Role of hormone-sensitive lipase in β-adrenergic remodeling of white adipose tissue

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Mottillo EP, Shen XJ, Granneman JG. Role of hormone-sensitive lipase in β-adrenergic remodeling of white adipose tissue. Am J Physiol Endocrinol Metab 293: E1188–E1197, 2007. First published August 21, 2007; doi:10.1152/ajpendo.00051.2007.— Free fatty acids (FFA) are important extracellular and intracellular signaling molecules and are thought to be involved in β-adrenergic-induced remodeling of adipose tissue, which involves a transient inflammatory response followed by mitochondrial biogenesis and increased oxidative capacity. This work examined the role of hormone-sensitive lipase (HSL), a key enzyme of acylglycerol metabolism, in white adipose tissue (WAT) remodeling using genetic inactivation or pharmacological inhibition. Acute treatment with the β3-adrenergic agonist CL-316,243 (CL) induced expression of inflammatory markers and caused extravasation of myeloid cells in WAT of wild-type (WT) mice. HSL-knockout (KO) mice had elevated inflammatory gene expression in the absence of stimulation, and acute injection of CL did not further recruit myeloid cells, nor did it further elevate inflammatory gene expression. Acute pharmacological inhibition of HSL with BAY 59-9435 (BAY) had no effect on inflammatory gene expression in WAT or in cultured 3T3-L1 adipocytes. However, BAY prevented induction of inflammatory cytokines by β-adrenergic stimulation in WAT in vivo and in cultured 3T3-L1 adipocytes. Chronic CL treatment stimulated mitochondrial biogenesis, expanded oxidative capacity, and increased lipid droplet fragmentation in WT mice, and these effects were significantly impaired in HSL-KO mice. In contrast to HSL-KO mice, mice with defective signaling of Toll-like receptor 4, a putative FFA receptor, showed normal β-adrenergic-induced remodeling of adipose tissue. Overall, results reveal the importance of HSL activity in WAT metabolic plasticity and inflammation.

Transdifferentiation; free fatty acids; Toll-like receptor 4

CHRONIC STIMULATION OF β3-ADRENERGIC RECEPTORS (AR) remodels white adipose tissue and produces a cellular phenotype with expanded mitochondrial mass, upregulated fatty acid oxidation gene expression, and elevated metabolic rate (6, 14). Histological, metabolic, and gene expression analyses of this plasticity indicate that β-adrenergic stimulation engages two interrelated physiological events: transient inflammation, which occurs within hours of drug treatment, followed by chronic expansion of oxidative capacity that takes place over several days (7, 25).

The pathways by which β3-AR activation triggers these processes are not well understood. Perhaps the most salient pathway activated by β3-adrenergic stimulation in adipocytes involves the hydrolysis of stored triglyceride (TG) into free fatty acids (FFA) and glycerol. Of these two products, there is growing appreciation that FFA (reviewed in Refs. 1 and 2) and products of FFA metabolism (3, 16) can serve as intra- and extracellular signaling molecules. We previously hypothesized that the acute and chronic responses to β3 receptor stimulation in WAT are adaptations to the excessive efflux of FFA (25). In this model, the mobilization of FFA in WAT triggers an acute inflammatory response, which wanes over time as fat cells expand mitochondrial mass and upregulate genes involved in FFA oxidation through the action of the peroxisome proliferator-activated receptors (PPARs).

Adipose tissue is the major site of TG storage and catabolism, wherein FFA are released via β-adrenergic-stimulated lipolysis through the function of multiple lipases (reviewed in Ref. 17). Adipose triglyceride lipase (ATGL) (10, 36), also known as desnutrin and patatin-like phospholipase domain containing 2, is a newly discovered lipase that acts specifically on TG and appears to be critical in the initiation of lipolysis. ATGL activity appears to be indirectly regulated by protein kinase A (PKA) activation through the phosphorylation-dependent release of its coactivator α/β hydrolase domain containing protein 5 (Abhd5) from perilipin (8, 35). Hormone-sensitive lipase (HSL) acts on both TG and diacylglycerol (DAG), and phosphorylation of HSL by PKA selectively increases its TG hydrolase activity (reviewed in Ref. 20). Last, monoacylglycerol lipase catalyzes the final breakdown into glycerol and FFA.

To investigate the role of FFA in β3-AR-induced tissue remodeling, we used genetic and pharmacological inactivation of HSL to reduce FFA mobilization during β3-AR activation. HSL can hydrolyze cholesteryl esters and retinyl esters (20), although its main function in adipose tissue is to act as a triglyceride/diglyceride lipase (17, 32). Consistent with its role as a major lipase, genetic deletion of HSL reduces circulating FFA levels and cuts β-adrenergic-stimulated lipolysis in half (11, 29, 34). In addition, deletion of HSL promotes adipocyte hypertrophy but does not result in obesity, possibly due to increased fat cell apoptosis (13, 29, 34). Our results indicate that genetic or selective pharmacological inhibition of HSL prevents upregulation of inflammatory cytokine expression by β-AR activation, supporting a role of mobilized FFA as signaling molecules in adipose tissue. The effects of β3-AR activation on inflammatory signaling, however, were not affected in mice with genetic disruption of Toll-like receptor 4 (Tlr4) signaling, indicating that induction of inflammatory signaling via CL-316,243 (CL) does not require this putative receptor of proinflammatory FFA (31). In addition, the expansion of mitochondrial mass and induction of fatty acid oxidation genes seen in wild-type (WT) mice were significantly impaired in HSL-KO mice, indicating that the acute and chronic responses to β3 receptor stimulation in WAT are adaptations to the excessive efflux of FFA (25). In this model, the mobilization of FFA in WAT triggers an acute inflammatory response, which wanes over time as fat cells expand mitochondrial mass and upregulate genes involved in FFA oxidation through the action of the peroxisome proliferator-activated receptors (PPARs).

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delayed in HSL-knockout (KO) mice. The results indicate that HSL-mediated lipolysis and subsequent downstream FFA signaling are important components of adipose tissue remodeling following acute and chronic β3-AR activation.

METHODS

Animals, surgery, and drug treatment. HSL-KO mice (29) were supplied by Dr. F. Kraemer (Stanford University). HSL+/– mice on a C57Bl/6 background were bred at Wayne State University. Mice were genotyped by PCR using primers HSL-A, 5’AGAGAGACCATCAGGCTTCCACC-3’; HSL-B, 5’CAAGGTGCTCTGCTGTGAGGCTT-3’; and SI-166, 5’AGATTGGAGAAGACATGCGAT-3’; where the WT allele corresponds to a 269-base pair (bp) band and the mutant allele to a 320-bp band. Heterozygous (Het) mice were phenotypically identical to WT mice (34) and displayed similar levels of inflammatory markers to that of WT mice (data not shown). Therefore, Het mice were grouped with WT as controls for analysis where indicated. Mice with inactivated Tlr4 (Thr229–230) and WT congenic controls (BALB/cAnPt) were obtained from Jackson Laboratories. All mice were weaned at 3 wk of age and fed rodent chow (Test Diet, no. 5001) and tested when 8–9 wk old. Both male and female mice were used in experiments. Experimental procedures involving animals were approved by the Wayne State University Animal Investigation Committee.

To examine the effects of β3-AR stimulation on acute inflammatory responses, WT and HSL-KO mice were injected intraperitoneally with 10 nmol of CL and killed 6 h later. To examine the effects of pharmacological inhibition of HSL, WT mice (n = 7–8) were pretreated with 30 mg/kg of the selective HSL inhibitor BAY 59-4355, also known as 4-isopropyl-3-methyl-2-{1-[3-(S)-methylpiperidin-1-yl]-methanoyl}-2H-isoxalo-5-one (BAY), and suspended in 0.5% methylcellulose or methylcellulose alone via oral gavage (5). After 1 h, mice were injected intraperitoneally with 10 nmol of CL or H2O and killed 3 h later, which encompassed the maximal effect of BAY and maximal expression of chemokine ligand 2 (Ccl2; data not shown). Epididymal white adipose tissue (EAT) pads were removed and processed for mRNA analysis as described below. In experiments with Thr229–230 and WT BALB/c mice, acute CL treatment lasted 3 h.

To examine the effects of chronic β3-AR activation, mice were anesthetized with halothane and implanted with osmotic minipumps that delivered vehicle (control) or CL at a rate of 0.75 nmol/h for 3 or 7 days. After treatments, animals were killed and EAT was removed and processed for histological, biochemical, and molecular analyses as detailed below.

Cell culture and in vitro assays. 3T3-L1 cells were differentiated as previously described (28), with >90% of cells becoming adipocytes. At 2 to 3 days postdifferentiation, cells were cultured overnight in DMEM (Invitrogen) without serum. The next morning, medium was replaced with serum-free, phenol red-free, DMEM-F-12 medium (Invitrogen) containing 1% fatty acid-free bovine serum albumin (BSA; Roche Diagnostics), and cells were pretreated with 2 µM BAY dissolved in DMSO or DMSO alone for 1 h. After pretreatment, cells were stimulated with 10 µM isoproterenol or H2O (control) for 2 h. Medium was subsequently collected for FFA analysis, and RNA extraction was performed using Trizol reagent (Invitrogen) or an RNasy mini kit (Qiagen). FFA released into medium were quantified using a NEFA C kit (Wako Chemicals USA) and read on a Versamax tunable microplate reader (Molecular Devices).

Isolation of adipocytes from mouse adipose tissue was carried out as described (30). Briefly, EAT was removed and finely minced with scissors. The tissue was then digested with 2 mg/ml collagenase (306 U/mg; Worthington Biochemical) in Krebs-Ringer buffer (Sigma) supplemented with 10 mM HEPES buffer (HKRB), pH 7.4, and 3% BSA for 45 min at 37°C with vigorous shaking. Adipocytes were separated from stromal cells by centrifugation, and the floating adipocytes were then washed three times with HKRB 3% BSA and resuspended in HKRB 1% BSA as a 10% suspension. Cells were aliquoted in triplicate into a 48-well plate, where they were pretreated with DMSO or 1 µM BAY for 15 min. After pretreatment, cells were stimulated with 2 µM CL or H2O (control) for 1 h. Medium was subsequently assayed for FFA as described above and for glycerol using Glycerol Reagent from Sigma.

Lipase assay. Lipase activity was assayed on recombinant protein extracts, as previously described (22), except that radiolabeled substrate was not used. Briefly, COS-7 cells were transiently transfected with fluorescently tagged HSL, Abhd5, ATGL, or EGFP alone using Lipofectamine and Plus Reagent (Invitrogen). Twenty-four to forty-eight hours later, cells were collected by trypsinization, rinsed with phosphate buffered saline, and sonicated on ice in buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol) plus protease inhibitor (Roche Diagnostics). The cell lysate was centrifuged at 1,000 g to clear unbroken cells and nuclei, and 25–30 µg of protein from each supernatant was combined in 5-m glass tubes and preincubated with 2 µM BAY or DMSO (control) for 30 min at room temperature in a total volume of 100 µl of buffer A. The TG substrate was prepared by emulsifying 330 µM triolein (Sigma) with 45 µM phosphatidylcholine (Sigma)/phosphatidylinositol (Avanti Polar Lipids) (3:1) in 100 mM potassium phosphate buffer, pH 7.0, and 5% fatty acid-free BSA (Roche). One volume of substrate was then added, and the reaction was incubated in a 37°C water bath for 1 h. Released FFA were quantified using a Roche FFA Kit (Roche Diagnostics) and read on a Versamax tunable microplate reader (Molecular Devices).

Histology. Adipose tissue was fixed and embedded in paraffin as detailed previously (25). Mitochondrial electron transport activity of WAT was determined by measuring the reduction of 2,3,5-triphenyltetrazolium chloride in tissue explants (7). Adipocyte mitochondria were stained in whole mount adipose tissue with streptavidin-Alexa 555 and imaged by spinning disc confocal microscopy (25).

mRNA expression. For analysis of mRNA, EWAT was placed in RNAlater (Ambion) and held at −80°C until processed. RNA from EWAT was extracted in Trizol (Invitrogen) and then purified with a RNaseasy mini kit (Qiagen). The expression pattern of various genes was quantified by qRT-PCR analysis, as previously described (25). Briefly, RNA (0.5–1.0 µg) was reverse transcribed into cDNA by using Superscript II (Invitrogen) and oligo(dT) primers. Twenty to fifty nanograms of cDNA was subjected to qPCR analysis, using SYBR Green as the fluorophore, and expression levels were normalized to that of peptideylprolyl isomerase A mRNA. PPARα cDNA was amplified using forward and reverse primers 5′-CTAAACCTTGGGC-CACACCT-3′ and 5′-CGGTAAACCTGAGTCTGA-3′, respectively, and matrix metalloproteinase 12 (MMP12) cDNA were quantified with 5′-GAGCTCATGGACCTGCTTA-3′ and 5′-ACGGG ACTGCTATT-3′. All other qPCR primers were previously described (25).

Tissue fractionation and immunoblotting. EWAT was homogenized in HEPES-EDTA-sucrose (HES; 10 mM HEPES, pH 7.8, 250 mM sucrose, 1 mM EDTA) and centrifuged at 500 g for 10 min to create a postnuclear supernatant. The postnuclear supernatant was then centrifuged at 18,000 g for 25 min to create a crude mitochondrial pellet and crude cytosol. The mitochondrial pellet was rinsed with HES and centrifuged again at 18,000 g. The pellet was then resuspended in HES, vortexed, and sonicated briefly on ice, and protein quantification was performed using the bicinchoninic acid method (Pierce).

Antibodies used were prohibitin (Abcam, ab2996, diluted 1:1,000) and nuclear receptor binding factor-1 (NRF1; diluted 1:1,000). SDS-PAGE was performed under standard conditions using 10% minigels (Invitrogen). Resolved proteins were transferred to polyvinylidene difluoride, and membranes were blocked for 1 h at room temperature in 5% powdered skim milk, probed with respective
primary antibodies at room temperature for 1 h, incubated with a secondary goat anti-rabbit horseradish peroxidase (diluted 1:3,000; Cell Signal Technologies), and quantified using a Bio-Rad Quantity One imaging system.

**Statistical analysis.** Data were evaluated by one- or two-way ANOVA. Post hoc comparisons were performed using the Bonferroni t-test. Two-tailed t-tests were performed where indicated.

**RESULTS**

HSL-KO mice are refractory to the acute induction of inflammation by CL. Infusion of CL triggers a transient inflammatory response in WAT that involves elevated expression of chemotactic chemokines and the recruitment and extravasation of myeloid cells (7). In the present work, we found that a single injection of CL was sufficient to provoke the expression of proinflammatory cytokines Ccl2 and Ccl9 in WT and Het mice (Fig. 1). CL treatment slightly elevated the expression of the macrophage marker EMR1 (also known as F4/80) over this time frame but did not increase low levels of the macrophage-specific MMP12. HSL-KO mice had significantly elevated expression of all proinflammatory markers in the unstimulated state, including pronounced elevation of the macrophage markers EMR1, as previously reported (4, 12), and MMP12. Acute CL treatment did not trigger a further increase in the expression of these markers.

Histological examination showed pronounced differences in the appearance of WT and KO WAT. Adipose tissue of WT mice is a mixture of adipocytes and stromal cells that contain very few macrophages and monocytes in the basal state (Fig. 2A). In contrast, HSL-KO mice exhibited numerous “crown structures” (4) in unstimulated WAT (Fig. 2C). These structures contain activated macrophages, as evidenced by intense F4/80 staining (4, 12), and have been hypothesized to be localized sites of adipocyte necrosis and clearance (4). Injection of CL triggered pronounced myeloid cell recruitment and extravasation within EWAT of WT mice and heterozygous mice (Fig. 2B). CL had no additional effect on HSL-KO mice, nor did mice at any time demonstrate myeloid cell recruitment and extravasation patterns seen in WT/Het mice (Fig. 2D).

Pharmacological inhibition of HSL prevents induction of inflammatory cytokines in vivo and in vitro. The above results suggest that myeloid cell recruitment by CL requires HSL. Nonetheless, the results are potentially complicated by the persistent upregulation of inflammatory markers associated with activated macrophages in WAT of HSL-KO mice. To address this issue we examined the effects of BAY, a potent and selective pharmacological inhibitor of HSL (5, 21, 26), on CL-induced inflammation.

BAY has previously been shown to be inactive on several lipases, including ATGL (21, 26), and has been reported to have no effect on the residual lipase activity seen in HSL-KO mice (21). However, these data are not entirely unequivocal since they did not test ATGL that was activated by its coactivator Abhd5 (22), nor did they examine HSL-KO mice under conditions where ATGL activity could be observed (21). Therefore, we examined the effects of BAY on FFA and glycerol efflux from adipocytes of WT and HSL-KO mice as well as the effects on recombinant ATGL and HSL.

![Fig. 1. Effect of acute CL-316,243 (CL) on mRNA levels of inflammatory markers. Effect of acute CL on epididymal white adipose tissue (EWAT) mRNA levels of chemokine ligand 2 (Ccl2), chemokine ligand 9 (Ccl9), EMR1 (also known as F4/80), and matrix metalloproteinase 12 (MMP12) of wild-type (WT)/heterozygote (Het) and hormone-sensitive lipase-knockout (HSL-KO) mice (n = 8–11). One-way ANOVA indicates a significant drug effect (*P < 0.05) and a significant genotype effect (#P < 0.05; ##P < 0.01; ###P < 0.001). Two-tailed t-test shows a significant difference between HSL-KO control (Ctl) and CL treatment vs. WT/Het Ctl for Ccl2. Two-tailed t-test (&) indicates a significant drug treatment effect for EMR1 in WT/Het animals. PPIA, peptidylprolyl isomerase A.](https://www.ajpendo.org/316243)
In WT adipocytes, BAY pretreatment reduced CL-induced FFA efflux by 55% (Fig. 3A) and virtually eliminated glycerol release (Fig. 3B). As expected, deletion of HSL reduced, but did not eliminate, CL-stimulated FFA efflux (Fig. 3A) while virtually abolishing induced glycerol release (Fig. 3B). Importantly, BAY had no effect on residual FFA or glycerol efflux in HSL-KO animals, demonstrating the molecular specificity of BAY. To test whether BAY affects ATGL activity stimulated by its coactivator Abhd5 (22), we looked at the effects of BAY on recombinant HSL, ATGL alone, and Abhd5-ATGL in an in vitro assay. As expected, BAY eliminated HSL-dependent lipase activity (Fig. 3C). ATGL exhibited little lipase activity on its own, whereas incubation with Abhd5 increased its activity to ~50% of HSL activity. BAY had no significant effect on ATGL alone or in the presence of Abhd5.

With the specificity of BAY clearly demonstrated, we treated mice with BAY to study the role of HSL in the acute response to CL. Acute pharmacological inhibition of HSL via BAY had no effect on inflammatory cytokine Ccl2 expression in unstimulated WAT (Fig. 4A). Induction of proinflammatory Ccl2 by CL, however, was nearly completely prevented by chemical inhibition of HSL. Along with data from KO mice, these data strongly indicate that CL induction of WAT inflammation requires HSL activity.

We next examined whether β3-AR activation was sufficient to upregulate inflammatory gene expression in cultured adipocytes and whether this effect required HSL activity. Figure 4C shows that, as expected, isoproterenol strongly elicited mobilization of FFA from 3T3-L1 adipocytes, and this effect was sharply attenuated by inhibition of HSL with BAY. Selective β3-AR stimulation of 3T3-L1 adipocytes significantly elevated expression of Ccl2, and this effect was completely abolished by pretreatment with BAY (Fig. 4B). Acute pharmacological inhibition of HSL had no effect on Ccl2 expression in unstimulated adipocytes.

Induction of proinflammatory signaling by CL does not require Tlr4 signaling. The above data indicate that HSL-dependent mobilization of FFA acutely upregulates proinflammatory cytokine expression in white adipocytes. The nature of the mechanism(s) that detects mobilized FFA is not known; however, it has been suggested that the Tlr4 is an FFA receptor that is capable of triggering proinflammatory responses like those induced by CL (23, 24, 31). To examine the role of Tlr4, we challenged WT mice and congenic mice with disrupted Trl4 signaling with CL. As shown in Fig. 5, Tlr4-defective mice had extremely low levels of proinflammatory markers in the unstimulated state. Nonetheless, CL induced these markers to an extent that equaled or exceeded that seen in WT mice.

Induction of mitochondrial oxidative capacity by CL is significantly delayed in HSL-KO mice. Chronic stimulation of β3-AR triggers mitochondrial biogenesis, induces expression of genes involved in fatty acid oxidation, and elevates tissue metabolic rate (25). Previous work demonstrated that the nuclear receptor PPARα is essential for this process, and we hypothesized that FFA might be important endogenous agonists of PPARα during β3-AR simulation. In WT/Het mice, infusion of CL for 3 days reduced fat cell size and strongly fragmented the central lipid droplet of adipocytes such that most cells acquired a multilocular appearance with enhanced eosin staining (indicative of mitochondria) between the fragmented droplets (Fig. 6). In contrast, HSL-KO mice showed virtually no signs of adipocyte remodeling after 3 days of CL infusion, and cells retained their unilocular appearance. After 7 days of CL treatment, EWAT remodeling was further pronounced in WT/Het mice, with greater eosin staining and an increased degree and number of multilocular fat cells. By 7 days of CL, WAT of HSL-KO mice exhibited signs of lipid droplet fragmentation and elevated eosin staining.

We next examined the importance of HSL on the regulation of genes involved in mitochondrial biogenesis and oxidative capacity (Fig. 7). Expression of PPARα was similar between
genotypes under control conditions. After 3 days of CL infusion, PPARα mRNA levels were upregulated fourfold in WT/Het mice but were unchanged in HSL-KO mice. However, by 7 days of CL, expression of these genes in KO mice was similar to controls.

Immunoblot analysis confirmed that, by 7 days of CL, the mitochondrial markers prohibitin and NRBF1 were similarly upregulated in both genotypes (Fig. 8, A and B), as was the ability to reduce triphenyltetrazolium, a marker of mitochondrial electron transport activity. We also examined the appearance and distribution of mitochondria in fixed whole mount fat pads by fluorescence confocal microscopy, using streptavidin-Alexa 555 to selectively label mitochondria. Virtually all

Both genes were expressed at similar levels under control conditions and were sharply upregulated at 3 days in control mice but not in KO mice. However, by 7 days of CL, expression of these genes in KO mice was similar to controls.

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Fig. 3. BAY 59-9435 (BAY) specificity for HSL. Isolated WT and HSL-KO adipocytes were pretreated with BAY or DMSO and stimulated with CL. Free fatty acid (FFA; A) and glycerol (B) levels were quantified and expressed initially as nmol/h from 3 independent experiments and finally expressed as %basal for each genotype. C: effect of BAY on lipase activity of HSL and adipose triglyceride lipase (ATGL). Cell extracts from transfected COS-7 cells were preincubated with or without BAY, incubated for 1 h with substrate, and assayed for released FFA. Data were initially expressed as nmol of FFA/h from 4 individual experiments and expressed as a %HSL/enhanced green fluorescent protein (EGFP) (4.89 ± 0.64 nmol/h).

Fig. 4. Effect of in vivo and in vitro BAY treatment on β-AR-induced Ccl2 mRNA levels. A: C57BL/6 mice (n = 7–8) were pretreated with BAY or methylcellulose (MC) for 1 h, followed by a treatment of CL (10 nmol) or water (H2O) for 3 h. Two-way ANOVA reveals a significant CL effect (P < 0.005), significant BAY effect (P < 0.001), and an interaction effect (P < 0.001). B: 3T3-L1 adipocytes were pretreated with BAY or DMSO control for 1 h, followed by a treatment of 10 μM isoproterenol (Iso) for 2 h. RNA samples were collected and mRNA for Ccl2 quantified by RT-qPCR. Data are expressed as %control and are representative of 3 separate experiments. C: media samples from B were collected, and released FFA were quantified using a NEFA C kit.
mitochondrial staining was associated with mature adipocytes, with little or no staining observed in stromal cells (Fig. 8D). Under control conditions, adipocyte mitochondrial staining was sparse, with heaviest concentrations immediately surrounding the nucleus. After 7 days of CL treatment, nearly all WT cells assumed a multilocular morphology, with intense streptavidin staining surrounding the fragmented lipid droplets. Adipocytes of KO mice also exhibited pronounced elevation of mitochondrial staining; however, because of the relative lack of lipid droplet fragmentation, the expanded mitochondrial staining was concentrated in the thin cytoplasmic space between the core lipid droplet and the plasma membrane. Interestingly, adipose tissue of CL-treated HSL-KO mice remained inflamed, as indicated by the macrophage marker EMR1, despite expanded oxidative capacity (Fig. 8E).

DISCUSSION

Chronic stimulation of β3-AR remodels white adipose tissue and produces a cellular phenotype with expanded mitochondrial mass, upregulated fatty acid oxidation gene expression, and elevated metabolic rate (6, 14). Analyses of this metabolic plasticity indicate that β3-AR stimulation engages two interrelated physiological events: transient inflammation, which occurs at the onset of drug treatment, followed by expansion of oxidative capacity that takes place over several days (7, 25). We hypothesized that tissue remodeling is an adaptive response to the excessive efflux of fatty acids provoked by β3-AR stimulation (9, 14, 25). In this model, excessive FFA induce adipose tissue inflammation, which is countered over time by the expanded capacity of the fat cells to oxidize lipid. Aside from the reciprocal temporal relationship between inflammation and oxidative capacity, the model is supported by the fact that PPARα knockout mice, which are incapable of expanding mitochondrial oxidative capacity during β3-AR stimulation, exhibit sustained inflammation during CL treatment (25). In this model, mobilized FFA function as signaling molecules that trigger inflammation and activate PPARα, which control expansion of mitochondrial oxidative capacity.

Recent work (8, 35) indicates that PKA-dependent lipolysis in fat cells involves the complementary activities of ATGL and HSL. Hormone-sensitive lipase is a major triglyceride/diglyceride lipase in adipose tissue, and its inhibition substantially reduces PKA-stimulated mobilization of FFA. Although fat cell FFA production is not entirely dependent on HSL, genetic
PKA activation of HSL leads to increased hydrolytic production of FFA, and FFA are known to be proinflammatory mediators in several cell types (1, 2). HSL can hydrolyze cholesteryl esters as well (20); however, the magnitude to which this occurs in fat cells in response to PKA activation is not clear, nor is it clear whether the possible products (free cholesterol and retinol) are inflammatory mediators. Similarly, it is unlikely that ATGL-mediated production of DAG is involved in the acute effects of CL since BAY reduced inflammation rather than increasing it, as would be expected if DAG were the proinflammatory mediator.

We were surprised to find that inflammatory gene markers were persistently elevated in adipose tissue of HSL-null mice, a result that was reported while this work was in progress (4, 12). Interestingly, CL challenge did not increase expression of inflammatory markers in knockout mice, nor did it lead to extravasation of immune cell sites observed in wild-type mice, suggesting distinct mechanisms for the inflammation. These observations indicate that HSL is required to increase inflammatory gene expression after acute CL challenge and are consistent with the hypothesis that CL-induced inflammation requires local generation of FFA. Surprisingly, the persistent inflammation observed in EWAT of the HSL-KO animal did not abate under chronic CL administration as it does in wild-type mice (7, 25). These results indicate that reduction in cell size and elevation of FFA oxidation are not sufficient to normalize inflammation in the HSL-KO mice.

The mechanism of persistent WAT inflammation in HSL-KO mice is currently not understood and could be due to any of several signaling events. HSL-KO mice have clear foci of activated macrophages that are likely to be a major source of inflammatory gene expression (4). It is possible that macrophage recruitment in HSL-null mice involves the chronic loss of anti-inflammatory products like retinoic acid (12) or the accumulation of proinflammatory substrates like DAG. Regardless, the chronic inflammation in HSL-KO mice differs in several respects from that triggered acutely by CL, and these almost certainly involve distinct signaling pathways.

The discordance between genetic and pharmacological inhibition of HSL with regard to levels of inflammatory cytokines is likely related to acute vs. chronic/developmental inhibition. Pharmacological inhibition with BAY occurred over a short period, whereas the KO the animal is defective for HSL throughout development. Although promising as an acute anti-inflammatory therapeutic, additional experiments are needed to assess the long-term effects of BAY.

It is uncertain how mobilized FFAs are detected so as to trigger an inflammatory response. Tlr4 has been proposed (31) to be a receptor that links excessive FFA to innate inflammatory responses. We found that basal levels of inflammatory markers were extremely low in mice with deficient Tlr4 signaling, consistent with a role of Tlr4 inflammatory signaling in adipose tissue. Nonetheless, the induction of inflammatory markers by CL was fully intact in Tlr4-defective mice, indicating that Tlr4 is not required to link mobilized FFA to CL-induced inflammatory responses. Our results do not exclude the existence of another FFA sensor/receptor but suggest that molecules linked to FFA metabolism, such as ceramide (15) or DAG, lead to the activation of other pathways, such as atypical protein kinase C isoforms (16).
The induction of mitochondrial biogenesis and expression of fatty acid oxidation genes by CL were significantly delayed in HSL-KO mice. We (25) previously reported that PPARα is required for the expansion of oxidative capacity in white adipose tissue during CL treatment. FFA are potent activating ligands for PPARα (18, 19), and FFA themselves can induce PPARα expression (33). It seems likely that reduced FFA release in HSL-KO animals contributes to the delay in adaptive metabolic remodeling. Nonetheless, HSL-KO mice still mobilize FFA, presumably through the action of other lipases, such as ATGL (10, 36), and this likely explains the residual metabolic remodeling that is apparent by 7 days of treatment in the animals. It has been recently shown that chronic administration of all-trans retinoic acid induces a brown adipose tissue-like phenotype in white adipose tissue (27). Presently, it is unclear whether CL treatment affects local generation of retinoic acid, which may have pro-oxidative effects. Regardless, CL-mediated production of retinoic acid would not explain the remodeling that occurs in HSL-KO mice after 7 days of CL treatment, since these mice would be expected to have reduced retinoic acid levels due the absence of retinyl esterase activity.

In summary, the present data indicate that HSL plays an important role in adipose tissue plasticity during acute and chronic activation of β3-AR. These data suggest that HSL-dependent generation of FFA initiates the proinflammatory effects of acute β3-AR activation and contributes to the adaptive upregulation of mitochondrial oxidative capacity during sustained stimulation. Nonetheless, the observation that acute and chronic inhibition of HSL can have pronounced, yet opposite, effects on inflammation suggests that the balance of HSL products and substrates is critical to the inflammatory status of fat tissue. Adipose tissue inflammation is thought to

**Fig. 8.** Induction of mitochondrial biogenesis in EWAT of WT and HSL-KO mice. Quantification of Western blot analysis for prohibitin (A) and nuclear receptor binding factor-1 (NRBF1; B) protein levels in EWAT extracts from 4 WT and KO mice. Blots were probed with respective antibodies, quantified via densitometry, and normalized per mg of tissue. CL significantly increases protein levels of both prohibitin and NRBF1 in WT and KO mice (**p < 0.01; ***p < 0.001). C: quantification of 2,3,5-triphenyltetrazolium chloride (TTC) reductase activity in EWAT minces of 5–6 WT/Het and HSL-KO mice following 7 days (7D) of control or CL treatment. CL significantly increased TTC reduction (**p < 0.05) in both WT/Het and HSL-KO mice. D: EWAT of control and CL-treated (CL-7d) mice stained with streptavidin-Alexa 555 and imaged by spinning disc confocal microscopy. CL treatment increased streptavidin staining in both genotypes, although only WT mice show extensive lipid droplet fragmentation. E: effect of chronic CL on mRNA expression of EMR1 in WT/Het and HSL-KO mice. mRNAs were determined by qRT-PCR in 5–7 individual mice. Two-way ANOVA demonstrates a significant genotype effect (**p < 0.001).
affect systemic insulin sensitivity, and it will be an important goal of future work to identify the signaling mechanisms involved during β3-AR-induced inflammation.

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