-TG and Liquick Cor-Chol (Cormay). PL amounts were estimated from total phosphorous content (32). TG, Chol, and PL amounts were related to tissue weight used for each extract. PL classes were separated by thin layer chromatography (TLC) using silica gel plates with concentrating zone 2.5 x 20 cm (Merck) (32) and identified using standards (Avantis). PLs were extracted with chloroform/methanol (2:1, v/v) for mass spectrometry (MS) analysis. Identification of molecular structure was carried out by tandem MS (MS/MS) analysis as previously described (33). Analysis of TG was carried out by mass spectrometry using ESI ionization obtained in an electrospray Q-ToF 2 (Micromass) as follows: 3 kV electrospray voltage in the positive mode with a 30 V cone voltage. The source temperature was 80°C, and the desolvation temperature was 150°C. MS/MS spectra were performed using argon as collision gas, with energy range of 30–40 V. Data acquisition was carried out with a Mass Lynx data system (V4.0). Molecular species were quantified by integration of MS peak area and normalized to the total TG area. Spectra can be provided upon request. Degree of saturation of the corresponding fatty acids (FA) was confirmed by gas chromatography with flame ionization detector (GC-FID) in a Clarus 400 (Perkin Elmer) (34). Analysis was carried out in triplicate in at least three samples from each group.

Statistical analysis
All values are expressed as means ± SEM. GraphPad Prism (San Diego) was used for all statistical calculations. Differences between GW3965 and vehicle-treated animal groups were determined by two-tailed Student t-test with the exception of the lipidomic analysis, in which one-tailed Student t-test was performed. P < 0.05 was considered significant.

RESULTS
Pharmacological LXR activation changes fat distribution in ob/ob mice
Magnetic resonance imaging (MRI) was used to investigate the impact of long-term LXR activation on whole body fat content (TF) and distribution in female ob/ob mice. Five weeks of treatment with the synthetic LXR agonist GW3965 did not affect food and water intake (data not shown) or body weight (BW) gain (Fig. 1A). MRI revealed that GW3965 elicited a change in body fat distribution: GW3965-treated mice stored less VS fat and more in the SC region than did control mice (Fig. 1A). As a consequence, the ratio of VS/SC fat was decreased in treated animals, whereas the ratio TF/BW was unchanged. Biochemical analysis confirmed that lipid levels, TG, PL, and Chol, were significantly reduced in VS fat but not in SC fat in GW3965-treated mice (Fig. 1B). Hematoxylin and eosin staining of histological sections of liver showed an increase of lipid droplet size in an already steatotic liver after GW3965 treatment compared with control (Fig. 1C). No obvious differences in cell size and number could be observed on VS and SC fat histological sections between control and treated mice (Fig. 1C).

GW3965 treatment modifies expression of genes and proteins involved in lipid homeostasis in adipose tissue
The protein levels of the main lipolysis markers in adipose tissue, adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), were significantly increased in VS fat and, inversely, significantly decreased in SC fat upon GW3965 treatment (Fig. 2A). GW3965 did not change the ratio of the activated form of HSL, p563HSL, to total HSL (p563HSL/tHSL) in either VS or in SC fat. The increased level of lipases in VS fat in response to GW3965 is consistent with the reduction in the amount of VS. In both VS and SC fat, the levels of Srebplc mRNA, the master regulator of lipogenesis, and Abeg1, a cellular cholesterol efflux mediator, were upregulated by GW3965 (Fig. 2B). However, only in SC fat were the expression levels of fatty acid synthase (Fas), stearoyl CoA desaturase 1
Effect of LXR activation in adipose tissue in ob/ob mice

Regulated by LXR. Activation of LXR by synthetic agonist has been shown to promote cholesterol efflux via the regulation of ApoE expression (22) and an enhanced production of ApoE-rich large HDL particles (35). Although expression level of \textit{ApoE} was not significantly upregulated in either VS or SC fat-treated mice compared with vehicle mice, a trend toward increased expression was observed.

\textit{Sod1}, and elongation of long chain fatty acids family member 6 (\textit{Elovl6}) increased by GW3965. The mRNA levels of peroxisome proliferator-activated receptor (\textit{Ppara}), \textit{Ppara}2, cluster of differentiation 36 (\textit{Cd36}), and lipoprotein lipase (\textit{Lpl}) were not changed by GW3965 treatment in either fat depot. ApoE is a class of apolipoprotein present in intermediate-density lipoprotein (IDL) that is regulated by LXR.
Upon separation of the PL classes by TLC, it was found that i) in both VS and SC fat, phosphatidylserine and phosphatidylinositol were barely detectable (data not shown) and ii) no significant difference in the proportion of the PL classes sphingomyelin (SM), PC, PE, and cardiolipin (CL) was detected between treated and control mice or between VS and SC fat within the group (supplementary Fig. I). Next, we analyzed the PC and PE classes by MS/MS. In both the PC class (Fig. 3B and supplementary Table II) and the PE class (Fig. 3C and supplementary Table III), similar molecular species were identified in VS and SC fat. The most striking effect of GW3965 treatment on PC molecular species was observed in SC fat: the plasmalogens [PC(C34:1-o) and PC(C36:2-o)] and PCs containing saturated or monounsaturated C18 FA [PC(C36:0) and PC(36:1)] were significantly reduced by GW3965, while PCs containing PUFA [PC(C38:5), PC(C38:6), PC(C40:5), PC(C40:6), and PC(C40:7)] were increased. Only a few PCs were altered by GW3965 in VS fat: PC(C36:3) was increased by GW3965, and PC(C32:2), PC(C36:5), and PC(C40:6) were decreased. In contrast to PC, PE molecular species were particularly altered by GW3965 in VS fat; GW3965 decreased C18:1 plasmalogens PE(C34:2-o) and (C34:1-o) as well as the PE species PE(C40:7) and PE(C40:9), both containing the PUFA DHA(C22:6). On the other hand, GW3965 increased the C16:1 PEs PE(C34:4) and PE(C34:2) as well as unsaturated C18 containing PE species such as PE(C36:5), PE(C36:4), and PE(C36:3) in VS fat (Fig. 3C). It is noteworthy that the plasmalogen PE(C36:3-o) was oppositely regulated by GW3965 in VS and SC fat. The lipidomic analysis revealed that LXR activation by GW3965

GW3965 treatment affects lipid composition in adipose tissue

During obesity, adipose tissue undergoes lipid remodeling to maintain adipocyte function (36). Such changes are also evident in the fat tissue from ob/ob mice in which there are alterations in TG, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) molecular profiles compared with lean WT animals (37). To investigate whether LXR activation leads to alterations in the molecular species profile of TG, PC, and PE in VS and SC adipose tissue in obesity, the different lipid fractions were separated and analyzed by MS/MS.

In the TG fraction of untreated mice, there was no difference in the quantity or composition of lipid species (supplementary Table I and Fig. 3A, gray bars). However, GW3965 treatment modified the lipid composition differently in VS and SC fat (Fig. 3A). In VS fat, GW3965 treatment significantly reduced TG species containing (FA) palmitate (C16:0), [TG(48:0), TG(50:1), and TG(52:2)], while it increased the amount of species enriched in C18 FA, such as stearate (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and γ-linolenic acid (C18:3) [TG(50:4), TG(52:0), TG(54:6), and TG(54:1)]. In SC fat, GW3965 treatment caused a significant increase in TG containing C16:0, C18:0, and C18:1 FAs [TG(50:0), TG(52:1), TG(52:0), TG(54:2) and TG(54:1)].

Upon separation of the PL classes by TLC, it was found that i) in both VS and SC fat, phosphatidylserine and phosphatidylinositol were barely detectable (data not shown) and ii) no significant difference in the proportion of the PL classes sphingomyelin (SM), PC, PE, and cardiolipin (CL) was detected between treated and control mice or between VS and SC fat within the group (supplementary Fig. I). Next, we analyzed the PC and PE classes by MS/MS. In both the PC class (Fig. 3B and supplementary Table II) and the PE class (Fig. 3C and supplementary Table III), similar molecular species were identified in VS and SC fat. The most striking effect of GW3965 treatment on PC molecular species was observed in SC fat: the plasmalogens [PC(C34:1-o) and PC(C36:2-o)] and PCs containing saturated or monounsaturated C18 FA [PC(C36:0) and PC(36:1)] were significantly reduced by GW3965, while PCs containing PUFA [PC(C38:5), PC(C38:6), PC(C40:5), PC(C40:6), and PC(C40:7)] were increased. Only a few PCs were altered by GW3965 in VS fat: PC(C36:3) was increased by GW3965, and PC(C32:2), PC(C36:5), and PC(C40:6) were decreased. In contrast to PC, PE molecular species were particularly altered by GW3965 in VS fat; GW3965 decreased C18:1 plasmalogens PE(C34:2-o) and (C34:1-o) as well as the PE species PE(C40:7) and PE(C40:9), both containing the PUFA DHA(C22:6). On the other hand, GW3965 increased the C16:1 PEs PE(C34:4) and PE(C34:2) as well as unsaturated C18 containing PE species such as PE(C36:5), PE(C36:4), and PE(C36:3) in VS fat (Fig. 3C). It is noteworthy that the plasmalogen PE(C36:3-o) was oppositely regulated by GW3965 in VS and SC fat. The lipidomic analysis revealed that LXR activation by GW3965
regulates the lipid molecular profile composition differently in VS and SC adipose tissue. However, in both depots, the observed alteration in lipid species composition might indicate an overall change toward less lipotoxic lipids.

**GW3965 treatment decreases immune cells infiltration in fat tissue**

As mentioned above, obesity is characterized by a low-grade inflammation in WAT with macrophage infiltration and local production of proinflammatory factors. To determine whether LXR activation influences macrophage infiltration and/or modulates the inflammation process, flow cytometry was used to analyze the cellular composition of the SVF of VS and SC fat depots (supplementary Fig. II and Fig. 4). In VS fat, we found that, upon GW3965 treatment, the total macrophage population (CD11b^+ F4/80^+ cells) and both anti-inflammatory M2 (CD206^+ ) and proinflammatory M1 (CD11c^+ ) macrophages were decreased (Fig. 4A). In line with this, the mRNA expression of CD206 and CD11c was decreased in VS fat (Fig. 4B). In SC fat, GW3965 treatment had no significant effect on the number of infiltrating CD11b^+ F4/80^+ macrophages (Fig. 4A).

Lymphocytes are targets of LXR (38, 39), and CD3^+ CD8^+ T cells are considered important for the initiation and propagation of the inflammatory process in adipose tissue from obese mice (10). Analysis of the lymphocyte population infiltrating the SC fat showed that GW3965 caused a significant decrease in CD3^+ CD4^+ , CD3^+ CD8^+ T cells, B220^+ B cells, and CD3^+ NKp46^+ NK cells (Fig. 4C). The CD3^+ CD8^+ population was also significantly decreased in blood (supplementary Fig. II), whereas no change was observed in the VS fat (Fig. 4C). These data show that GW3965 treatment primarily affects infiltration of macrophages in VS fat and infiltration of lymphocytes in SC fat in obese mice.

**GW3965 treatment mitigates the expression of proinflammatory factors in fat tissue**

Cytokine expression is a hallmark of macrophage activity. Analysis of mRNA expression and/or protein level of several cytokines in VS and SC fat (Fig. 5) revealed that in both VS and SC fat the levels of mRNA of the proinflammatory cytokine Il6 and the chemokine Mcp1 were significantly downregulated in GW3965-treated mice. Expression of the gene encoding the acute phase response protein Saa-3 was downregulated in VS fat. On the other hand, expression of Ym1, a gene that is expressed in activated macrophages and suggested to be involved in inflammation, was significantly reduced in SC fat but not in VS fat in response to GW3965 (Fig. 5A).

The spontaneous release of IL-6, MCP-1, and adiponectin, an adipokine involved in metabolic regulation, was measured by ELISA in adipose tissue explant culture media.
GW3965 exposure in vivo reduced the spontaneous release of IL-6 from SC fat explants and of MCP-1 from both VS and SC explants, whereas no differences were observed in spontaneous secretion of adiponectin (Fig. 5B). The qPCR results corroborated the proinflammatory cytokine secretion profile observed with the adipose tissue explant culture (Fig. 5A). Serum levels of MCP-1, SAA-3, and adiponectin were also measured; the MCP-1 level was lower in serum of animals treated with GW3965, and no significant difference was observed in circulating SAA-3 or adiponectin (supplementary Fig. III). Our results show that GW3965 treatment can modulate the production and secretion of proinflammatory cytokines, such as IL-6 and MCP-1, in WAT from obese mice.

**GW3965 treatment partially improves insulin sensitivity**

Decreased accumulation of VS fat, induction of lipogenic genes in WAT, as well as reduced secretion of proinflammatory cytokines, possibly as a consequence of modulation of macrophage infiltration in the WAT, would be consistent
DISCUSSION

Long-term pharmacological activation of LXR in the ob/ob mouse did not impact total body fat content; however, it did change the ratio between VS and SC fat. While VS fat storage was reduced, SC fat storage was increased. These effects were associated with opposite changes of the lipolytic markers ATGL and HSL, suggesting a differential regulatory effect of LXR on lipid catabolism in VS and SC fat. Furthermore, TG, PL, and Chol content was significantly reduced in VS fat and tended to increase in SC fat following LXR agonist treatment. We conclude that long-term pharmacological activation of LXR in the ob/ob mouse model of obesity leads to redistribution of fat deposits from the VS to the SC region and that this occurs by differential regulation of lipid turnover and/or transport in the two types of fat.

In addition to being a source of energy, lipids are structural components essential for membrane integrity. During obesity, adipose tissue undergoes expansion to increase its lipid storage capacity. This involves major remodeling of the lipid membrane of the growing adipocyte to maintain its functionality. In human studies, a combination of

![Graph](image-url)
lipidomic analysis and computational modeling has revealed that adipose tissue expansion in obesity leads to changes in PL membrane composition (36). Furthermore, Pietiläinen et al. showed that the lipid remodeling was associated with increased vulnerability to inflammation due to higher level of plasmalogens containing arachidonate (C20:4). In the ob/ob mouse model, we observed a difference between VS and SC fat in the change of PL molecular species after GW3965 treatment; PE molecular species were mainly affected in VS fat, whereas PC molecular species were affected in SC fat. In neither VS nor SC fat was arachidonate PL composition significantly changed upon GW3965 treatment. However, we observed an increase of EPA (C20:5) PC and DHA (C22:6) PC species in SC fat and a decrease of DHA PEs in VS fat in response to the treatment. In both SC and VS fat, the mRNA expression of Elovl6 was significantly induced by GW3965 treatment. ELOVL6 has been identified as a major regulator of membrane PL remodeling in adipose tissue (36). These lipidomic data are likely to reflect different roles and functions of the two depots.

In contrast to the n-6 PUFA arachidonate that gives rise to inflammatory molecules, such as leukotrienes and prostaglandins, the n-3 PUFAs EPA and DHA are considered as precursors of anti-inflammatory mediators (40). Although the main source of long-chain PUFA is nutritional, mammals are able to synthesize endogenous arachidonate, EPA, and DHA from dietary essential fatty acids C18:2 n-6 (linoleic acid) and C18:3 n-3 (ω-linolenic acid) by combined action of fatty acid desaturases and elongases. It has been proposed that an increase of DHA species might contribute to both maintenance of membrane integrity and diminished adipose tissue vulnerability to inflammation during obesity (36). Thus, LXR activation, by modulating specifically EPA PL and DHA PL species in WAT, may influence the inflammation process and affect adipose tissue expansion as observed in VS fat after GW3965 treatment. Further investigations, including computational modeling, will be required to determine to what extent changes in PL lipid composition induced by pharmacological LXR activation impact biophysical properties of membranes in SC and VS adipose tissue during obesity.

Epidemiological, clinical, and experimental observations have linked proinflammatory mediators, such as IL6 and tumor necrosis factor (TNF)-α, to complications associated with obesity and IR (6). Indeed, the low-grade inflammation occurring in obese adipose tissue has been suggested as one of the decisive processes leading to development of pathologies related to obesity (for review, see Ref. 41). Recently, it was shown that the inflammatory process in obese adipose tissue is initiated and driven by a dynamic change in T-lymphocyte population that induces macrophage accumulation and maintains local inflammation (10). Although inflammation is increased in both VS and SC adipose tissues during obesity (42), VS fat appears to be more prone to inflammation with a higher level of macrophage infiltration than SC fat (18, 19). We found that in ob/ob mice, the number of macrophages per gram of tissue, particularly the M1 type of macrophage (CD11c+CD11b+), was higher in VS than SC fat, and inversely that T-cell number per gram of tissue infiltrating the WAT (CD3+CD4− and CD3+CD8−) was higher in SC fat. Interestingly, GW3965 treatment of ob/ob mice resulted in a differential effect on the recruitment of macrophages and lymphocytes in the two types of fat deposit. In VS fat, the macrophage population was significantly decreased by GW3965 treatment, whereas no change was observed in T-cell populations. In contrast, in SC adipose tissue, no significant change in the macrophage population was detected, but the T-lymphocyte population was markedly decreased by GW3965. Thus, also in this respect SC and VS fat responded differently to LXR activation. The impact of LXR on immune cell migration has been previously described; Walcher et al. have shown that synthetic LXR
ligands, T091317 and GW3965, inhibit chemokine-induced migration of human CD4+ T lymphocytes in early atherogenesis by perturbation of PI3K and Rac1 signaling pathways (39). Moreover, recent findings have demonstrated an antiproliferative role of LXR in T cells (20) and macrophages (43). LXR activation has been shown to repress proinflammatory gene expression after lipopolysaccharide (LPS) or TNF-α stimulation (27). In T lymphocytes, the antiproliferative effect of LXR has been linked to its ability to promote cholesterol export by upregulating Abcg1 expression (20). Abcg1 was induced in both VS and SC fat by GW3965 treatment; however, whether changes in cholesterol transport are coupled to differences in immune cell infiltration into the adipose tissue remains to be investigated. We conclude that pharmacological LXR activation during obesity regulates the recruitment of immune cells into adipose tissue in a fat tissue-dependent manner and contributes to decreased inflammation by repression of genes encoding proinflammatory cytokines.

Lipids play a crucial role as signaling mediators for initiating and maintaining an immune response. FA composition has been reported to vary according to the anatomical location of the adipose tissue, and several studies have pointed out the relation between FA composition of the adipose tissue and obesity (44, 45). FAs released from adipocytes have been shown to induce recruitment of macrophages secreting TNF-α and thereby amplify the inflammatory response via a paracrine loop (46). Certain saturated fatty acids (SFA), such as laurate (C12:0), myristate (C14:0), and palmitate (C16:0) [but not stearate (C18:0)], stimulate inflammatory gene expression in adipocytes (46, 47). In our study, we observed a switch from C16:0-enriched TGs to C18:0-enriched TGs in both SC and VS fat after GW3965 treatment, consistent with the increased expression of Elavd6, an enzyme responsible for the conversion of C16:0 to C18:0. Thus, LXR activation may modulate adipose tissue inflammation by specifically promoting C18:0 TG species and thereby limiting the expression of proinflammatory genes by the adipocytes in both VS and SC fat.

The Toll-like receptor (TLR)4 is well recognized for its implication in adipose tissue inflammation and insulin sensitivity (48, 49). TLRs activate several proinflammatory pathways, such as MAPK, JNK, and NFκB signaling cascades, during innate immune response, which in turn leads to production of proinflammatory cytokines (50). Although it is still debated (51), SFAs, such as C16:0 and C18:0, have been shown to stimulate adipose tissue inflammation through the TLR4 localized at the surface of macrophages and adipocytes, whereas unsaturated fatty acids (USFA) may block the activation of the receptor (52–54). We found no difference in TLR4 gene expression in adipose tissue or in the SVF fraction obtained from vehicle or GW3965-treated ob/ob mice (data not shown). However, GW3965 treatment increased USFA-containing PEs in VS fat and PUFA-containing PCs in SC fat that could potentially antagonize the activation of TLR4 by C16:0 and C18:0 in macrophages. Thus, GW3965 treatment could modulate adipose tissue inflammation in ob/ob mice via the TLR4 pathways.

Decrease of VS fat deposit is clearly beneficial for obese human subjects, and therefore, reduced VS fat accumulation in GW3965-treated ob/ob mice could be expected to have a positive effect on insulin sensitivity. However, as previously demonstrated by Grefhorst et al., GW3965 treatment has only a moderate effect on general insulin sensitivity in ob/ob mice, most likely because of increased hepatic TG content and lipotoxicity (55). Thus, reduced VS fat accumulation, which can be induced by LXR activation, is not necessarily beneficial. This is further supported by the fact that PPARy2-deficient ob/ob mice, despite a 65% decrease in adipose tissue mass compared with control ob/ob mice, accumulate reactive lipid species in liver, pancreas, and muscle, leading to severe insulin resistance and diabetes (56). On the other hand, LXR activation induced a selective decrease of immune cell infiltration accompanied by a decrease in expression/secretion of proinflammatory markers in SC and VS adipose tissue that could be considered positive in terms of limiting the development of insulin resistance. There is thus an intricate balance of advantageous and disadvantageous effects elicited by LXR activation, and the net outcome is difficult to predict.

The authors thank Peter Damberg for excellent technical support to perform MRI; Prof. Paolo Parini and Dr. Jurga Laurencikiene for valuable discussions and comments on the manuscript; and Christina Thulin-Anderson and Noémie Denyer for excellent technical help.

REFERENCES

1. Mokdad, A. H., B. A. Bowman, E. S. Ford, F. Vinicor, J. S. Marks, and J. P. Koplan. 2001. The continuing epidemics of obesity and diabetes in the United States. JAMA 286: 1195–1200.

2. Whitlock, G., S. Lewington, P. Sherliker, R. Clarke, J. Emberson, J. Halsey, N. Qizilbash, R. Collins, and R. Peto. 2009. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. Lancet 373: 1093–1096.

3. Lelliott, C., and A. J. Vidal-Puig. 2004. Lipotoxicity, an imbalance between lipogenesis de novo and fatty acid oxidation. Int. J. Obes. Relat. Metab. Disord. 28(Suppl. 4): S22–S28.

4. Unger, R. H. 2003. Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. Endocrinology 144: 5159–5165.

5. Bastard, J. P., M. Maachi, C. Lagathu, M. J. Kim, M. Caron, H. Vidal, J. Capeau, and B. Feve. 2006. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur. Cytokine Netw. 17: 4–12.

6. Hotamisligil, G. S. 2006. Inflammation and metabolic disorders. Nature 444: 860–867.

7. Hellmann, J., Y. Tang, M. Kosuri, A. Bhatnagar, and M. Spite. 2011. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. FASEB J. 25: 2399–2407.

8. Kurokawa, J., H. Nagano, O. Ohara, N. Kubota, T. Kadowaki, S. Arai, and T. Miyazaki. 2011. Apoptosis inhibitor of macrophage (AIM) is required for obesity-associated recruitment of inflammatory macrophages into adipose tissue. Proc. Natl. Acad. Sci. USA. 108: 12072–12077.

9. Lumeng, C. N., J. L. Bodzin, and A. R. Saltiel. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest. 117: 177–188.

10. Nishimura, S., I. Manabe, M. Nagasaki, K. Eto, H. Yamashita, M. Oh sugi, M. Otsu, K. Hara, K. Usuki, S. Sugiyura, et al. 2009. CD8+
effect on T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 15: 914–920.

11. O’Rourke, R. W., M. D. Metcalf, A. E. White, A. Madala, B. R. Winters, I. I. Maizlin, B. A. Jobe, C. T. Roberts, Jr., M. K. Silika, and D. L. Marks. 2009. Depots-specific differences in inflammatory mediators and a role for NK cells in IFN(gamma) inflammation in human adipose tissue. *Int. J. Obes. (Lond.)* 33: 978–990.

12. Meng, K., C. H. Lee, and F. Sarem. 2010. Metabolic syndrome and ectopic fat deposition: what can CT and MR provide? *Acad. Radiol.* 17: 1302–1312.

13. Savage, D. B., K. F. Petersen, and G. I. Shulman. 2007. Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol. Rev.* 87: 507–520.

14. Virtue, S., and A. Vidal-Puig. 2010. Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome—an allostatic perspective. *Biochem. Biophys. Acta.* 1801: 338–349.

15. Hellmér, J., C. Marcus, T. Sonnenfeld, and P. Arner. 1992. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J. Clin. Endocrinol. Metab.* 75: 15–20.

16. Fujikawa, S., Y. Matsuzawa, K. Tokunaga, T. Kawamoto, T. Kobatake, Y. Keno, K. Kotani, S. Yoshida, and S. Tarui. 1991. Improvement of glucose and lipid metabolism associated with selective reduction of intra-abdominal visceral fat in premenopausal women with visceral fat obesity. *Int. J. Obes.* 15: 853–859.

17. Grootveld, B. R., D. E. Kelch, R. W. Ring, A. Meier, and F. L. Thaete. 1999. Effects of weight loss on regional fat distribution and insulin sensitivity in obesity. *Diabetes.* 48: 839–847.

18. Bruun, J. M., A. S. Lihn, S. B. Pedersen, and B. Richelsen. 2005. Monocyte chemotactic protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J. Clin. Endocrinol. Metab.* 90: 2895–2899.

19. Cancello, R., J. Tordjman, C. Poitou, G. Guillem, J. L. Bouillot, D. Hugol, C. Cousioss, A. Basdevant, A. Bar Hen, P. Bedossa, et al. 2006. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes.* 55: 1554–1561.

20. Bensingjäger, S. J. M. Beadle, S. B. Joseph, N. Zelcer, E. M. Janssen, M. A. Hausner, R. Shih, J. S. Parks, P. A. Edwards, B. D. Jamieson, et al. 2008. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell.* 134: 97–111.

21. Zelcer, N., and P. Tontonoz. 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest.* 116: 607–614.

22. Laffitte, B. A., J. J. Repa, S. B. Joseph, D. C. Wilpitz, H. R. Kast, D. J. Mangelsdorf, and P. Tontonoz. 2001. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA.* 98: 507–512.

23. Janowski, B. A., P. J. Willy, T. R. Devi, J. R. Falcè and D. Mangelsdor. 2005. An oxysterol signaling pathway mediated by the nuclear receptor LXR alpha. *Nature.* 433: 729–731.

24. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. M. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldenste, and D. Mangelsdor. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXaRalpha and LXbRbeta. *Genes Dev.* 14: 2891–2893.

25. Laffitte, B. A., L. C. Chao, J. Li, R. Walczak, S. Hummasti, S. B. Joseph, A. Castrillo, D. C. Wilpitz, D. J. Mangelsdor, and J. L. Collins, et al. 2003. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proc. Natl. Acad. Sci. USA.* 100: 5149–5154.

26. Castrillo, A. S., B. Joseph, C. Marathe, D. J. Mangelsdor, and P. Tontonoz. 2005. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *J. Biol. Chem.* 278: 10433–10449.

27. Joseph, S. B., A. Castrillo, B. A. Laffitte, D. J. Mangelsdor, and P. Tontonoz. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat. Med.* 9: 213–219.

28. Grootveld, K., K. R. Milson, G. U. Schuster, T. M. Stalnig, K. Dahlman-Wright, and J. A. Gustafsson. 2003. Gene expression profiling in adipose tissue indicates different transcriptional mechanisms of liver X receptors alpha and beta, respectively. *Biochem. Biophys. Res. Commun.* 301: 589–593.

29. Inouye, K. E., H. Shi, J. K. Howard, C. H. Daly, G. M. Lord, B. J. Rollins, and S. J. Flier. 2007. Absence of CC chemokine ligand 2 does not limit associated inflammation of macrophages into adipose tissue. *Diabetes.* 56: 2242–2250.

30. Korach-André, M., A. Archer, C. Gabb, R. P. Barros, M. Pedrelli, K. R. Steffen, A. T. Pettersson, J. Laurentiiceni, P. Parini, and A. G. Gustafsson. 2011. Liver X receptors regulate de novo lipogenesis in a tissue-specific manner in C57BL/6 female mice. *Am. J. Physiol. Endocrinol. Metab.* 301: E210–E222.

31. Riggs, J. T., J. A. Antequera, A. I. Andres, M. J. Petron, and E. Muriel. 2004. Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. *Anal. Chem. Acta.* 520: 201–205.

32. Doria, M. L., Z. Cotrim, B. Macedo, C. Simoes, P. Domingues, L. Helguero, and M. R. Domingues. 2012. Lipidomic approach to identify patterns in phospholipid profiles and define class differences in mammary epithelial and breast cancer cells. *Breast Cancer Res.* 133: 635–649.

33. Virtue, S., and A. Vidal-Puig. 2010. Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome—an allostatic perspective. *Biochem. Biophys. Acta.* 1801: 338–349.

34. Hellmé, J., C. Marcus, T. Sonnenfeld, and P. Arner. 1992. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J. Clin. Endocrinol. Metab.* 75: 15–20.

35. Laffitte, B. A., L. C. Chao, J. Li, R. Walczak, S. Hummasti, S. B. Joseph, A. Castrillo, D. C. Wilpitz, D. J. Mangelsdor, and J. L. Collins, et al. 2003. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proc. Natl. Acad. Sci. USA.* 100: 5149–5154.

36. Castrillo, A. S. B. Joseph, C. Marathe, D. J. Mangelsdor, and P. Tontonoz. 2005. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *J. Biol. Chem.* 278: 10433–10449.

37. Joseph, S. B., A. Castrillo, B. A. Laffitte, D. J. Mangelsdor, and P. Tontonoz. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat. Med.* 9: 213–219.

38. Grootveld, K., K. R. Milson, G. U. Schuster, T. M. Stalnig, K. Dahlman-Wright, and J. A. Gustafsson. 2003. Gene expression profiling in adipose tissue indicates different transcriptional mechanisms of liver X receptors alpha and beta, respectively. *Biochem. Biophys. Res. Commun.* 301: 589–593.

39. Inouye, K. E., H. Shi, J. K. Howard, C. H. Daly, G. M. Lord, B. J. Rollins, and S. J. Flier. 2007. Absence of CC chemokine ligand 2 does not limit associated inflammation of macrophages into adipose tissue. *Diabetes.* 56: 2242–2250.
Effect of LXR activation in adipose tissue in ob/ob mice

49. Tsukumo, D. M., M. A. Carvalho-Filho, J. B. Carvalheira, P. O. Prata, S. M. Hirabara, A. A. Schenka, E. P. Araujo, J. Vassallo, R. Curi, L. A. Velloso, et al. 2007. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes.* 56: 1986–1998.

50. Kumar, H., T. Kawai, and S. Akira. 2009. Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.* 388: 621–625.

51. Erridge, C., and N. J. Samani. 2009. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. *Arterioscler. Thromb. Vasc. Biol.* 29: 1944–1949.

52. Lee, J. Y., A. Plakkidas, W. H. Lee, A. Heikkinen, P. Chanmugam, G. Bray, and D. H. Hwang. 2003. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *J. Lipid Res.* 44: 479–486.

53. Lee, J. Y., K. H. Sohn, S. H. Rhee, and D. Hwang. 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J. Biol. Chem.* 276: 16683–16689.

54. Lee, J. Y., J. Ye, Z. Gao, H. S. Youn, W. H. Lee, L. Zhao, N. Sizemore, and D. H. Hwang. 2003. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J. Biol. Chem.* 278: 37041–37051.

55. Grefhorst, A., T. H. van Dijk, A. Hammer, F. H. van der Sluijs, R. Havinga, L. M. Havekes, J. A. Romijn, P. H. Groot, D. J. Reijngoud, and F. Kuipers. 2005. Differential effects of pharmacological liver X receptor activation on hepatic and peripheral insulin sensitivity in lean and ob/ob mice. *Am. J. Physiol. Endocrinol. Metab.* 289: E829–E838.

56. Medina-Gomez, G., S. L. Gray, L. Yetukuri, K. Shimomura, S. Virtue, M. Campbell, R. K. Curtis, M. Jimenez-Linan, M. Blount, G. S. Yeo, et al. 2007. PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. *PLoS Genet.* 3: e64.