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Chapter 2

12/15-Lipoxygenase Deficiency Promotes the Development of Adipose Tissue Inflammation and Insulin Resistance

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Submitted
Abstract

Pro-inflammatory pathways are thought to causally contribute to insulin resistance. 12/15-lipoxygenase (12/15-LO) is expressed in macrophages and adipocytes and is an important modulator of inflammation potentially generating pro- as well as anti-inflammatory mediators. The aim of the present study was to establish the impact of 12/15-LO on the development of obesity and insulin resistance in mice. Insulin sensitivity in 12/15-LO$^{-/-}$ and wild-type (WT) control mice fed either standard chow or high-fat diet (HFD) was assessed using hyperinsulinemic-euglycemic clamp studies and adipose tissue was examined for macrophage content and polarization. In addition, bone marrow transplantations were performed to address the role of macrophage 12/15-LO.

12/15-LO$^{-/-}$ mice were more insulin resistant compared to controls, particularly when fed a HFD. Insulin resistance in HFD-fed 12/15-LO$^{-/-}$ mice was associated with pronounced obesity and adipose tissue macrophage infiltration. Interestingly, adipose tissue macrophages in 12/15-LO$^{-/-}$ mice were skewed towards the pro-inflammatory M1 phenotype. Additional in vitro experiments revealed impaired polarization towards the anti-inflammatory M2 phenotype in 12/15-LO$^{-/-}$ macrophages. Importantly, insulin resistance was increased in WT mice transplanted with 12/15-LO$^{-/-}$ compared to WT bone marrow, indicating that macrophage 12/15-LO is important for maintenance of insulin sensitivity in HFD-fed mice. Decreased insulin sensitivity in mice with bone marrow-specific deletion of 12/15-LO occurred in the absence of differences in body weight gain, thereby dissociating insulin resistance from obesity. The fact that transplantation of WT bone marrow in 12/15-LO$^{-/-}$ mice increased insulin sensitivity compared to 12/15-LO$^{-/-}$ mice transplanted with 12/15-LO$^{-/-}$ bone marrow further emphasizes the importance of macrophage 12/15-LO for maintaining insulin sensitivity in HFD-fed mice.

In conclusion, macrophage 12/15-LO is a key mediator involved in the maintenance of insulin sensitivity, conceivably by promoting polarization of macrophages towards an anti-inflammatory M2 phenotype.
Introduction

Developed societies are facing a rapid increase in the prevalence of obesity and associated disorders such as insulin resistance and type 2 diabetes mellitus (1). Elucidation of the mechanisms underlying obesity-associated disorders is therefore urgently needed for the development of novel treatment strategies. Traditionally, adipose tissue has been regarded to function solely as storage site for excess energy. Yet, current concepts consider adipose tissue an important endocrine organ that produces a wide variety of (adipo)cytokines that impact local as well as whole body metabolism (2). Interestingly, many of these (adipo)cytokines are linked to inflammation, either directly or by promoting the influx of inflammatory cells into adipose tissue. In general, the contribution of pro-inflammatory pathways, and particularly adipose tissue inflammation, to the etiology of insulin resistance has gained a lot of attention lately. Adipose tissue of obese mice (3;4) and humans (5) is infiltrated with macrophages secreting pro-inflammatory factors that promote insulin resistance in adipocytes (6). Furthermore, adipocytokines that are produced by inflamed adipose tissue have been linked to the development of systemic insulin resistance (7;8). In addition, anti-inflammatory drugs were shown to improve insulin sensitivity in animal models and in insulin-dependent type 2 diabetes patients (8;9). Although the mechanisms underlying adipose tissue inflammation and the relation to insulin resistance are still incompletely understood, inflammation-related pathways conceivably represent promising therapeutic targets for the future development of anti-diabetic drugs.

12/15-lipoxygenase (12/15-LO) is an enzyme that catalyzes the oxygenation of polyunsaturated fatty acids, resulting in the formation of the lipid mediators 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-HPETE, amongst others (10). Arachidonic acid, that is released from membrane phospholipids during inflammation, is an important substrate for 12/15-LO (10). Consequently, 12/15-LO has been implicated in the modulation of the course of a variety of inflammatory diseases such as atherosclerosis (11-14) and rheumatoid arthritis (15). Interestingly, these studies have indicated that 12/15-LO is not per se pro-inflammatory but that it can have pro- as well as anti-inflammatory activities, depending on the metabolic context. Therefore, the present study used 12/15-LO-deficient mice to assess the role of this enzyme in the development of diet-induced obesity, adipose tissue inflammation and insulin resistance. In addition, bone marrow transplantation experiments were performed to address the impact of macrophage-specific 12/15-LO deficiency on the development of insulin resistance.
Materials and Methods

Animals
Male wild-type (WT) C57BL/6J and 12/15-LO-deficient mice were obtained via the Jackson Laboratories exclusive European distributor Charles River (Maastricht, The Netherlands). All mice were housed under climate-controlled conditions and a 12 hour light-dark cycle and had free access to food and water. Mice were fed either a standard laboratory chow or a hypercaloric high-fat diet (HFD) containing 60% (energy) fat and low cholesterol (0.02%) (AB-diets, Woerden, The Netherlands) for 12 weeks. After this period, experiments were carried out as indicated below. All animal experiments were performed according to the Dutch law on the Welfare of Laboratory Animals and experimental procedures were approved by the responsible ethics committee for animal experiments of the University of Groningen.

Blood parameters
Blood samples were obtained at the end of the respective studies by cardiac puncture using heparinized syringes and were immediately placed on ice. Blood was centrifuged at 8000 rpm at 4 °C for 10 min and plasma was stored at -80 °C until further analysis. Plasma triglycerides, free fatty acids, total cholesterol and free cholesterol were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and Wako Pure Chemical Industries, Neuss, Germany). Plasma insulin levels were measured with an ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden).

Analysis of liver lipid composition
To determine hepatic triglyceride content, liver tissue was homogenized and lipids were extracted according to the general method of Bligh and Dyer essentially as described previously (16). Triglycerides were measured as detailed above.

Analysis of gene expression by real-time quantitative PCR
RNA was extracted from tissue samples using Tri-reagent (Sigma, St. Louis, MO, USA) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Sigma) according to the manufacturers instructions. Multi-exon spanning primer/probe sets were synthesized by Eurogentec (Seraing, Belgium). Real-time qPCR analysis was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). Tissue gene expression levels were normalized to cyclophilin, except for the adipose tissue macrophage polarization markers Mgl-1 and Mgl-2. Expression levels of those genes were normalized to the macrophage-specific marker Cd68 to correct for the amount of macrophages present in adipose tissue. Gene expression in in vitro experiments was normalized to 18S. Results were further normalized to the mean expression level of the respective control group.
**Immunohistochemistry**
Tissues were fixed in 4% formalin and embedded in paraffin. Sections were incubated for 2 hours with a monoclonal rat anti-F4/80 antibody (CI:A3-1, Abcam, Cambridge, UK) to stain macrophages and a mouse monoclonal anti-caveolin-1 antibody (clone 2297, BD Pharmingen, San Diego, CA, USA) to visualize all cells. After washing, the slides were incubated with the appropriate fluorescently-labeled secondary antibodies for 30 min. Images were acquired using a Leica confocal AOBS microscope.

**Assessment of insulin sensitivity**
Determination of insulin sensitivity by means of hyperinsulinemic euglycemic clamp in conscious unrestrained mice was performed as described elsewhere (17). Briefly, a catheter was placed via the jugular vein into the right atrium of the heart and connected to a metal piece containing two inlets which was fixed on the head at least 4 days before the clamp. Mice were placed in experimental cages at 11 p.m. and fasted for 9 h before start of the infusions with access to water. Mice were allowed to move freely during the whole course of the clamp experiment. After glucose was measured and a blood spot (see below) was taken at t=0, two infusion lines were connected to the catheter inlets. Through one, a mixture containing bovine serum albumin (1% w/v, Sigma), insulin (44 mU/ml, Actrapid, Novo Nordisk, Bagsvaerd, Denmark), somatostatin (40 μg/ml, UCB, Breda, The Netherland), unlabeled glucose (194 mg/ml) and [U-13C]glucose (6 mg/ml, 99% atom percent excess, Cambridge Isotope Laboratories, Andover, MA, USA) was infused at a constant rate of 0.135 ml/h. The second infusion line contained only a glucose solution (194 mg/ml unlabeled glucose and 6 mg/ml [U-13C]glucose). The administration rate of glucose through this line was variable and was adjusted to maintain euglycemia. Blood glucose levels were measured every 15 min by tail bleeding and a blood spot was taken every 30 min. Glucose was extracted from blood spots by a 30 min incubation with 50 μl H2O followed by addition of 0.5 ml ethanol and, after thorough mixing, overnight incubation at room temperature. The next day, samples were centrifuged at 14,000 rpm for 10 min, supernatants were transferred to derivatization tubes and dried under a stream of nitrogen at 60 °C. Glucose was derivatized to glucose penta-acetate by the addition of 100 μl pyridine and 200 μl acetic acid anhydride followed by an overnight incubation at room temperature. Subsequently, samples were dried again under a stream of nitrogen at 60 °C and taken up in ethyl acetate. Enrichment with [U-13C]glucose was measured using GC-MS. Glucose appearance and disposal rates were calculated as described (18).

**In vitro polarization of bone marrow-derived macrophages**
Bone marrow cells were collected from mouse femurs and tibias and cultured as described elsewhere (19). Briefly, bone marrow cells were flushed out and plated in RPMI medium supplemented with 10% FCS, 10 mM HEPES, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 15% L929-conditioned medium (as a source of Csf1). Extra medium was added on day 3 and medium was replaced.
on day 6. On day 8, cells were lifted and replated for the actual experiments. M2-polarization was induced by incubation with IL-4 (20 ng/ml, Biovision, Mountain View, CA, USA) for 18 h. Subsequently, cells were lysed in tri-reagent (Sigma) and RNA was isolated as described above.

**Bone marrow transplantation**
Transplantations were performed from CD45.2+ donor mice to recipients CD45.1+ in order to allow assessment of chimerism formation after transplantation. Recipient mice were γ-irradiated with a dose of 9.5 Gy using a 137Cs source (IBL 639, CIS Bio International, Gif-sur-Yvette, France). Donor mice were sacrificed and femurs and tibias were harvested. The bones were opened and flushed with DMEM containing 2% fetal calf serum (FCS, Invitrogen). Recipient mice were transplanted with 3 million cells by i.v. injection. Neomycin sulfate (3.5 g/l) was added to the drinking water of the mice for the first two weeks after transplantation. Chimerism formation was assessed 8 weeks post transplantation in a venous blood sample. Therefore, erythrocytes were lysed using a buffer containing 0.156 M ammoniumchloride, 0.1 mM EDTA and 17 mM NaCl. After washing, the remaining cells were stained with anti-CD45.1 (PE) and anti-CD45.2 (FITC) antibodies (BD Pharmingen) for 40 minutes and analyzed on a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA, USA).

**Statistics**
Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, SPSS Inc., Chicago, IL). Data are presented as means ± SEM. Differences between groups were compared using the Mann-Whitney U-test. P values <0.05 were considered statistically significant.

**Results**

*Insulin sensitivity is reduced in chow-fed 12/15-LO−/− mice*
Body weight of 5 months old 12/15-LO−/− mice fed a chow diet was not different from that of WT mice (table I). Plasma levels of triglycerides, free fatty acids and cholesterol were similar (table I) and there were also no differences in fasting blood glucose (4.6 ± 0.1 vs. 5.0 ± 0.2 mmol/l, fig. 1A) and plasma insulin levels (0.39 ± 0.11 vs. 0.33 ± 0.09 ng/ml, fig. 1B). Furthermore, no significant differences were found between the groups regarding the weight of the epididymal, omental and retroperitoneal fat pads (data not shown), and also liver weights were similar (table I). However, the livers of 12/15-LO−/− mice contained less triglycerides compared with those of WT mice (p<0.01, table I).

To examine insulin sensitivity, mice were subjected to a hyperinsulinemic euglycemic clamp. Chow-fed 12/15-LO−/− mice displayed a reduced insulin sensitivity as indicated by a 35% lower glucose infusion rate during the
Table I. Characteristics of wild-type and 12/15-LO⁻/⁻ mice on chow diet

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>12/15-LO⁻/⁻</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>32.1 ± 1.0</td>
<td>33.7 ± 1.1</td>
</tr>
<tr>
<td>Plasma triglycerides (mg/dl)</td>
<td>22.7 ± 4.4</td>
<td>21.5 ± 7.7</td>
</tr>
<tr>
<td>Plasma free fatty acids (mmol/l)</td>
<td>1.12 ± 0.11</td>
<td>0.79 ± 0.17</td>
</tr>
<tr>
<td>Plasma free cholesterol (mg/dl)</td>
<td>20 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Plasma total cholesterol (mg/dl)</td>
<td>54 ± 5</td>
<td>48 ± 9</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.30 ± 0.06</td>
<td>1.18 ± 0.10</td>
</tr>
<tr>
<td>Liver triglycerides (µmol/liver)</td>
<td>46.8 ± 8.5</td>
<td>20.8 ± 4.0b</td>
</tr>
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</table>

b p<0.01 compared to WT. Data are presented as means ± SEM (n=5-6 per group).

Fasting clamp (272 ± 41 vs. 420 ± 48 µmol kg⁻¹ min⁻¹, p<0.05, fig. 1C). The decreased glucose disposal rate (322 ± 49 vs. 459 ± 44 µmol kg⁻¹ min⁻¹, fig. 1D) suggested that peripheral tissues are less insulin sensitive, although this difference did not reach statistical significance (p=0.086). Despite the decreased triglyceride content of the livers of 12/15-LO⁻/⁻ mice, there was no difference in hepatic insulin sensitivity, as indicated by similar hepatic glucose production rates under hyperinsulinemic conditions (50 ± 11 vs. 39 ± 15 µmol kg⁻¹ min⁻¹, fig. 1E). Taken together, these results suggest that insulin sensitivity is lower in chow-fed 12/15-LO⁻/⁻ mice.

Figure 1. Chow-fed 12/15-LO⁻/⁻ mice are insulin resistant. (A) Fasted blood glucose levels and (B) fasted plasma insulin levels in wild-type (WT) and 12/15-LO⁻/⁻ mice (n=5-7 per group). (C) Glucose infusion rate (GIR), (D) glucose disposal rate (GDR) as a measure of peripheral insulin sensitivity and (E) hepatic glucose production (HGP) as a measure of hepatic insulin sensitivity in WT and 12/15-LO⁻/⁻ mice. Data in panels C-E are derived from hyperinsulinemic euglycemic clamp experiments performed as detailed in materials and methods (n=6-7 per group). All data are given as means ± SEM. a p<0.05 compared to WT controls.
12/15-LO<sup>−/−</sup> mice fed a high-fat diet are severely insulin resistant

After feeding a HFD (for FA composition, see supplemental table) for 12 weeks, 12/15-LO<sup>−/−</sup> mice were significantly heavier as compared to WT controls (p<0.05, table II), although food consumption did not differ between the groups (data not shown). Plasma triglycerides, free fatty acids and cholesterol levels were not affected by the absence of 12/15-LO (table II). Although liver weights did not differ significantly between the groups (table II), livers of HFD-fed 12/15-LO<sup>−/−</sup> mice contained 46% more triglycerides than livers from WT controls (p<0.05, table II). 12/15-LO<sup>−/−</sup> mice also had more abdominal adipose tissue compared to WT mice. While the amount of epididymal fat was not different between the groups, 12/15-LO<sup>−/−</sup> mice had 51% (p<0.05) more omental fat and 74% (p<0.05) more retroperitoneal fat than WT mice (table II).

### Table II. Characteristics of wild-type and 12/15-LO<sup>−/−</sup> mice after feeding a high-fat diet for 12 weeks

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>12/15-LO&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>41.4 ± 1.7</td>
<td>47.3 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma triglycerides (mg/dl)</td>
<td>35.6 ± 4.5</td>
<td>24.8 ± 3.6</td>
</tr>
<tr>
<td>Plasma free fatty acids (mmol/l)</td>
<td>1.10 ± 0.12</td>
<td>1.21 ± 0.14</td>
</tr>
<tr>
<td>Plasma free cholesterol (mg/dl)</td>
<td>49 ± 2</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>Plasma total cholesterol (mg/dl)</td>
<td>174 ± 9</td>
<td>162 ± 10</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.71 ± 0.17</td>
<td>2.16 ± 0.24</td>
</tr>
<tr>
<td>Liver triglycerides (μmol/liver)</td>
<td>183 ± 18</td>
<td>268 ± 34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal WAT (g)</td>
<td>2.09 ± 0.26</td>
<td>2.10 ± 0.24</td>
</tr>
<tr>
<td>Omental WAT (g)</td>
<td>0.51 ± 0.06</td>
<td>0.76 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Retroperitoneal WAT (g)</td>
<td>0.72 ± 0.08</td>
<td>1.25 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>p<0.05 compared to WT. Data are presented as means ± SEM (n=6-7 per group). WAT, white adipose tissue.

Fasting glucose levels were not different (6.0 ± 0.4 vs. 6.0 ± 0.4 mmol/l, fig. 2A), but fasting insulin levels were on average almost 4-fold higher in the 12/15-LO<sup>−/−</sup> mice (5.4 ± 2.5 vs. 1.4 ± 0.3 ng/ml, p=0.063, fig. 2B).

Hyperinsulinemic euglycemic clamp experiments revealed that HFD-fed 12/15-LO<sup>−/−</sup> mice had a severely impaired insulin sensitivity compared to HFD-fed WT mice as indicated by a 58% reduction in the glucose infusion rate (61.4 ± 2.0 vs. 145.4 ± 15.5 μmol kg<sup>−1</sup> min<sup>−1</sup>, p<0.01, fig. 2C). Decreased insulin sensitivity was detected in peripheral tissues as well as in livers of 12/15-LO<sup>−/−</sup> mice, as reflected by 34% lower glucose disposal rates (136.7 ± 11.6 vs. 206.7 ± 12.5 μmol kg<sup>−1</sup> min<sup>−1</sup>, p<0.01, fig. 2D) and a 23% higher hepatic glucose production under hyperinsulinemic conditions (75.3 ± 12.7 vs. 61.2 ± 3.6 μmol kg<sup>−1</sup> min<sup>−1</sup>, p<0.05, fig. 2E). Supporting the finding of
decreased insulin sensitivity of the liver, expression of glucose-6-phosphatase (G6pc), the enzyme that catalyzes the final and rate-controlling step in gluconeogenesis, was more than 3-fold increased in insulin-stimulated livers of 12/15-LO<sup>−/−</sup> mice collected directly after the hyperinsulinemic clamp (p<0.01, fig. 2F).

**Adipose tissue inflammation is increased in 12/15-LO<sup>−/−</sup> mice**

To explore the mechanistic basis of decreased insulin sensitivity in HFD-fed 12/15-LO<sup>−/−</sup> mice, we first investigated adipose tissue macrophage content and phenotype. Increased Cd68 mRNA expression readily indicated that increased numbers of macrophages were present in adipose tissue of 12/15-LO<sup>−/−</sup> mice (4.6-fold, p<0.01, fig. 3A). Furthermore, adipose tissue mRNA expression of Mcp-1, a chemokine considered to play a pivotal role in promoting adipose tissue macrophage infiltration (20;21), was also approximately 3-fold higher in 12/15-LO<sup>−/−</sup> compared to WT mice (p<0.05, fig. 3B). Immunohistochemical staining for the macrophage marker F4/80 confirmed the higher presence of macrophages, in characteristic crown-like structures, in the adipose tissue of 12/15-LO<sup>−/−</sup> mice (13.8 ± 0.8 % vs. 5.5 ± 0.6 %, p<0.01, fig. 3C and D).

Next we investigated whether not only macrophage numbers, but also macrophage polarization was altered in the adipose tissue of 12/15-LO<sup>−/−</sup> mice. Polarization of adipose tissue macrophages towards the pro-inflammatory M1 phenotype is considered to contribute to insulin resistance whereas polarization towards the
anti-inflammatory M2 phenotype is considered to be beneficial in this respect (22). The mRNA expression of the two major markers of alternatively activated (M2) macrophages, Mgl-1 and Mgl-2, was considerably decreased in 12/15-LO\(^{-/-}\) mice compared to controls when normalized to the macrophage marker Cd68 (-62%, p<0.01 and -78%, p<0.01, respectively, fig. 3E and F). Importantly, also when normalized to F4/80, another macrophage marker, these differences remained markedly significant (data not shown). Normalization of the expression levels of the M2-markers to the macrophage-specific markers Cd68 and F4/80 is feasible because Mgl-1 and Mgl-2 expression in adipocytes is very low and absent, respectively (data not shown). These results suggest that, in response to HFD feeding, adipose tissue macrophages of 12/15-LO\(^{-/-}\) mice display a more pro-inflammatory phenotype.

To assess a potential causal relation between 12/15-LO expression and macrophage polarization, additional in vitro experiments were performed in which bone marrow-derived macrophages were stimulated with IL-4, a known inducer of the M2 phenotype (23). These experiments showed that, compared to WT controls, 12/15-LO\(^{+/+}\) macrophages had a lower expression of Mgl-1 (-43%, p<0.05, fig. 4A) and Mgl-2 (-47%, p<0.05, fig. 4B), indicating an impaired ability of macrophages to polarize towards the anti-inflammatory (M2) phenotype in the absence of functional 12/15-LO.

Figure 3. Increased adipose tissue inflammation in high-fat diet-fed 12/15-LO\(^{+/+}\) mice. Mice were fed a high-fat diet for 12 weeks. Adipose tissue mRNA expression of (A) Cd68 and (B) Mcp-1 (n=6-7 per group). Representative photographs of adipose tissue from (C) wild-type (WT) and 12/15-LO\(^{+/+}\) mice stained for the macrophage marker F4/80 (green) and the general membrane marker caveolin-1 (red). Images were obtained using a confocal laser scanning microscope (original magnification 400x). (D) Quantification of the number of crown-like structures in adipose tissue (n=6-7 per group). mRNA expression of the M2 polarization markers (E) Mgl-1 and (F) Mgl-2 in adipose tissue macrophages (n=6-7 mice per group). Data are presented as means ± SEM. \(^a\) p<0.05, \(^b\) p<0.01 compared to WT controls.
Insulin sensitivity is decreased in mice specifically lacking macrophage 12/15-LO expression

To delineate the mechanistic contribution of the lack of 12/15-LO specifically in macrophages to increased whole body insulin resistance, a bone marrow transplantation experiment was performed in which either 12/15-LO⁻/⁻ or WT bone marrow was transplanted into WT recipients. Chimerism was confirmed 8 weeks after bone marrow transplantation (data not shown). Subsequently, the
mice were fed a HFD for 12 weeks. In contrast to what was observed in the total body 12/15-LO-deficient mice, no differences in body weight or hepatic triglyceride content were observed between the groups at the end of the diet period (table III). Moreover, liver and fat pad weights in both groups were similar as were plasma levels of triglycerides, free fatty acids and cholesterol (table III). Although fasting glucose and insulin levels were not significantly different (data not shown), data obtained from the hyperinsulinemic euglycemic clamp showed that mice transplanted with 12/15-LO⁻/⁻ bone marrow were less insulin sensitive than the mice that had received WT bone marrow as demonstrated by a 45% reduced glucose infusion rate (172 ± 31 vs. 312 ± 17 μmol kg⁻¹ min⁻¹, p<0.05, fig. 5A). A reduction in the glucose disposal rate by 36% (250 ± 36 vs. 388 ± 11 μmol kg⁻¹ min⁻¹, p<0.05, fig. 5B) revealed that mainly peripheral tissues were insulin resistant, while hepatic insulin sensitivity was not different between the groups as indicated by the similar hepatic glucose production rates during the clamp (78 ± 7 vs. 76 ± 9 μmol kg⁻¹ min⁻¹, fig. 5C). The increased insulin resistance in the mice transplanted with 12/15-LO⁻/⁻ bone marrow was accompanied by an approximately 2-fold increase in adipose tissue Cd68 mRNA expression (fig 5D), albeit this did not reach statistical significance.

Figure 5. High-fat diet-fed wild-type (WT) mice transplanted with 12/15-LO⁻/⁻-BM are insulin resistant compared to WT mice receiving WT bone marrow. WT mice were transplanted with either WT or 12/15-LO⁻/⁻-BM, allowed to recover for 8 weeks, and subsequently fed a high-fat diet for 12 weeks. (A) Glucose infusion rate (GIR), (B) glucose disposal rate (GDR), and (C) hepatic glucose production (HGP) during the hyperinsulinemic euglycemic clamp performed as detailed in materials and methods (n=4-5 per group). Adipose tissue mRNA expression of the macrophage marker Cd68 (D) and adipose tissue macrophage mRNA expression of the M2 polarization markers Mgl-1 (E) and Mgl-2 (F) in bone marrow transplanted mice (n=6-7 per group). Data are presented as means ± SEM. * p<0.05 compared to mice receiving WT control bone marrow.
Similar to what was observed in the HFD-fed total body 12/15-LO⁻/⁻ mice, adipose tissue macrophages in WT mice that received 12/15-LO⁻/⁻ bone marrow also showed a reduced anti-inflammatory M2 polarization compared to controls as demonstrated by a 36% decreased Mgl-1 expression (p<0.05, fig. 5E) and a 30% lower Mgl-2 expression (p<0.05, fig. 5F). Moreover, transplantation of WT bone marrow into 12/15-LO⁻/⁻ recipient mice partially rescued the insulin resistant phenotype of the whole body 12/15-LO⁻/⁻ mice (fig. 6A-C). Together, these data underscore the crucial role of macrophage 12/15-LO expression for maintenance of insulin sensitivity in HFD-fed mice.

Discussion

This study demonstrates that lack of functional 12/15-LO in mice results in reduced insulin sensitivity. The insulin resistant phenotype was already apparent on a chow diet but became more pronounced after feeding a HFD. Mechanistically, we identified increased adipose tissue inflammation, due to an impaired polarization of macrophages lacking 12/15-LO towards the anti-inflammatory M2 phenotype, as a major contributing factor to the increased insulin resistance observed in 12/15-LO⁻/⁻ mice. Results from the bone marrow transplantation experiments also indicated a protective role for macrophage 12/15-LO against the development of insulin resistance. Interestingly, the reduced insulin sensitivity in WT mice transplanted with 12/15-LO⁻/⁻ bone marrow compared to those receiving WT bone marrow occurred in the absence of effects on body-, liver-, and fat pad weights. Thereby, insulin resistance due to absence of 12/15-LO appears to be dissociated from increased obesity as observed in the total body 12/15-LO⁻/⁻ mice. Macrophage infiltration and subsequent adipose tissue inflammation are regarded
important contributors to the development of insulin resistance (4;8). Our initial finding in the HFD-fed whole body 12/15-LO−/− mice was that their adipose tissue contained more macrophages than that of WT mice. Consistently, adipose tissue expression of *Mcp-1*, an important stimulus for macrophage attraction (20;21;24), was almost 3-fold higher in 12/15-LO−/− mice compared to WT mice after 12 weeks of high-fat diet feeding.

It is increasingly recognized that, apart from the total number of macrophages present in adipose tissue, also the polarization state of these cells might be relevant. Resident macrophages in lean adipose tissue typically display an anti-inflammatory phenotype, known as M2. However, when adipose tissue expands, as in obesity, a switch in macrophage polarization occurs towards the more inflammatory M1 phenotype (25;26). It has been demonstrated that skewing the M1/M2 balance of adipose tissue macrophages towards the M2 phenotype has the potential to improve insulin sensitivity (22). A similar phenomenon has been described for Kupffer cells regarding hepatic insulin sensitivity (27). It is noteworthy that 12/15-LO expression is more than 10-fold higher in M2 compared to M1 macrophages (28), already indicating that this enzyme is of functional relevance in M2 macrophages. Furthermore, 12/15-LO is a well-recognized mediator in the IL-4 signaling cascade (29) that represents a major pathway in M2 polarization (23). Lack of 12/15-LO might therefore result in a shift in macrophage polarization towards the pro-inflammatory M1 phenotype. *In vitro* stimulation of bone marrow-derived macrophages with IL-4 confirmed that 12/15-LO−/− macrophages indeed have a reduced ability to polarize towards the M2 phenotype compared to macrophages derived from WT mice. In accordance with the results from the *in vitro* experiments, mRNA expression of M2-markers by adipose tissue macrophages was reduced in 12/15-LO−/− mice, consistent with a shift towards M1 polarization.

Since 12/15-LO is expressed by macrophages as well as by adipocytes (11), bone marrow transplantation experiments were performed to assess the specific contribution of functional 12/15-LO in macrophages to the development of insulin resistance observed in the whole body 12/15-LO−/− mice. Hyperinsulinemic euglycemic clamp studies showed a markedly reduced insulin sensitivity in HFD-fed WT mice that received 12/15-LO−/− bone marrow compared to those that received WT bone marrow, notably in absence of differences in the degree of obesity. Moreover, 12/15-LO−/− mice transplanted with WT bone marrow were more insulin sensitive compared to controls that received 12/15-LO−/− bone marrow after feeding a HFD. These data indicate that macrophage 12/15-LO is indeed important for the maintenance of insulin sensitivity, although an additional contribution of 12/15-LO expression in other cells like adipocytes or skeletal muscle cells (30) can not be excluded. Consistent with our data in the whole body 12/15-LO−/− mice, the more severe insulin resistance in the mice transplanted with 12/15-LO−/− bone marrow was accompanied by a shift in adipose tissue macrophage polarization towards the M1 phenotype. These data further support the concept (22;26) that changing the M1/M2 balance of macrophages in adipose tissue leads to altered insulin sensitivity.
Data published thus far suggest multiple and even divergent roles for 12/15-LO during inflammation. On the one hand, 12/15-LO might promote inflammation by producing mediators that stimulate the inflammatory reaction (31;32). However, there is also evidence that 12/15-LO has anti-inflammatory properties and that it might be a key mediator involved in the resolution of inflammation (12;15;33). The pro- or anti-inflammatory activities of 12/15-LO conceivably depend on the metabolic context, which might be the underlying reason for the apparent contradictory results that have been published thus far on the role of 12/15-LO in various disease models in which inflammation plays a prominent role (11-15). With regard to atherosclerosis for example, one group reported that 12/15-LO exacerbates vascular inflammation and atherosclerosis (14;34-36), while another group found that 12/15-LO plays a crucial role in the resolution of inflammation in the vascular wall and protects against the development of atherosclerosis by the production of anti-inflammatory lipid mediators (12).

Extending this discussion to the field of obesity and insulin resistance, two other studies reported that 12/15-LO<sup>−/−</sup> mice might be protected against the development of diet-induced insulin resistance and adipose tissue inflammation (37;38), which is in contrast to our present results. The differences in outcome of our present study as compared to those two others (37;38) might be explained by differences in dietary cholesterol content (37) or in the duration of the experiment (38). In addition, since the type of substrates available determines which mediators are produced by 12/15-LO, dietary fatty acid composition might be important in determining whether 12/15-LO acts pro- or anti-inflammatory. The fatty acid composition of the HFD used in this study is provided in the supplemental table. However, this information is not available from the previously mentioned studies (37;38) making direct comparisons difficult. In addition to fatty acid substrate availability via the diet, not utilized substrates, that accumulate due to the absence of 12/15-LO, might be fueled into other pathways. Interestingly, we found that the levels of the 12/15-LO substrates linoleic and arachidonic acid were significantly increased in the adipose tissue phospholipid fraction of 12/15-LO<sup>−/−</sup> compared to WT mice (data not shown). Although these data do not reveal whether increased production or decreased metabolic conversion cause the increased arachidonic acid levels found in the adipose tissue of 12/15-LO<sup>−/−</sup> mice, this accumulation of substrate may impact on other, possibly pro-inflammatory, pathways such as the cyclooxygenase pathway. More research will, however, be required to further investigate this hypothesis.

In summary, our data demonstrate that the lack of functional 12/15-LO results in severe insulin resistance in HFD-fed mice. Mechanistically, the absence of 12/15-LO expression in macrophages appears to be an important pathophysiological mediator of this effect, conceivably by inducing a shift in adipose tissue macrophage polarization from an anti-inflammatory M2 towards a more pro-inflammatory M1 phenotype. These results might have important consequences, not only for the 12/15-LO pathway as a potential therapeutic target for insulin resistance and type 2
diabetes mellitus but also in general when 12/15-LO inhibition is considered to treat various other chronic inflammatory diseases.

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References


Supplemental tabel chapter 2
### Supplemental table. Fatty acid composition of high-fat diet

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