Fig. 1

A

Body Weight (g)

WT M14

* 0 10 20 30 40

HFD

B

Fat Mass (g)

WT M14

* 0 5 10 15

HFD

C

Lean Mass (g)

WT M14

* 0 5 10 15 20 25 30

HFD

D

Basal Glucose (mM)

WT M14

* 0 2 4 6 8 10 12 14

HFD

E

Basal Insulin (pM)

WT M14

* 0 50 100 150 200

HFD

Fig. 1
Fig. 5

**White Adipose Tissue**

(A) Glucose Uptake (nmol/g/min)

(B) Glucose Uptake (nmol/g/min)

**Brown Adipose Tissue**

Fig. 5
Fig. 7

- **A**: Body Weight (g)
- **B**: Whole Body Fat Mass (g)
- **C**: Basal Glucose (nM)
- **D**: Food Intake (kcal/day)
- **E**: Physical Activity (counts/day)
- **F**: Energy Expenditure (kcal/kg/hr)

**VO₂ Consumption Rate (ml/kg/hr)**
Altered Interleukin-10 Signaling in Skeletal Muscle Regulates Obesity-Mediated Inflammation and Insulin Resistance

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Running Head: IL-10 Regulates Skeletal Muscle Glucose Metabolism

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ABSTRACT

Skeletal muscle insulin resistance is a major characteristic of obesity and type 2 diabetes. Although obesity-mediated inflammation is causally associated with insulin resistance, the underlying mechanism is unclear. Here, we examined the effects of chronic obesity in mice with muscle-specific overexpression of interleukin-10 (M<sup>IL10</sup>). After 16 weeks of high-fat diet (HFD), M<sup>IL10</sup> mice became markedly obese but showed improved insulin action as compared to wild-type mice, which was largely due to increased glucose metabolism and reduced inflammation in skeletal muscle. Since leptin regulates inflammation, the beneficial effects of interleukin-10 (IL-10) were further examined in leptin-deficient ob/ob mice. Muscle-specific overexpression of IL-10 in ob/ob mice (MCK-IL10<sup>ob/ob</sup>) did not affect spontaneous obesity, but MCK-IL10<sup>ob/ob</sup> mice showed increased glucose turnover as compared to ob/ob mice. Lastly, mice with muscle-specific ablation of IL-10 receptor (M-IL10R<sup>−/−</sup>) were generated to determine whether IL-10 signaling in skeletal muscle is involved in IL-10 effects on glucose metabolism. After HFD, M-IL10R<sup>−/−</sup> mice developed insulin resistance with reduced glucose metabolism as compared to wild-type mice. Overall, these results demonstrate IL-10 effects to attenuate obesity-mediated inflammation and improve insulin sensitivity in skeletal muscle, and our findings implicate a potential therapeutic role of anti-inflammatory cytokine in treating insulin resistance and type 2 diabetes.
INTRODUCTION

Obesity has emerged as a global issue in recent decades and is associated with numerous human diseases including insulin resistance, type 2 diabetes, and cardiovascular diseases (1, 2). The underlying mechanism by which obesity induces numerous health problems remains poorly understood. In that regard, increasing evidence suggests an important role of dysregulated immune system in obesity-mediated insulin resistance (3, 4). Obesity is characterized by altered levels of circulating cytokines, and adipose tissue macrophage and inflammation have been causally associated with insulin resistance (5, 6). However, recent studies have challenged this earlier notion on the causal role of adipose tissue macrophage and inflammation in the development of insulin resistance (7, 8).

While adipose tissue is widely viewed as the epicenter of obesity-mediated inflammation, it is not the only organ shown to develop macrophage infiltration and inflammation in obesity. In fact, recent studies indicate that obesity-mediated inflammation and macrophage accumulation develop in multiple organs including skeletal muscle, liver, pancreas, heart, and brain (9-13). In that regard, our recent study found that exercise-mediated weight loss improved insulin action without affecting adipose tissue inflammation in diet-induced obese mice (7). Additionally, improved insulin action following weight loss was associated with reduced local inflammation in skeletal muscle, suggesting an important role of muscle inflammation in obesity-mediated insulin resistance (7). These findings clearly contest the “adipocentric” view of insulin resistance.

Interleukin (IL)-10 is a Th2-type cytokine that inhibits the synthesis and activity of pro-inflammatory cytokines and counteracts Toll-like receptor-mediated inflammation (14-16). We have previously shown that transgenic overexpression of IL-10 selectively in skeletal muscle improved glucose metabolism in mice after 3 weeks of high-fat diet (HFD) (9). While our
previous data suggest a potential therapeutic role of IL-10 in type 2 diabetes, our interpretation is limited due to a short-term feeding of HFD with the mice not having developed type 2 diabetes phenotypes (i.e., hyperglycemia). Therefore, the current study was designed to specifically examine the role of IL-10 in markedly obese and diabetic mice after 16 weeks of HFD, a better representation of obese type 2 diabetic human subjects. Additionally, leptin is an important adipocyte-derived hormone that is elevated in obesity and regulates numerous physiological functions including energy balance and inflammation (17). Thus, the present study also examined the effects of muscle-specific transgenic expression of IL-10 on glucose metabolism in leptin-deficient ob/ob mice. Lastly, we examined whether IL-10 signaling in skeletal muscle is directly responsible for IL-10 effects on muscle glucose metabolism using a newly generated mouse model lacking IL-10 receptor 1 type chain selectively in skeletal muscle. Our findings indicate that selective targeting of IL-10 signaling in skeletal muscle improves glucose metabolism in obese and diabetic mice following chronic HFD or deficient in leptin and further demonstrate that these effects are mediated by direct activation of IL-10 signaling in skeletal muscle.
MATERIALS AND METHODS

Chronic HFD in M\textsuperscript{IL10} mice  Male transgenic mice with muscle-specific overexpression of IL-10 (M\textsubscript{IL10}) and wild-type (WT) littermates were fed with HFD (55% fat by calories; Harlan Teklad TD93075, Madison, WI) or standard chow diet (Labdiet Prolab Isopro RMH 3000 5P75, St. Louis, MO) ad libitum for 16 weeks (n=6/group). During chronic high-fat feeding, we performed a weekly measurement of body composition to determine the changes in whole body fat and lean mass.

Generation of MCK-IL10\textsuperscript{ob/ob} mice  We have generated leptin-deficient mice with muscle-specific overexpression of IL-10 (MCK-IL10\textsuperscript{ob/ob}) by cross-breeding M\textsuperscript{IL10} mice with \textit{ob/}+ heterozygous mice (purchased from the Jackson Laboratory, Bar Harbor, ME). The F1 female MCK-IL10\textsuperscript{ob/}+ mice were then intercrossed with male \textit{ob/}+ mice to generate MCK-IL10\textsuperscript{ob/ob} mice. The metabolic studies were conducted in MCK-IL10\textsuperscript{ob/ob} mice that have been backcrossed for more than 5 generations (\textit{ob/ob}, n=7; MCK-IL10\textsuperscript{ob/ob}, n=12).

Generation of M-IL10R\textsuperscript{−/−} mice  Mice lacking IL-10 signaling in skeletal muscle (M-IL10R\textsuperscript{−/−}) were generated by cross-breeding MCK-Cre expressing mice (kindly donated by Dr. Roger J. Davis) and floxed IL10R1\textsuperscript{−/−} mice (kindly donated by Dr. Werner Muller). The metabolic studies were conducted in M-IL10R\textsuperscript{−/−} mice that have been backcrossed for several generations. M-IL10R\textsuperscript{−/−} mice and MCK-Cre mice (referred to as WT) serving as controls were fed with HFD or chow diet for 6 weeks (n=6-11/group). All mice were housed under controlled temperature (23°C) and light/dark cycle with free access to food and water. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.
**Body composition and energy balance**  Whole body fat and lean mass were non-invasively measured using $^1$H-MRS (Echo Medical Systems, Houston, TX). Indirect calorimetry and energy balance parameters including food/water intake, energy expenditure, respiratory exchange ratio, and physical activity were non-invasively assessed for 3 days using metabolic cages (TSE-Systems Inc., Bad Homburg, Germany). We used the TSE-Systems LabMaster platform with easy-to-use calorimetry featuring fully-automated monitoring for food and water and XYZ activity. Labmaster cages that are most similar to facility home cages were used, thereby allowing the use of bedding in the cage and minimizing any animal anxiety during the experimental period. The system provides intuitive software with flexibility for experimental setup and data utilization.

**Hyperinsulinemic-euglycemic clamp**  Following chow or HFD, a survival surgery was performed at 5–6 days before clamp experiments to establish an indwelling catheter in jugular vein. On the day of clamp experiment, mice were fasted overnight (~17 hrs), and a 2-hr hyperinsulinemic-euglycemic clamp was conducted in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 2.5 mU/kg/min; Humulin, Eli Lilly, IN) (18). To maintain euglycemia, 20% glucose was infused at variable rates during clamps. Whole body glucose turnover was assessed with a continuous infusion of [3-$^3$H]glucose (PerkinElmer, Waltham, MA), and 2-deoxy-D-[1-$^{13}$C]glucose (2-$^{13}$C)DG (PerkinElmer, Waltham, MA) was administered as a bolus (10 µCi) at 75 min after the start of clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis.

**Biochemical analysis and calculation**  Glucose concentrations during clamps were analyzed using 10 µl plasma by a glucose oxidase method on Analox GM9 Analyser (Analox...
Instruments Ltd., London, UK). Plasma concentrations of [3-^3H]glucose, 2-[^14C]DG, and ^3H2O were determined following deproteinization of plasma samples as previously described (18). For the determination of tissue 2-[^14C]DG-6-Phosphate content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-[^14C]DG-6-P from 2-[^14C]DG. Plasma insulin levels were measured using an ELISA kit (ALPCO Diagnostics, Salem, NH). Intramuscular triglyceride concentrations were determined by homogenizing muscle samples (quadriceps) in chloroform-methanol and using a triglyceride assay kit (Sigma, St. Louis, MO). Plasma non-esterified fatty acids levels were measured by NEFA kit (Zenbio, Durham, NC) according to manufacturer’s protocol.

Rates of basal hepatic glucose production (HGP) and insulin-stimulated whole body glucose turnover were determined as previously described (18). Insulin-stimulated rate of HGP was determined by subtracting the glucose infusion rate from whole body glucose turnover. Whole body glycolysis and glycogen plus lipid synthesis from glucose were calculated as previously described (18). Insulin-stimulated glucose uptake in individual tissues was assessed by determining the tissue (e.g., skeletal muscle) content of 2-[^14C]DG-6-phosphate and plasma 2-[^14C]DG profile.

**Molecular analysis for insulin signaling and inflammation** Skeletal muscle (quadriceps) and liver samples were collected at the end of clamp experiments to assess insulin signaling by immunoblotting with rabbit monoclonal antibodies against Akt and p-Akt-Ser^473 (Cell signaling, Danvers, MA). Muscle samples were homogenized, and plasma and homogenized muscle samples were used to measure levels of interleukin (IL)-6, IL-10, interferon (IFN)-γ, and IL-1α using enzyme-linked immunosorbent assay (ELISA) with Luminex 200 Multiplex (Millipore,
Darmstadt, Germany). Plasma IL-10 levels were measured using an ELISA kit (Abcam, Cambridge, UK).

For quantitative real-time (RT)-PCR, RNA isolation was performed with homogenized muscle (gastrocnemius and quadriceps), liver and white adipose tissue samples using Trizol (Life Sciences, Carlsbad, CA) according to manufacturer’s protocols. cDNA was synthesized from 2 μg of total RNA by use of Omniscript cDNA synthesis kit (Qiagen, Venlo, Netherlands). cDNA and cyber green supermix (Bio-rad, Hercules, CA) was run in Biorad-CFX96 real time system with the primers (Supp. Table 1). Relative gene expression was calculated compared to housekeeping gene.

**Histologic analysis**  Skeletal muscle samples (gastrocnemius) were collected, fixed in 10% neutral formalin for 48 hours, and embedded in paraffin blocks. Sections (5 μm) were taken and stained with hematoxylin-eosin. Images were taken under 20X magnification.

**Statistical analysis**  Data are expressed as mean ± SE values. The significance of difference in mean values was determined using two-way ANOVA with Newman-Keuls and Games-Howell tests for post hoc analysis and Student’s t test where applicable. The statistical significance was at the *P*<0.05 level.
RESULTS

Chronic feeding of HFD in M^{IL10} mice  Starting at 6–7 weeks of age, male M^{IL10} and WT
mice were fed with chow diet or HFD ad libitum for 16 weeks. At the end of feeding respective
diet, all mice were age-matched at the time of metabolic studies. M^{IL10} and WT mice showed
similar body weights on chow diet, and after 16 weeks of HFD, both groups of mice became
obese with a comparable increase in body weights (Fig. 1A). Consistent with this, whole body fat
mass, measured using ^1H-MRS, was not different between chow-fed M^{IL10} and WT mice, and fat
mass increased by 3–4-fold after HFD in both groups of mice (Fig. 1B). Although whole body
lean mass was statistically different between the groups on chow diet (25.1±0.3 in WT mice vs.
23.5±0.6 in M^{IL10} mice; \( P=0.03 \)), this difference of 1.5 g of lean mass is within the range of
variability for lean mass of C57BL/6 mice. Whole body lean mass after HFD did not differ
between groups (Fig. 1C). Metabolic cage analysis showed no significant difference in daily
food intake, VO_{2} consumption, and physical activity between M^{IL10} and WT mice after 16 weeks
of HFD (Supp. Fig. 1).

M^{IL10} mice are protected from HFD-induced insulin resistance  Basal glucose levels
increased after 16 weeks of HFD in both groups of mice (i.e., hyperglycemia), and plasma
insulin levels were also elevated by more than 6-fold in both groups of mice after HFD (Fig. 1D
& E). To determine the effects on whole body glucose metabolism, a 2-hr hyperinsulinemic-
euglycemic clamp was conducted in awake mice. During clamp, plasma glucose levels were
maintained at euglycemia (~7 mM), and plasma insulin levels were raised to ~130 pM and ~230
pM in both groups of chow and HFD-fed mice, respectively (Fig. 2A & B).

After 16 weeks of HFD, the WT mice developed insulin resistance as shown by ~60%
decrease in glucose infusion rates during clamp as compared to chow-fed WT mice (Fig. 2C).
Although glucose infusion rates also decreased in the M^{IL,10} mice after HFD, the glucose infusion rates in the HFD-fed M^{IL,10} mice were significantly higher as compared to HFD-fed WT mice (Fig. 2C). Radioactive isotope labeling data during the clamp showed a markedly increased whole body glucose turnover in the HFD-fed M^{IL,10} mice as compared to the HFD-fed WT mice (Fig. 2D), indicating that M^{IL,10} mice were more insulin sensitive than WT mice after 16 weeks of HFD.

Increased insulin sensitivity in HFD-fed M^{IL,10} mice was largely due to a 30% increase in glucose uptake in skeletal muscle (quadriceps) (Fig. 2E). Insulin-stimulated glucose uptake in gastrocnemius muscle also increased in HFD-fed M^{IL,10} mice as compared to HFD-fed WT mice, but this difference did not reach a statistical significance (Fig. 2F). To further examine muscle insulin action, we performed Western blots using skeletal muscle to assess insulin signaling and found that Ser-473 phosphorylation of Akt did not differ between WT and M^{IL,10} mice on chow diet, consistent with comparable muscle glucose uptake in these mice (Supp. Fig. 2A). After 16 weeks of HFD, muscle Akt phosphorylation was decreased by ~70% in WT mice (P=0.1), and muscle Akt phosphorylation tended to be higher in HFD-fed M^{IL,10} mice as compared to HFD-fed WT mice (Fig. 3A). Hematoxylin-eosin stained sections of skeletal muscle from WT and M^{IL,10} mice showed no obvious anomaly in the overall structure (Fig. 3B). Additionally, intramuscular triglyceride levels tended to be elevated in chow-fed M^{IL,10} mice as compared to chow-fed WT mice (Fig. 3C). After HFD, intramuscular triglyceride levels were comparable between M^{IL,10} and WT mice. The IL-10 mRNA levels in skeletal muscle were approximately 2-fold higher in HFD-fed M^{IL,10} mice as compared to HFD-fed WT mice (Fig. 3D).

**Obesity-mediated inflammation in skeletal muscle is attenuated in M^{IL,10} mice** We have previously shown that inflammation develops in skeletal muscle after 3 weeks of HFD, and that
this local inflammation may be causally associated with insulin resistance (9). To that end, we
assessed inflammation profile in skeletal muscle by performing quantitative RT-PCR using
samples obtained from HFD-fed M\textsuperscript{IL10} and WT mice. Skeletal muscle mRNA levels of F4/80 and
CD68, as markers of macrophage infiltration, were decreased significantly in HFD-fed M\textsuperscript{IL10}
mice as compared to HFD-fed WT mice (Fig. 4A & B). Muscle MCP-1 mRNA levels also
decreased in HFD-fed M\textsuperscript{IL10} mice (Fig. 4C). Using Luminex analysis, we found that local levels
of IFN-\(\gamma\), IL-1\(\alpha\) and IL-6 were increased by 2- to 4-fold in muscle samples from WT mice after
16 weeks of HFD (Fig. 4D-F). In contrast, muscle samples from HFD-fed M\textsuperscript{IL10} mice showed
completely normal levels of IFN-\(\gamma\), IL-1\(\alpha\) and IL-6 indicating that muscle IL-10 overexpression
protected against diet-induced inflammation in skeletal muscle (Fig. 4D-F). Plasma levels of IL-
10 and IL-6 did not significantly differ between WT and M\textsuperscript{IL10} mice on chow or HFD (Supp. Fig.
2B & C). Plasma IL-1\(\alpha\) levels tended to increase by more than 2-fold in WT mice after 16 weeks
of HFD, but they were not affected by HFD in M\textsuperscript{IL10} mice. (Supp. Fig. 2D).

**Obesity-mediated inflammation and insulin resistance in adipose tissue and liver** Insulin-
stimulated glucose uptake in white and brown adipose tissues did not differ between chow-fed
WT and M\textsuperscript{IL10} mice (Fig. 5A & B). After 16 wks of HFD, white and brown adipose tissue
developed in both groups of mice (Fig. 5A & B). Macrophage markers, F4/80 and CD68 mRNA
levels, also increased in white adipose tissue by chronic HFD but were not different between
HFD-fed WT and M\textsuperscript{IL10} mice (Fig. 5C & D).

Basal HGP did not differ between WT and M\textsuperscript{IL10} mice on chow or HFD (Fig. 6A). During
the clamp, insulin decreased HGP in both chow-fed WT and M\textsuperscript{IL10} mice (Fig. 6B). HFD-fed WT
mice developed insulin resistance in liver as indicated by increased clamp HGP, but HFD-fed
M\textsuperscript{IL10} mice showed lower clamp HGP as compared to HFD-fed WT mice (Fig. 6B). Western blot
analysis showed that neither phospho-Akt nor total Akt protein levels were altered between WT
and M(IL10) mice on chow or HFD (Fig. 6C). However, liver mRNA levels of glucose-6-
phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) tended to be lower in
HFD-fed M(IL10) mice as compared to HFD-fed WT mice (Fig. 6D & E). Macrophage markers,
CD68 and F4/80 mRNA levels in liver were not significantly altered in HFD-fed M(IL10) mice,
indicating that local inflammation was selectively suppressed in skeletal muscle (Supp. Fig. 2E
& F).

**IL-10 expressing leptin-deficient mice are protected from insulin resistance** Recent
studies have shown deleterious effects of HFD on intestinal epithelium and the role of gut
microbes on obesity-mediated inflammation (19). Thus, we have generated muscle-selective IL-
10 expressing, spontaneously obese mice by cross-breeding M(IL10) mice with heterozygous leptin-
deficient ob/+ mice. The offspring from this cross were further mated to obtain MCK-IL10(ob/ob)
mice. As expected, ob/ob mice became obese spontaneously on chow diet, and by 16 weeks of
age, whole body fat mass accounted for more than 50% of body weight in ob/ob mice (Fig. 7A &
B). MCK-IL10(ob/ob) mice also became spontaneously obese on chow diet and reached a
comparable degree of obesity as ob/ob mice at 16 weeks of age (Fig. 7A & B). Basal plasma
glucose and fatty acids levels were similar in both groups of mice (Fig. 7C & Supp. Fig. 3A). A
3-day metabolic cage analysis showed that daily food intake and physical activity did not differ
between groups (Fig. 7D & E). In contrast, MCK-IL10(ob/ob) mice showed a modest but significant
increase in energy expenditure that was largely due to elevated VO2 consumption selectively at
night cycle as compared to ob/ob mice (Fig. 7F & G).

Hyperinsulinemic-euglycemic clamp study showed a significantly increased whole body
glucose turnover in MCK-IL10(ob/ob) mice as compared to ob/ob mice, suggesting increased
insulin sensitivity in these mice (Fig. 8A). Consistent with this notion, insulin-stimulated glucose uptake in skeletal muscle tended to increase in MCK-IL10<sup>ob/ob</sup> mice as compared to <i>ob/ob</i> mice although this difference did not reach a statistical significance (Fig. 8B). Glucose uptake in white adipose tissue and hepatic insulin action did not differ between groups (Fig. 8C & D).

**Mice lacking muscle IL-10 signaling are insulin resistant after HFD** To determine the cell type responsible for the downstream effects of IL-10, we have generated mice with muscle-specific ablation of IL-10 receptor (M-IL10R<sup>−/−</sup>) (Supp. Fig. 3B & C). Male M-IL10R<sup>−/−</sup> mice and MCK-Cre mice (as controls; WT) were fed HFD or chow diet ad libitum starting at 7 weeks of age. Metabolic studies were performed after 6 weeks of HFD, prior to the onset of overt hyperglycemia that may affect glucose metabolism (i.e., glucose toxicity). Both groups of M-IL10R<sup>−/−</sup> and WT mice gained similar fat mass after 6 weeks of HFD (Fig. 9A). Basal glucose levels were higher after HFD in M-IL10R<sup>−/−</sup> mice as compared to WT mice (Fig. 9B). During hyperinsulinemic-euglycemic clamp, plasma glucose levels were maintained at euglycemia (~7 mM) in both groups of mice (data not shown). Strikingly, whole body glucose turnover was significantly decreased, and glucose infusion rates were lower in HFD-fed M-IL10R<sup>−/−</sup> as compared to HFD-fed WT mice (Fig. 9C & D). Hepatic insulin action during clamp did not differ between WT and M-IL10R<sup>−/−</sup> mice fed chow or HFD (Fig. 9E). Quadriceps and gastrocnemius muscle glucose uptake tended to be lower in M-IL10R<sup>−/−</sup> mice as compared to WT mice after HFD (Supp. Fig. 3D).

Furthermore, local IL-10 levels in skeletal muscle tended to decrease in M-IL10R<sup>−/−</sup> mice as a possible feedback loop of IL-10 signaling (Supp. Fig. 3E). Muscle mRNA levels of F4/80 (macrophage marker) and TNF-α were markedly increased in WT mice after HFD (Fig. 9F & G). Importantly, HFD-fed M-IL10R<sup>−/−</sup> mice showed a significant further increase in muscle
mRNA levels of F4/80, TNF-α, IL-6, and IL-1β as compared to HFD-fed WT mice, indicating that muscle deletion of IL-10 receptor exacerbated HFD-induced local inflammation in skeletal muscle (Fig. 9F-I).
DISCUSSION

Although adipose tissue macrophage and inflammation are well-described characteristics of obesity-mediated insulin resistance, recent studies refute the cause-and-effect relationship between adipose tissue inflammation and insulin resistance (5-8). Lee et al. have shown that insulin resistance develops in the absence of adipose tissue inflammation after 3 days of high-fat feeding (8). Our recent study also found that a short-term weight loss induced by low-caloric diet or exercise improves insulin sensitivity without altering adipose tissue inflammation (7). These findings indicate that adipose tissue inflammation develops in obesity, but it may not be causally associated with insulin resistance in skeletal muscle, a major organ responsible for glucose disposal.

Obesity is a state of systemic inflammation, and local inflammation develops in multiple organs including skeletal muscle and liver (9, 10). Importantly, recent studies have suggested a direct and causal role of skeletal muscle inflammation and muscle-derived cytokines in the development of insulin resistance (20-25). Our previous study has found that after 3 weeks of high-fat feeding, skeletal muscle was characterized by inflammation, insulin resistance and reduced glucose metabolism (9). Thus, these findings are consistent with a notion that HFD-mediated local inflammation in skeletal muscle is causally associated with insulin resistance. However, the metabolic and inflammatory process developing in metabolic organs after a short-term high-fat feeding may differ from the events following chronic obesity. Additionally, chronic obesity models (e.g., long-term HFD) that are better reflecting type 2 diabetes conditions potentially involve increased insult from immune cells and inflammatory signaling in skeletal muscle, adipose tissue and kidney as well as more compromised lipid metabolism and inflammation in liver (8, 26, 27). Therefore, our current study addresses the effects of anti-
inflammatory cytokine IL-10 in chronically obese conditions induced by a 16-week feeding of HFD. Moreover, leptin is known to regulate inflammation and immunity with its effects of T cell and macrophage-secreted cytokines (17, 28, 29). Thus, leptin-deficient model used in the current study rules out the potential leptin effects on inflammation. Our newly generated MCK-IL10\(\text{ob/ob}\) mice also circumvent direct effects of excess dietary lipid uptake and lipid-induced inflammation/insulin resistance because those mice develop spontaneous obesity on chow diet.

As a model of diet-induced obesity and type 2 diabetes, we examined the effects of long-term HFD on glucose metabolism and local inflammation in M\(\text{IL10}\) mice. After 16 weeks of HFD, WT mice became markedly obese with a 4-fold increase in whole body fat mass. Intramuscular levels of IL-6, IFN-\(\gamma\), and IL-1\(\alpha\) were low in chow-fed mice as expected, but after 16 weeks of HFD, local cytokine levels in skeletal muscle were elevated by 3 to 4-fold in WT mice. Some of these inflammatory cytokines may be released by locally infiltrating macrophages and/or surrounding adipocytes in skeletal muscle fiber. IL-6 was previously shown to induce insulin resistance by activating STAT3 and increasing intracellular levels of SOCS3, which may target insulin signaling proteins for ubiquitin-mediated degradation, causing insulin resistance (30, 31). In contrast, IL-6 has also been shown to be released by post-exercise skeletal muscle and to promote glucose metabolism, but this cellular effect involves a much higher level of IL-6 than what is typically observed in obesity (32). Furthermore, IL-1\(\alpha\) was shown to inhibit insulin signaling by inducing serine phosphorylation of IRS-1 in adipocytes (33). Consistent with this notion, insulin-stimulated Akt phosphorylation was reduced in the skeletal muscle of HFD-fed WT mice, supporting the cause-and-effect relationship between obesity-mediated increase in local macrophage and inflammatory cytokines and skeletal muscle insulin resistance.
Despite marked obesity after 16 weeks of HFD, M^{IL10} mice were significantly more insulin sensitive than WT mice, which was largely due to increased insulin signaling and glucose metabolism in skeletal muscle. Intramuscular lipid content was also similar between WT and M^{IL10} mice after HFD, indicating that IL-10 mediated suppression of muscle inflammation can improve insulin sensitivity without altering intramuscular lipid content in obese mice. IL-10 is an anti-inflammatory cytokine previously known as “cytokine synthesis inhibiting factor (CSIF)” and produced by many immune cell types including CD4-T helper cells and macrophages (34, 35). Similar to other cytokines, such as IL-6 and tumor necrosis factor (TNF)-α, myocytes have also been shown to express IL-10 and IL-10 receptors (36, 9). IL-10 suppresses local inflammation by inhibiting synthesis and action of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, as well as inhibiting macrophage activation (37, 38). To that end, intramuscular injection of IL-10 DNA was shown to be effective in suppressing inflammation and preventing autoimmune diabetes in mice (39).

Our findings that obesity-mediated increase in IL-6, IFN-γ, and IL-1α in skeletal muscle was normalized in M^{IL10} mice support the anti-inflammatory action of IL-10 in these mice. Also HFD-fed M^{IL10} mice had decreased levels of macrophage markers, F4/80 and CD68, as well as reduced MCP-1 mRNA levels. Importantly, these data indicate that improved muscle glucose metabolism may be due to IL-10 effects to rescue from local inflammation in response to chronic high-fat feeding in mice. Additionally, the inflammation was suppressed only in skeletal muscle of HFD-fed M^{IL10} mice; there was no difference in liver and adipose tissue inflammation in these mice. Plasma IL-10 levels were also not different in HFD-fed M^{IL10} mice. Furthermore, contrary to skeletal muscle, white and brown adipose tissues and liver remained insulin resistant after HFD in M^{IL10} mice, supporting the muscle-specific expression of IL-10 and its effects on muscle
glucose metabolism. However, we found that HGP during clamp was significantly reduced in M^{IL\,10} mice as compared to WT mice after 16 weeks of HFD, suggesting improved hepatic insulin action in HFD-fed M^{IL\,10} mice. Since IL-10 has been shown to be released by myocytes and its level to modestly increase in circulation (9), IL-10 may also be responsible for suppressing gluconeogenesis in these mice. In that regard, we did not see alterations in liver Akt phosphorylation in HFD-fed M^{IL\,10} mice, but gluconeogenic gene expression tended to decrease in M^{IL\,10} mice, consistent with reduced HGP in these mice.

Recent studies have shown that HFD with selective composition of fatty acids may be directly responsible for systemic inflammation possibly by altering intestinal permeability or gut microbe population (19). We and others have also suggested that obesity-mediated inflammation is due to excess nutrient availability, imbalanced nutrient flux and metabolism, and activation of intracellular endoplasmic reticulum and oxidative stress (3, 12, 30). In order to delineate the important question pertaining to the source of obesity-mediated inflammation, we determined the effects of IL-10 in spontaneously obese mice by cross-breeding M^{IL\,10} mice with leptin-deficient ob/ob mice. As expected, IL-10 expressing ob/ob mice (MCK-IL10^{ob/ob}) became profoundly obese while on a chow diet with fat mass accounting for more than 50% of their body weight. Despite being markedly obese and having the same level of plasma FFA, MCK-IL10^{ob/ob} mice were more insulin sensitive than ob/ob mice, further demonstrating IL-10’s potent insulin-sensitizing effects associated with its anti-inflammatory effect in skeletal muscle. Thus, these data indicate that obesity-mediated inflammation and insulin resistance in skeletal muscle are not dependent on dietary composition. Our findings also suggest that IL-10’s insulin sensitizing effects are independent of leptin signaling.
The beneficial effects of IL-10 to protect against obesity-mediated insulin resistance may be due to IL-10’s ability to suppress local inflammation and the deleterious action of pro-inflammatory cytokines in skeletal muscle. Gao et al. have recently shown that hydrodynamic delivery of mouse IL10 protects against HFD-mediated glucose intolerance that was associated with reduced inflammation in adipose tissue (40). However, since IL-10 receptors are expressed in multiple cell types including immune cells and myocytes (9, 41), IL-10’s direct action on myocytes cannot be ruled out. To further determine the mechanism of IL-10’s insulin-sensitizing effects, we generated mice with muscle-specific ablation of IL-10 receptor. Since deletion was specific to IL-10 receptor type 1, we excluded only IL-10 signaling and not all IL-10 cytokine subfamily members. After 6 weeks of HFD, M-IL10R−/− mice became more insulin resistant than WT mice, indicating that absence of IL-10 signaling in skeletal muscle exacerbates diet-induced insulin resistance. Also, mRNA levels of macrophage markers and inflammatory cytokines were profoundly elevated in HFD-fed M-IL10R−/− mice. These results suggest that IL-10 effects may be mediated by intracellular IL-10 signaling in skeletal muscle. To that end, we have previously found that obesity-induced signal to activate local inflammation may involve oxidative stress and altered Ca2+ homeostasis in skeletal muscle (42). Muscle IL-10 levels also tended to be lower in M-IL10R−/− mice that might be result of feedback mechanism. Thus, IL-10 signaling in myocytes may relieve oxidative stress and suppress local inflammation, resulting in improved glucose metabolism in skeletal muscle. Further studies are needed to understand how IL-10 may affect oxidative stress in skeletal muscle.

In conclusion, our findings demonstrate that obesity due to chronic high-fat feeding or leptin deficiency causes local inflammation with marked increases in IL-6, IL-1α, and IFN-γ levels in skeletal muscle. Obesity-mediated muscle inflammation is completely attenuated by a
local expression of IL-10, and this anti-inflammatory action of IL-10 protects against muscle
insulin resistance. Additionally, IL-10 signaling in myocytes may relieve obesity-induced
oxidative stress and inflammation in skeletal muscle. Taken together, these results support our
previous notion that IL-10 may be a potential therapeutic target to treat insulin resistance and
type 2 diabetes. Although it has short half-life in vivo, local delivery of IL-10 is safe and may
have positive effects in skeletal muscle insulin sensitivity (35). While eventual clinical
application and method of delivery need further investigation, the role of IL-10 as an anti-
inflammatory and insulin-sensitizing agent may open new doors in type 2 diabetes research.

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DUALITY OF INTEREST

No potential conflicts of interest relevant to this article were reported.
REFERENCES


25
FIGURE LEGENDS:

**Figure 1.** Metabolic profiles of muscle-specific IL-10 overexpressing mice (M^{IL10}) and WT mice on chow and after 16 weeks of HFD. **A:** Body weight. **B:** Whole body fat mass. **C:** Whole body lean mass. **D, E:** Basal plasma glucose and insulin levels following overnight fast (~17 hrs).
Values are means ± SE for 6 mice in each group. *P<0.05.

**Figure 2.** A 2-hour hyperinsulinemic-euglycemic clamp was performed in awake WT and M^{IL10} mice after 16 weeks of HFD to assess insulin sensitivity. **A, B:** Plasma glucose and insulin levels during clamp. **C:** Steady-state glucose infusion rates during clamps. **D:** Whole body glucose turnover. **E, F:** Insulin-stimulated glucose uptake in skeletal muscle (quadriceps and gastrocnemius). Values are means ± SE for 6 mice in each group. *P<0.05.

**Figure 3.** Insulin signaling, muscle morphology, triglyceride and IL-10 levels in skeletal muscle. **A:** Insulin stimulated Akt phosphorylation in quadriceps muscle. **B:** Hematoxylin-eosin staining of gastrocnemius muscles of M^{IL10} and WT mice fed chow or HFD. **C:** Intramuscular triglyceride content in quadriceps muscles of M^{IL10} and WT mice fed chow or HFD (n= 3-5/group). **D:** IL-10 mRNA levels in gastrocnemius muscles of HFD-fed M^{IL10} and WT mice measured by qRT-PCR (n= 4-5/group). Values are means ± SE for each group. *P<0.05.

**Figure 4.** Local inflammation in skeletal muscles of M^{IL10} and WT mice on chow or after 16 weeks of HFD. **A, B, C:** F4/80, CD68, and IL-6 mRNA levels in quadriceps muscles of HFD fed M^{IL10} and WT mice measured by qRT-PCR (n=3/group). **D, E, F:** IFN-γ, IL-6, and IL-1α levels in gastrocnemius muscles of HFD and chow-fed M^{IL10} and WT mice measured by multiplex ELISA (n= 4-6/group). Values are means ± SE for each group. *P<0.05.
Figure 5. Insulin-stimulated glucose uptake and inflammation in adipose tissues. A, B: Insulin-stimulated glucose uptake in white (epidymidal) and brown (intrascapular) adipose tissue in M^{IL,10} and WT mice fed chow or HFD (n=4-7/group). C, D: F4/80 and CD68 mRNA levels in white adipose tissues of M^{IL,10} and WT mice fed chow or HFD (n=5-6/group). Values are means ± SE for each group. *P<0.05.

Figure 6. Hepatic glucose metabolism and insulin signaling in M^{IL,10} and WT mice fed chow or HFD for 16 weeks. A, B: Hepatic glucose production (HGP) at basal state and during hyperinsulinemic-euglycemic clamp in mice (n=5-6/group). C: Insulin-stimulated Akt phosphorylation in liver. D, E: Liver G6Pase and PEPCK mRNA levels (n=4-6/group). Values are means ± SE for each group. *P<0.05.

Figure 7. Metabolic effects of muscle-specific IL-10 overexpression in leptin-deficient ob/ob mice (MCK-IL10^{ob/ob}). A, B, C: Body weight, whole body fat mass using 1H-MRS, and fasting glucose levels were measured from 7 MCK-IL10^{ob/ob} and 11 ob/ob mice. D, E, F, G: Daily food intake, physical activity, energy expenditure, and average of hourly VO_{2} consumption were measured during a 3-day analysis of metabolic cages in 3 MCK-IL10^{ob/ob} and 4 ob/ob mice. Values are means ± SE for each group. *P<0.05.

Figure 8. A 2-hour hyperinsulinemic-euglycemic clamp in awake MCK-IL10^{ob/ob} and ob/ob mice. A: Whole body glucose turnover in MCK-IL10^{ob/ob} (n=7) and ob/ob (n=11) mice. B: Insulin-stimulated glucose uptake in gastrocnemius and quadriceps muscles. C: White adipose tissue glucose uptake in MCK-IL10^{ob/ob} (n=7) and ob/ob (n=12) mice. D: Hepatic insulin action reflected as insulin-mediated percent suppression of HGP in MCK-IL10^{ob/ob} (n=7) and ob/ob mice (n=11). Values are means ± SE for each group. *P<0.05.
Figure 9. Metabolic profile, insulin action, and skeletal muscle inflammation were assessed in M-IL10R−/− mice and WT mice after 6 weeks of HFD or chow. A: Whole body fat mass measured using 1H-MRS. B: Basal glucose levels. C: Glucose infusion rates during hyperinsulinemic-euglycemic clamp in awake mice (n=6-11). D: Whole body glucose turnover. E: Hepatic insulin action expressed as insulin-mediated percent suppression of HGP. F, G, H, I: F4/80, TNF-α, IL-1β, and IL-6 mRNA levels in skeletal muscles of WT and M-IL10R−/− mice fed chow or HFD (n=3-7/group). Values are means ± SE for each group. *P<0.05.