High-Fat Diet-Mediated Lipotoxicity and Insulin Resistance Is Related to Impaired Lipase Expression in Mouse Skeletal Muscle

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Elevated expression/activity of adipose triglyceride lipase (ATGL) and/or reduced activity of hormone-sensitive lipase (HSL) in skeletal muscle are causally linked to insulin resistance in vitro. We investigated here the effect of high-fat feeding on skeletal muscle lipolytic proteins, lipotoxicity, and insulin signaling in vivo. Five-week-old C3H mice were fed normal chow diet (NCD) or 45% kcal high-fat diet (HFD) for 4 weeks. Wild-type and HSL knockout mice fed NCD were also studied. Whole-body and muscle insulin sensitivity, as well as lipolytic protein expression, lipid levels, and insulin signaling in skeletal muscle, were measured. HFD induced whole-body insulin resistance and glucose intolerance and reduced skeletal muscle glucose uptake compared with NCD. HFD increased skeletal muscle total diacylglycerol (DAG) content, protein kinase C membrane translocation, and impaired insulin signaling as reflected by a robust increase of basal Ser1101 insulin receptor substrate 1 phosphorylation (2.8-fold, \( P < .05 \)) and a decrease of insulin-stimulated v-Akt murine thymoma viral oncogene homolog Ser473 (−37%, \( P < .05 \)) and AS160 Thr642 (−47%, \( P < .01 \)) phosphorylation. We next showed that HFD strongly reduced HSL phosphorylation at Ser660. HFD significantly up-regulated the muscle protein content of the ATGL coactivator comparative gene identification 58 and triacylglycerol hydrolase activity, despite a lower ATGL protein content. We further show a defective skeletal muscle insulin signaling and DAG accumulation in HSL knockout compared with wild-type mice. Together, these data suggest a pathophysiological link between altered skeletal muscle lipase expression and DAG-mediated insulin resistance in mice. (Endocrinology 154: 1444–1453, 2013)

Obesity has become a major health problem worldwide. It constitutes a strong risk factor for other diseases, such as type 2 diabetes mellitus and cardiovascular diseases (1, 2). Obesity has been associated with ectopic fat deposition, and in sedentary populations, the level of intramyocellular triacylglycerol (TAG) (IMTG) is negatively correlated with insulin sensitivity (3, 4). However, studies on endurance athletes revealed a paradoxically elevated IMTG content in highly insulin sensitive subjects (5). It is now well established that skeletal muscle insulin resistance is mechanistically linked to accumulation of lipotoxic lipid species. Several studies show the detrimental

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Abbreviations: AAC, area above the curve; AMPK, 5′-AMP protein kinase; Akt, v-Akt murine thymoma viral oncogene homolog; ATGL, adipose triglyceride lipase; AU, arbitrary unit; AUC, area under the curve; CGI58, comparative gene identification 58; DAG, diacylglycerol; GGT, glucose tolerance test; HFD, high-fat diet; HSL, hormone-sensitive lipase; IMTG, intramyocellular TAG; IRS1, insulin receptor substrate-1; ITT, insulin tolerance test; NCD, normal chow diet; PKC, protein kinase C; PUN, perilipin; TAG, triacylglycerol; TAGH, TAG hydrolase.
role of diacylglycerols (DAGs) and ceramides in cultured myotubes (6, 7), as well as in vivo in mice and human skeletal muscle (8–11).

DAG, a byproduct of lipolysis, is derived from TAG hydrolysis by adipose triglyceride lipase (ATGL) and is further hydrolyzed by the DAG hydrolase hormone-sensitive lipase (HSL) (12). Thus, HSL knockout mice accumulate DAG in several organs, such as skeletal muscle (13), whereas ATGL null mice appear more glucose tolerant (14). We recently demonstrated a causal relationship between defective lipolysis, lipotoxicity, and insulin resistance in human primary myotubes. Up-regulation of ATGL expression and/or inhibition of HSL activity both promoted DAG accumulation and impaired insulin signaling (7). Besides lipases, lipid droplet-associated proteins of the perilipin (PLIN) family (PLIN1–PLIN5) have been shown to play a major role in the control of TAG hydrolysis and lipolysis in multiple tissues (15, 16). It is still unclear whether skeletal muscle PLIN protein expression is disturbed in obesity and insulin-resistance states.

The aim of the present study was to test in vivo in mice the pathophysiological relevance of altered skeletal muscle lipolytic protein expression. We investigated here the impact of a high-fat diet (HFD) on the expression of lipases, lipidotoxicity, and insulin resistance in skeletal muscle of C3H mice. This model has been previously used as a relevant model of diet-induced obesity and diabetes (17). We further examined this relationship in HSL knockout mice.

**Materials and Methods**

**Animals**

Animal protocols were performed according to Institut National de la Santé et de la Recherche Médicale and Institut des Maladies Métaboliques et Cardiovasculaires Animal Care Facility guidelines. All protocols were reviewed and approved by an Institutional Animal Care and Use Committee. Wild-type males C3H/HeOuJ and females B6D2 HSL null mice (kindly provided by Dr Cecilia Holm) were used and maintained on a 12-hour light, 12-hour dark cycle. They were housed 4 per cage, with ad libitum water and food. HFD (45% fat, Research Diets D12451; Research Diets, Inc, New Brunswick, New Jersey) or normal chow diet (NCD) (10% fat, D12450B) were initiated for 4 weeks right after weaning (5 wk of age) in C3H mice. HSL null mice were fed for 7 weeks under NCD.

**Body mass and body composition**

Body weight was measured weekly. For C3H mice, body composition was evaluated by quantitative nuclear magnetic resonance imaging (EchoMRI 3-in-1 system; Echo Medical Systems, Houston, Texas) before and at 4 weeks of each diet.

**Glucose tolerance test (GTT) and insulin tolerance test (ITT)**

For ip ITT and GTT, a bolus of insulin 0.5 mU/g of lean mass (Insuman Rapid, Sanofi Aventis, France) and D-glucose 2 mg/g of lean mass (Sigma-Aldrich, Saint-Quentin Fallavier, France) was injected to 6-hour-fasted mice, respectively. Blood glucose levels were monitored from the tip of the tail with a glucometer (Accucheck; Roche, Meylan, France) at 0, 15, 30, 45, 60, and 90 minutes after injection. Fasting insulin was measured using an ultrasensitive ELISA (ALPCO Diagnostics, Salem, New Hampshire). For ITTs, area above the curve (AAC) was calculated to account for differences in baseline fasting blood glucose concentrations as previously suggested (18).

**Tissue-specific [2-3H]deoxyglucose uptake during the GTT**

To determine muscle-specific glucose uptake, [2-3H]deoxyglucose (PerkinElmer, Boston, Massachusetts) (12 μCi/mouse) was mixed with 20% D-glucose to obtain a fixed specific activity before ip injection (2-mg/g lean body mass) as previously described with slight modifications (19). Animals were killed by cervical dislocation 45 minutes after injection, and soleus and gastrocnemius muscles were snap frozen in liquid nitrogen. Tissue-specific accumulation of 2-deoxyglucose-6-phosphate was determined as previously described, with minor modifications (20). The total of the [3H]-radioactivity found in 2-deoxyglucose-6-phosphate was divided by the mean specific activity of glucose at 45 minutes to obtain the tissue-specific clearance index (Kg) (mililiter per 100 g of tissue per minute) as described elsewhere (21).

**Tissue collection and preparation**

After an overnight fast, mice were killed by cervical dislocation, and tissues were rapidly extracted and freeze clamped in liquid nitrogen before being stored at −80°C. For insulin signaling experiments, fresh tissues of C3H mice were incubated at 37°C for 20 minutes in Krebs-Henseleit buffer with or without 100nM insulin. After incubation, tissues were freeze clamped in liquid nitrogen and stored at −80°C. B6D2 wild-type and HSL null mice were injected ip with 10 mU/g of insulin 10 minutes before being killed.

**Tissue fractionation**

Plasma membrane and cytosol fractions were prepared as previously described with slight modifications (22, 23). Briefly, 50 mg of gastrocnemius muscles were extracted in buffer A (50mM Tris [pH 8.0] and 0.5mM dithiothreitol) containing 10-μL/mL protease inhibitor (Sigma-Aldrich), 10-μL/mL phosphatase 1 inhibitor (Sigma-Aldrich), and 10-μL/mL phosphatase II inhibitor (Sigma-Aldrich). Tissues were homogenized using a polytron and centrifuged at 1000g for 10 minutes at 4°C. The supernatant 1 was collected and kept on ice. The pellet was rinsed once in buffer A, centrifuged at 1000g for 10 minutes at 4°C, and the new pellet was resuspended in buffer B (buffer A plus Nonidet P-40 1%) and stood on ice for 1 hour with occasional mixing. The tube was then centrifuged at 16 000g for 20 minutes at 4°C, and the supernatant was used as the crude membrane fraction. The supernatant 1 was centrifuged at 16 000g for 20 minutes at 4°C, and the new supernatant was used as the cytosol fraction. A representative blot of 1 tissue fractionation experiment is shown in Supplemental Figure 1, published on The

Western blot analysis

Soleus muscles were homogenized during 2 cycles of 30 seconds, 5500 rpm, 4°C using the Precellys 24 apparatus (Bertin Technologies, Montigny-le-Bretonneux, France) in a buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10-μL/mL protease inhibitor, 10-μL/mL phosphatase I inhibitor, and 10-μL/mL phosphatase II inhibitor. Tissue lysates were centrifuged at 14 000 g for 25 minutes, and supernatants were stored at −80°C. A total of 40 μg of solubilized proteins from muscle tissue were run on a 4%–20% gradient SDS-PAGE (Bio-Rad, Hercules, California), transferred onto nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Piscataway, New Jersey), and incubated with the primary antibodies: ATGL, HSL, pSer660-HSL, and pSer565-HSL (Cell Signaling Technology, Beverly, Massachusetts) and comparative gene identification 58 (CGI58) (Abnova Corp, Taipei, Taiwan) for lipases; pSer473-Akt, v-Akt murine thymoma viral oncogene homolog (Akt), pSer1101-insulin receptor substrate-1 (IRS1), pTyr612-IRS1, IRS1, pThr642-AS160, and AS160 for insulin signaling (Cell Signaling Technology). For protein kinase C (PKC) translocation assays, PKCα, PKCβ, and PKCε primary antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, California). α-Tubulin (Sigma-Aldrich) was used as cytosolic marker, and caveolin1 (Cell Signaling Technology) was used as plasma membrane marker. Gels were loaded with equal amount of membrane and cytosol proteins. For detection of PLIN, membranes were also probed with PLIN2 and PLIN3 (Thermo Scientific, Illkirch, France) and PLIN5 (Progen, Heidelberg, Germany) antibodies. Subsequently, immunoreactive proteins were determined by enhanced chemiluminescence reagent (SuperSignal West Dura or SuperSignal West Dura; Thermo Scientific) and visualized by exposure to Hyperfilm ECL (GE Healthcare, Princeton, New Jersey). Glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology) was used as internal control.

Lipase activity assays

TAG hydrolyase (TAGH) activity was measured on soleus lysates as previously described (24). Briefly, muscles were extracted in a lysis buffer containing 0.25M sucrose, 1mM EDTA, 1mM dithiothreitol, 20-μg/mL leupeptin, and 20-μg/mL antipain. [9, 10-3H(N)]triolein (PerkinElmer) and cold triolein were emulsified with phospholipids by sonication. The data are expressed in nanomoles of oleic acid released per hour per milligram of protein.

Neutral lipid molecular species analysis

Soleus muscles were homogenized in 1 mL of methanol/5mM EGTA (2:1, vol/vol) with FAST-PREP (MP Biochemicals, Solon, Ohio). Lipids corresponding to 2 mg of tissue were extracted according to Bligh and Dyer (25) in methanol/water/dichloromethane (1.5:1.5:2, vol/vol/vol), in the presence of internal standards: 3 μg of stigmastanol, 3 μg of 1,3-dimyristyl, 3 μg of cholesteryl heptadecanoate, and 20 μg of glyceryl trinonadecanoate. Dichloromethane phase was evaporated to dryness. Neutral lipid were separated on solid phase extraction columns (Macherey Nagel glass Chromabond pure silice, 200 mg) after washing cartridge with 2 mL of chloroform, crude extract was applied on the cartridge in 40 μL of chloroform, and neutral lipids were eluted in 2 mL of chloroform:methanol (9:1, vol/vol). The organic phase was evaporated to dryness and dissolved in 20 μL of ethyl acetate. One microliter of the lipid extract was analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using an Zebron-1 Phenomenex fused silica capillary columns (5 m × 0.32 mm inner diameter, 0.50 μm of film thickness) (26). Oven temperature was programmed from 200°C to 350°C at a rate of 5°C per minute, and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 315°C and 345°C, respectively. The equivalent of 0.3 mg of tissue was evaporated under nitrogen, the dry pellets were dissolved overnight in 0.2 mL of NaOH (0.1M), and proteins were measured with the Bio-Rad protein assay.

[1-14C]Palmitate incorporation

Whole solei were incubated for 2 hours in a modified sucrose-EDTA medium (250mM sucrose, 1mM EDTA, and 10mM Tris-HCl [pH 7.4]) with a combination of [1-14C]palmitate (1 μCi/ml PerkinElmer) and unlabeled palmitate (200μM final concentration). At the end of incubation, total lipids were extracted in chlorofrom/methanol (2volume/volume) and separated by thin layer chromatography as previously described (27). Incorporation rates were normalized to wet tissue weight.

Statistics

All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software, Inc, San Diego, California). Normal distribution of the data was tested with Kolmogorov-Smirnov tests. Unpaired Student’s t tests were performed to determine differences between groups. Two-way ANOVA followed by Bonferroni’s post hoc tests was applied when appropriate. Pearson correlations were applied when data were normally distributed and Spearman correlations for nonparametric data. All values in figures and tables are presented as mean ± SEM. Statistical significance was set at P < .05.

Results

Short-term HFD induces rapid weight gain and whole-body insulin resistance in C3H mice

As expected, 4 weeks of HFD 45% kcal from fat induced greater weight gain compared with NCD in C3H mice (NCD +25%, P < .001; HFD +60%, P < .001) mainly because of a robust increase in fat mass (NCD +61%, P < .01; HFD +382%, P < .001), whereas lean body mass was not significantly changed between NCD and HFD (Table 1). Importantly, 4 weeks of HFD in C3H mice were sufficient to induce a comparable body weight gain (about 60%) as is typically observed in C57Bl/6 mice after 8 weeks under the same diet (data not shown). In addition, HFD increased fasting blood glucose (+18%, P < .001) and fasting blood insulin (4.5-fold, P < .001) compared with NCD in C3H mice (Table 1). Whole-body insulin and glucose tolerance were measured (Figure 1, A
The ITT AAC was reduced by $-44\%$ ($P < .01$) (Figure 1A), whereas the GTT area under the curve (AUC) was significantly increased in HFD-fed mice (3-fold, $P < .01$) (Figure 1B). Interestingly, plasma insulin concentration at 15 minutes during the GTT was significantly elevated in HFD-fed mice (2.8-fold, $P < .001$) (Figure 1C). Thus, C3H mice were strongly insulin resistant after 4 weeks of HFD and represent an interesting model to study the effect of short-term high-fat feeding on the mechanisms of fat-induced insulin resistance.

**Short-term HFD promotes lipotoxicity and impairs insulin signaling in skeletal muscle**

Because skeletal muscle is an important site of glucose disposal in the body, we examined the effects of short-term HFD on skeletal muscle glucose uptake, lipotoxicity, and insulin signaling. We first showed a reduced glucose uptake in both oxidative soleus and glycolytic gastrocnemius muscles of HFD-fed mice in vivo (Figure 2A). Of interest, glucose uptake in soleus was inversely correlated to the log(AUC) of the GTT ($r = -0.73, P = .01$). The reduced glucose uptake was associated with significant disturbances in muscle insulin signaling. Indeed, we noticed an increased basal Ser1101 phosphorylation of the IRS1 (2.8-fold, $P < .05$) (Figure 2, B and F), associated with an expected decrease of its activating phosphorylation on Tyr612 upon insulin stimulation ($-68\% P < .01$) (Figure 2, C and F). Consistently, downstream insulin-stimulated phosphorylation of Akt at Ser473 residue ($-37\%, P < .01$) (Figure 2, D and F) and AS160 at Thr642 ($-88\%, P < .01$) (Figure 2, E and F) was reduced in HFD-fed mice, whereas their basal phosphorylation remained unaffected by the diet (Supplemental Figure 2). IRS1 phosphorylation at Ser1101 inhibits IRS1 function and is a primary target for DAG-activated PKC (28). This prompted us to examine PKC translocation to the plasma membrane and skeletal muscle lipid content. We found a higher membrane to cytosol ratio of all PKC isoforms (PKCpan) as well as of the main novel PKC isoforms, PKCb and PKCe, in the muscle of HFD-fed compared with NCD-fed mice (Figure 3, A and B, and Supplemental Figure 3). As expected, the higher novel PKC membrane translocation was associated

| Table 1. Body Composition and Metabolic Variables in Fasted C3H Mice |

<table>
<thead>
<tr>
<th></th>
<th>NCD</th>
<th>HFD</th>
<th>$P$ value</th>
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<tbody>
<tr>
<td>Weight (g)</td>
<td>27.9 ± 0.38</td>
<td>35.5 ± 0.50</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>2.6 ± 0.28</td>
<td>9.1 ± 0.33</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>19.5 ± 0.20</td>
<td>19.7 ± 0.22</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fasting insulin (ng/mL)</td>
<td>0.47 ± 0.08</td>
<td>2.1 ± 0.30</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>140 ± 3.87</td>
<td>166 ± 4.21</td>
<td>&lt;.001</td>
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N.S., not significant.
with a significantly elevated total DAG content (+39%, P < .05) (Figure 3C), whereas a trend for higher IMTG content (+35%, P = .12) (Figure 3D) was observed in HFD-fed mice. Interestingly, we noted a strong relationship between skeletal muscle DAG content and fasting blood insulin (r = 0.60, P = .01), as well as a negative correlation between muscle plasma membrane PKC\textsubscript{pan} and whole-body insulin sensitivity as measured by the ITT AAC (r = –0.57, P = .03). Additionally, IRS1 serine phosphorylation occurred without any significant change in c-Jun N-terminal kinase-1 phosphorylation (Supplemental Figure 4A), whereas 5\textsuperscript{’}-AMP protein kinase (AMPK) Thr172 phosphorylation was significantly reduced in HFD-fed mice (Supplemental Figure 4B).

Short-term HFD alters skeletal muscle lipolytic proteins

To gain further insight into the mechanisms of HFD-induced lipotoxicity, we examined lipase and PLIN protein expression in skeletal muscle. Interestingly, compared with NCD, HFD up-regulated the expression of the ATGL coactivator CGI58 both in soleus and gastrocnemius muscles (Figure 4B and Supplemental Figure 5B), whereas ATGL protein tended to decrease (Figure 4A and Supplemental Figure 5A). CGI58 protein level progressively
rose from the beginning of the HFD until 4 weeks, whereas ATGL expression was not significantly changed (Supplemental Figure 6). Importantly, we observed a very strong decrease in Ser660-HSL phosphorylation (NCD, 0.16 ± 0.02 arbitrary units [AU] vs HFD, 0.08 ± 0.01 AU, P < .01) (Figure 4C and Supplemental Figure 5C), whereas Ser565-HSL phosphorylation was not altered when adjusted to total HSL protein (NCD, 3.73 ± 0.76 AU vs HFD, 2.28 ± 0.62 AU, P = .18) (Figure 4C). The decrease in Ser660-HSL phosphorylation occurred despite a significant increase of total HSL protein (NCD, 1.21 ± 0.05 AU, P < .01) (Figure 4C). We also measured the protein content of PLINs that are lipid droplet related proteins involved in the control of lipid droplet dynamics and metabolism. PLIN2 and PLIN3 proteins were not affected by HFD (Figure 5, A and B), whereas PLIN5 was strongly up-regulated in HFD-fed mice compared with NCD (+86%, P < .01) (Figure 5C and Supplemental Figure 5D). Moreover, PLIN5 expression paralleled the increase of CGI58 protein during the time course of high-fat feeding (Supplemental Figure 7). In a larger cohort of mice, we found a very strong positive relationship between PLIN5 expression and total body weight (Figure 5D), as well as fat mass (r = 0.81, P < .001). Skeletal muscle lipase and PLIN protein expression responded similarly in B6D2 mice fed the same HFD for a longer period of 14 weeks (data not shown). These changes in lipolytic proteins were associated with a trend for increased TAGH activity (+53%) (NCD, 1.64 ± 0.24 nmol/h⁻¹·mg⁻¹ vs HFD, 2.51 ± 0.41 nmol/h⁻¹·mg⁻¹, P = .09).

**Increased DAG and disrupted insulin signaling in skeletal muscle of HSL knockout mice**

To examine the link between reduced HSL activity in skeletal muscle and insulin resistance, we also studied HSL null mice on NCD (Figure 6A). We performed ex vivo studies in whole-soleus muscle and showed a higher incorporation rate of palmitate into DAG (+93%, P < .05) (Figure 6B), whereas the rate of incorporation of palmitate into TAG was not significantly affected (Figure 6C). This was accompanied by a reduced insulin activation of Akt on its Ser473 residue (−19%, P < .05) (Figure 6D), whereas basal Akt phosphorylation remained unaffected (data not shown). Thus, HSL deficiency in skeletal muscle promotes DAG accumulation and impairs insulin signaling.

**Discussion**

It is now well established that insulin resistance is related to accumulation of DAG and ceramides in skeletal muscle (6, 29). However, the mechanisms underlying their accumulation during the development of obesity are still discussed. In this study, we show that short-term HFD impairs skeletal muscle lipases and PLIN proteins and concomitantly promotes DAG accumulation, PKC membrane translocation, and insulin resistance in skeletal muscle. These findings are consistent with recent in vitro data, where we demonstrated a causal relationship between altered lipase expression, DAG accumulation, and insulin resistance in human primary myotubes (7).

In the present study, we used C3H mice, which represent an interesting model to study the effect of short-term high-fat feeding on skeletal muscle lipotoxicity and insulin resistance (17). A robust and fast body weight gain is observed in C3H mice within 4 weeks of HFD. Under the same diet, 8 weeks are required for C57Bl/6 mice to reach the same level of weight gain. We observed a strong deterioration of whole-body insulin sensitivity, glucose tolerance, and skeletal muscle glucose uptake in C3H mice...
within 4 weeks of HFD. Importantly, skeletal muscle insulin signaling was impaired in HFD-fed mice as reflected by an increased Ser1101-IRS1 phosphorylation and a decrease of Tyr612-IRS1 phosphorylation despite a significant increase of IRS1 protein. The higher expression of IRS1 protein could be explained by relatively higher fasting plasma insulin in HFD-fed mice. Insulin was shown to acutely induce IRS1 expression in mouse and human skeletal muscle to compensate for serine phosphorylation-induced IRS1 proteosomal degradation (30). As expected, downstream IRS1 signaling was strongly impaired as evidenced by reduced insulin-stimulated Ser473-Akt and Thr642-AS160 phosphorylation. We also showed impaired muscle AMPK activation, which could, together with reduced Akt activity, contribute to reduced AS160 activation, which is a downstream target of both Akt and AMPK signaling (31). These alterations of insulin signaling are consistent with other reports and confirm that skeletal muscle is a primary site of glucose disposal and insulin action (32).

Increased Ser1101 phosphorylation of IRS1 has been shown to inhibit IRS1 function and could be a specific target of PKC (28). We thus show a higher translocation of both PKCα and PKCe at the plasma membrane of skeletal muscle in HFD-fed mice. DAGs are involved in activation of conventional PKC (α, βI, βII, γ) and novel PKC (δ, ε, η, θ), and DAG accumulation is known to be strongly associated with obesity and insulin resistance in humans (9, 24, 33) and mice (34). Interestingly, we noted that in parallel to insulin resistance, HFD-fed mice had elevated levels of DAG in skeletal muscle as previously described (35). HFD-induced impairments in skeletal muscle IRS1 function and insulin signaling were also independent of changes in c-Jun N-terminal kinase-1 activation in agreement with recent studies (36, 37). Our data are consistent with the view that HFD induces muscle insulin resistance through DAG-mediated novel PKC activation (9, 38).

Figure 5. HFD-induced modulation of skeletal muscle PLINs. (A–C) PLIN protein expression in mice fed with NCD or HFD for 4 weeks. (A) PLIN2 protein expression. (B) PLIN3 protein expression. (C) PLIN5 protein expression (n = 4–5). Insets show a representative blot of 2 NCD- and 2 HFD-fed mice. **P < .01 vs NCD. (D) Pearson correlation between body weight and PLIN5 protein expression (n = 28). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 6. Elevated DAG and impaired insulin signaling in skeletal muscle of HSL null mice. (A) Representative blots of HSL protein and loading control in 2 wild-type (WT) and 2 HSL-knockout (KO) mice. (B and C) Incorporation of [1-14C]palmitate in neutral lipid pools in soleus of WT and HSL-KO mice. (B) Incorporation rate into the DAG pool. (C) Incorporation rate into the TAG pool. (D) Insulin-stimulated Akt Ser473 phosphorylation in soleus of WT and HSL-KO mice (n = 5–6). *P < .05 vs WT. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Because DAG and TAG availability is highly regulated by lipolytic proteins (12), and that defective expression/activity of skeletal muscle lipases may promote DAG accumulation, we next investigated the influence of HFD on lipolytic proteins. This hypothesis is based on previous work, which demonstrate a strong association between obesity and defective skeletal muscle TAG and DAG hydrolysis (24) and related lipases in humans (7, 39). Although sn-1,2-DAG has been the most studied DAG in mammalian cells, it was shown that sn-1,3-DAG can promote PKCα binding to pure palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine membrane vesicles (40). Thus, both sn-1,3-DAG and sn-1,2-DAG could activate PKC translocation to plasma membrane. In addition, we and others have recently shown that DAG produced from TAGHs, such as ATGL, can enter the pathway of phospholipid synthesis in normal human fibroblasts (41) and primary human myotubes (42). This suggests that ATGL-mediated TAG hydrolysis can provide sn-1,2-DAG capable of activating PKC. Of interest, ATGL protein content tended to be down-regulated, whereas its coactivator CGI58 was significantly up-regulated in skeletal muscle of HFD-fed mice. This inverse relationship has been previously observed in human myotubes (42) and in a human adipocyte cell line (43) and suggests that CGI58 and ATGL are reciprocally regulated in skeletal muscle and possibly in other tissues. Up-regulation of CGI58 despite the decrease of ATGL was sufficient to increase TAGH activity and lipolysis. This result confirms in vivo the central and limiting role of CGI58 in the regulation of skeletal muscle lipolysis (42).

An important finding of this study is that HSL Ser660 phosphorylation was dramatically reduced in the muscle of HFD-fed mice. No change in the phosphorylation level of the inhibitory HSL Ser565 residue was observed. Interestingly, HSL Ser660 phosphorylation is closely related to HSL activity in vitro (44). This serine residue is activated by cAMP-dependent protein kinase A (44). A similar down-regulation of HSL phosphorylation has been observed in adipose tissue during high-fat feeding (45). This may be explained by a reduction of protein kinase A activity through a down-regulation of its α-subunit in the obese state (46). Moreover, a reduced HSL Ser660 phosphorylation and partial resistance to catecholamine-induced lipolysis have been observed in skeletal muscle of obese insulin-resistant subjects (39, 47). Together, these data suggest that obesity and high-fat feeding may be associated with a low HSL activity in skeletal muscle.

We further investigated HSL null mice to understand the mechanistic link between low HSL activity and insulin resistance in skeletal muscle. We could show that HSL deficiency increases the rate of DAG accumulation without significant changes in skeletal muscle TAG dynamics. This finding is in agreement with a previous report (13). The higher DAG accumulation was associated with a lower insulin-stimulated activation of Akt on its Ser473 residue in the muscle of HSL null mice. Our data are in agreement with other reports showing a reduced insulin-mediated glucose transport in skeletal muscle of chow diet-fed HSL null mice (48, 49). It is important to note here that whole-body HSL deficiency may give a more complex phenotype than muscle-specific HSL deficiency. Thus, several phenotypic discrepancies have been observed in HSL knockout mice under different backgrounds and diet (48–50). This is also in line with recent findings showing an increased DAG accumulation and mild insulin resistance in human primary myotubes treated with a selective HSL inhibitor (7). Thus, the observed changes in DAG and insulin signaling were muscle autonomous. Together, these data suggest a causal link between low HSL activity, DAG accumulation, and insulin resistance in skeletal muscle.

The access of lipases to lipid droplets is partly controlled by PLIN proteins. In adipose tissue, PLIN1 is strongly expressed, and its role in lipolysis has been well studied (51, 52). Recent studies highlight the role of another member of the PLIN family, PLIN5, especially in oxidative tissues where PLIN1 is virtually not expressed (53). Wang et al (54, 55) and Granneman et al (56) showed that PLIN5 regulates negatively lipolysis and can link ATGL and CGI58 (54–56). PLIN5 protein expression increases in parallel of body fat mass during the HFD. This may be seen as an adaptive response to buffer excess dietary lipids into lipid droplets. This could be driven by increased FA flux and activation of peroxisome proliferator activated receptor α, since PLIN5 is a well-known target of peroxisome proliferator activated receptor α (16, 59). Futures studies will be necessary to understand the physiological role of PLIN5 in skeletal muscle in vivo and its association with insulin resistance.

One study limitation is that besides DAG, we cannot exclude a role of ceramides in fat-induced insulin resistance. Several studies have shown that ceramides contribute to the development of insulin resistance in palmitate-treated myocytes (6) and in mice fed lard diet and/or during saturated oil infusions (8, 35, 60). However, to the best of our knowledge, a direct effect of ceramide-activated Ser/Thr kinases on IRS1 serine phosphorylation has never been reported. We cannot dismiss either that other mechanisms might contribute to skeletal muscle DAG accumulation in obesity and diabetes, such as reduced DAG kinase activity and/or DAG-acyl-transferase-1 activity (34, 61).
In summary, our data show a pathophysiological link between altered lipase expression, lipotoxicity, and insulin resistance in skeletal muscle in vivo. These findings strengthen the relevance of in vitro results showing a causal association between altered lipase expression and insulin resistance in myotubes. Restoring a proper lipolytic control in skeletal muscle may alleviate obesity-related lipotoxicity and insulin resistance.

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