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# Interleukin-6 Depletion Selectively Improves Hepatic Insulin Action in Obesity

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**Obesity and insulin resistance are considered chronic inflammatory states, in part because circulating IL-6 is elevated. Exogenous IL-6 can induce hepatic insulin resistance *in vitro* and *in vivo*. The importance of endogenous IL-6, however, to insulin resistance of obesity is unresolved. To test the hypothesis that IL-6 contributes to the inflammation and insulin resistance of obesity, IL-6 was depleted in Lep$^{ob}$ mice by injection of IL-6-neutralizing antibody. In untreated Lep$^{ob}$ mice, signal transducer and activator of transcription-3 (STAT3) activation was increased compared with that in lean controls, consistent with an inflammatory state. With IL-6 depletion, activation of STAT3 in liver and adipose tissue and expression of haptoglobin were reduced. Expression of the IL-6-dependent, hepatic acute phase protein fibrinogen was also decreased. Using the hyperinsulinemic-euglycemic clamp technique, insulin-dependent suppression of endogenous glucose production was 89% in IL-6-depleted Lep$^{ob}$ mice, in contrast to only 32% in Lep$^{ob}$ controls, indicating a marked increase in hepatic insulin sensitivity. A significant change in glucose uptake in skeletal muscle after IL-6 neutralization was not observed. In a direct comparison of hepatic insulin signaling in Lep$^{ob}$ mice treated with anti-IL-6 *vs.* IgG-treated controls, insulin-dependent insulin receptor autophosphorylation and activation of Akt (pSer$^{473}$) were increased by nearly 50% with IL-6 depletion. In adipose tissue, insulin receptor signaling showed no significant change despite major reductions in STAT3 phosphorylation and haptoglobin expression. In diet-induced obese mice, depletion of IL-6 improved insulin responsiveness in 2-h insulin tolerance tests. In conclusion, these results indicate that IL-6 plays an important and selective role in hepatic insulin resistance of obesity. (*Endocrinology* 146: 3417–3427, 2005)**

OBESITY IS AN increasing health concern that is highly correlated with the risk of developing insulin resistance and type 2 diabetes. Substantial evidence indicates that a low-level inflammatory state accompanies obesity. This is evidenced by elevated levels of inflammatory markers and inflammatory cytokines in these individuals. C-Reactive protein (CRP), plasminogen activator inhibitor-1, and fibrinogen are three such plasma markers (1–4). CRP production is induced in the liver at least in part by IL-6 and may predict the development of type 2 diabetes (2). The release of cytokines by adipose tissue probably contributes to the inflammatory state of obesity. Adipose tissue-derived hormones and cytokines (adipokines) may also mediate insulin resistance in insulin target tissues. It has been recently shown that monocytes infiltrate adipose tissue in proportion to adiposity and can be a source of adipose tissue-derived inflammatory cytokines, particularly TNF$\alpha$ (5). TNF$\alpha$, in turn, may induce the release of additional adipokines by adipocytes (6).

In support of the link among obesity, inflammation, and insulin resistance, IL-6 and TNF$\alpha$, both known to be released from adipose tissue, have been demonstrated to directly cause insulin resistance in model systems (6–11). Nonetheless, a clear cause and effect relationship between inflammatory cytokines and insulin resistance and the development of type 2 diabetes has not yet been completely resolved. TNF$\alpha$ is the most widely studied inflammatory cytokine to be implicated as a mediator of the insulin resistance of obesity. Neutralization of TNF$\alpha$ in obese rodent models with injection of a fusion protein containing a TNF$\alpha$ receptor-binding domain partially corrected insulin resistance in these animals (12). TNF$\alpha$ receptor-deficient mice also have improved insulin sensitivity and resistance to high fat diet-induced insulin resistance (13). Unfortunately, human studies have not confirmed a role for TNF$\alpha$ as a contributor to insulin resistance (14).

IL-6 is produced by adipocytes, monocytes, endothelial cells, and hepatocytes, among others. In humans, roughly 15–35% of circulating IL-6 can be accounted for by adipose tissue secretion (15). Furthermore, circulating IL-6 is strongly associated with obesity and is a predictor of the development of type 2 diabetes (16). IL-6 is an important mediator of the acute phase response in the liver (17) and controls the expression of CRP and fibrinogen. Thus, the correlation between the expression of these acute phase proteins and type 2 diabetes probably follows the dependence of expression on IL-6. Our group has shown that IL-6 is an inhibitor of insulin signaling and insulin action in isolated hepatocytes, hepatoma cell lines, and livers of experimental mice (10, 18). Importantly, IL-6 leads to insulin resistance *in vivo* when chronically administered to mice at levels similar to those found in obese individuals (18). IL-6 may affect insulin action in other insulin target tissues as well. Recent studies have reported IL-6 inhibition of insulin action in muscle (19) and 3T3L-1 adipocytes (6).

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Abbreviations: CRP, C-Reactive protein; CT, cycle threshold; DG, 2-deoxyglucose; DGP, 2-deoxyglucose phosphate; DIO, diet-induced obese; IR, insulin receptor; IRS, insulin receptor substrate-1; MCP-1, monocyte chemoattractant protein-1; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

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In the current study we demonstrate that IL-6 plays an important role in the hepatic insulin resistance of obesity. Two murine models of obesity are used. The leptin-deficient $Lep^{ob}$ mouse in a C57BL/6 background is a well-characterized model of murine obesity and insulin resistance. High fat diet-induced obese (DIO) C57BL/6J mice develop a milder insulin resistance and hyperglycemia. In both models of obesity, injection of an anti-IL-6-neutralizing antibody results in improved insulin sensitivity. Additionally, the improved insulin receptor (IR) signaling is consistent with the receptor being a target for obesity-associated insulin resistance mediated by IL-6.

## Materials and Methods

### *Animals*

Six-week-old male C57BL/6J$^{ob/ob}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained in a one-way isolation room at the University of Rochester. Animals were kept on a 12-h light, 12-h dark cycle and fed standard rodent chow (except where indicated). The university committee on animal resources approved all protocols used on animals. To induce insulin resistance in wild-type C57BL/6J, these mice were placed on a rodent diet containing 45% of calories from lard (Research Diets, New Brunswick, NJ) at 6 wk of age and were studied between 5–7 wk on the diet.

### *In vivo analysis of hepatic and adipose tissue insulin signaling*

Mice were fasted for approximately 16 h. For insulin stimulation, mice were anesthetized using an isofluorane vaporizer (Summit Medical, Salem, OR) and injected ip with 2.5 U/kg Novolin human insulin (Novo Nordisk Pharmaceuticals, Princeton, NJ). After 10 min, animals were anesthetized and killed. Liver and epididymal fat pads were then harvested and frozen in liquid nitrogen. Tissues were homogenized using a lysis buffer containing 100 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, protease inhibitor cocktail (Calbiochem, La Jolla, CA), 1 mM phenylmethylsulfonylfluoride, 10 mM benzamidine, 10 mM tetrasodium pyrophosphate, and 5 mM activated sodium orthovanadate. Homogenates were cleared by centrifugation at 20,000 × *g*, and protein content was quantitated by the Bradford method (20). Immunoprecipitation and immunoblotting were performed as previously described (10).

### *Antibodies and IL-6 neutralization*

IL-6 antibody and control goat IgG were purchased from R&D Systems, Inc. (Minneapolis, MN). pAkt (serine 473), signal transducer and activator of transcription-3 (STAT3), and pSTAT3 (tyrosine705) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Akt1/2 and IR β-chain antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Clone 4G10 phosphotyrosine-specific antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Mice were injected ip with 0.5 mg goat anti-IL-6 dissolved in 0.5 ml PBS. Control mice were similarly injected ip with 0.5 mg goat IgG in 0.5 ml PBS. Studies were conducted between 68–72 h after antibody injection.

### *Hyperinsulinemic-euglycemic clamp and metabolic tracer analysis*

Hyperinsulinemic-euglycemic clamp studies were performed at the Mouse Metabolic Phenotyping Center at Vanderbilt University (Nashville, TN). Six control mice and nine experimental group mice were successfully catheterized via the jugular vein several days before the study. Mice were allowed to recover for 1 wk before treatment with IgG (control) or anti-IL-6-neutralizing antibody (experimental). Studies were conducted in chronically catheterized, conscious mice. On the morning of the study (3 d after anti-IL-6 or IgG), overnight-fasted mice were placed in a rat restrainer to allow for tail vein sampling. A primed continuous infusion of [3-$^3$H]glucose was initiated at −120 min to measure whole-animal glucose turnover. At 0 min, a constant infusion of insulin (20 mU/kg·min) was initiated and continued for the duration of the study. Glucose levels were monitored by sampling from a severed tail vein every 10 min, and a variable glucose infusion was given to maintain isoglycemia. Plasma [$^3$H]glucose specific activity was assessed at 0, 80, 90, 100, and 120 min. The whole body glucose turnover rate was calculated as the ratio of the [$^3$H]glucose infusion rate (disintegrations per minute per kilogram per minute) to arterial plasma glucose specific activity (disintegrations per minute per milligram). Endogenous glucose production was calculated as the difference between the whole body glucose turnover rate and the exogenous glucose infusion rate.

Tissue-specific glucose uptake (or tissue glucose metabolic rate) was calculated as described by Kraegen *et al.* (21). In brief, a bolus of [$^{14}$C]2-deoxyglucose ([$^{14}$C]DG) was given at 120 min to assess tissue-specific glucose uptake. Plasma [$^{14}$C]DG was assessed at 122, 125, 130, 135, and 145 min. The animals were then anesthetized with an iv infusion of sodium pentobarbital, and the tissues (soleus, gastrocnemius, superficial vastus lateralis, liver, heart, brain, inguinal fat, and diaphragm) were removed, immediately frozen in liquid nitrogen, and stored at −70 C until further analysis. Plasma samples were deproteinized with $Ba(OH)_2$ (0.3 N) and $ZnSO_4$ (0.3 N) and dried, and radioactivity was assessed on a scintillation counter (TriCarb 2900 TR, Packard, Meriden, CT). Frozen tissue samples were homogenized in 0.5% perchloric acid, centrifuged, and neutralized. One supernatant was directly counted to determine radioactivity from both [$^{14}$C]DG and [$^{14}$C]DG phosphate ([$^{14}$C]DGP). A second aliquot was treated with $Ba(OH)_2$ and $ZnSO_4$ to remove [$^{14}$C]DG and any tracer incorporated into glycogen. Samples were then counted to determine radioactivity from free [$^{14}$C]DG (22). [$^{14}$C]DGP was calculated as the difference between the two aliquots. The accumulation of [$^{14}$C]DGP was normalized to tissue weight and tracer bolus to calculate tissue glucose uptake (21).

### *Insulin tolerance tests*

Mice were fasted for approximately 16 h. Mice were then briefly anesthetized under isofluorane. Insulin (Novo Nordisk Pharmaceuticals, Princeton, NJ), diluted in vehicle (0.9% NaCl), was injected ip at 1 U/kg. Blood glucose levels were determined by tail vein sampling at the indicated intervals using an Accu-Chek (Roche, Indianapolis, IN) glucose meter and glucose test strips.

### *Northern blot analysis*

Total RNA was isolated from fat or liver using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing 1× 4-morpholinepropanesulfonic acid buffer and 0.66 M formaldehyde. RNA was transferred to a ζ-Probe (Bio-Rad Laboratories, Hercules, CA) membrane overnight and X-linked in a Stratalinker (Stratagene, La Jolla, CA). Hybridization was performed at 68 C using Express-Hyb hybridization buffer (BD Clontech, Palo Alto, CA). Membranes were exposed in a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) and scanned in a Storm 840 PhosphorImager (Molecular Dynamics). Quantitation was performed using ImageQuant software (Molecular Dynamics).

### *Real-time PCR analysis*

One micrograms of total RNA was converted to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories). Thirty nanograms of total cDNA were used for each real-time PCR. TaqMan assays containing proprietary primer sets and labeled probes (Applied Biosystems, Foster City, CA) were used with TaqMan 2× Master Mix (Applied Biosystems) to detect mouse haptoglobin and β-actin gene expression. Real-time PCR was performed on an iCycler IQ multicolor real-time PCR detection system (Bio-Rad Laboratories). The iCycler software determines the cycle threshold (CT) by calculating the second derivative of each trace and establishes the point of maximum curvature. Individual PCRs were performed in triplicate on samples using β-actin (as a housekeeping gene) and haptoglobin assays to obtain average CT values for these genes. Average β-actin CT values were subtracted from haptoglobin CT

values to obtain ΔCT values. ΔΔCT values were then obtained by subtracting experimental sample ΔCT values from the control sample ΔCT. Relative haptoglobin expression was then calculated using $2^{-\Delta\Delta CT}$.

### *Statistical analysis*

Statistical analysis of the data was performed using SPSS 10.0 for Windows (SPSS, Inc., Chicago, IL). Experimental means were compared using an independent-samples *t* test when sample means from two groups were compared (Figs. 1, B and D, 2, and 5). Levene's test was performed to detect unequal variance between groups. One-way ANOVA was applied when means from more than two groups were compared (Figs. 1, A and C, 3, 4, and 6), and Fisher's protected least significant difference test was used for comparisons between pairs of groups. $P < 0.05$ was considered significant to reject the null hypothesis.

## Results

### *IL-6 contributes to a chronic inflammatory state in Lep$^{ob}$ mice*

Obesity is characterized by a low-level chronic inflammation state (23). A central mediator of the acute phase response during infection, particularly in the liver, is the proinflammatory cytokine IL-6. To determine whether this cytokine also has a role in the chronic inflammatory state of obesity, we examined hepatic IL-6 responses in genetically obese Lep$^{ob}$ mice. STAT3 phosphorylation on tyrosine 705 and expression of the acute phase proteins haptoglobin and fibrinogen are known to be under the influence of IL-6. STAT3 phosphorylation was found to be consistently, but modestly, elevated in the liver of Lep$^{ob}$ mice (Fig. 1A). In adipose tissue, however, STAT3 phosphorylation was markedly elevated in obese mice compared with lean controls (Fig. 1C). To determine whether IL-6 played a role in these apparent inflammatory responses in the liver and adipose tissue of obese mice, mice were injected with an IL-6-neutralizing antibody or control IgG. The injection protocol had been shown by others (24, 25) to effectively neutralize most IL-6 activity *in vivo* over a several-day treatment period. Three days after injection of the antibody, reduced hepatic and adipose tissue STAT3 phosphorylation was observed (Fig. 1, A and C, respectively). The results suggested that the IL-6 neutralization protocol was effective and indicated that IL-6 contributed to the elevated STAT3 phosphorylation in both the liver and adipose tissue of Lep$^{ob}$ mice. Haptoglobin and fibrinogen are hepatic acute phase proteins that are primarily under the control of IL-6. Consistent with a role for IL-6 in the expression of these proteins, IL-6 neutralization reduced haptoglobin and fibrinogen expression in the liver of Lep$^{ob}$ mice (Fig. 1B). Additionally, haptoglobin expression in adipose tissue was significantly reduced after IL-6 depletion. Together, these results indicate that IL-6 contributes to inflammatory responses in liver and adipose tissue of Lep$^{ob}$ mice and confirm that the antibody neutralization protocol effectively reduces these IL-6-mediated responses.

### *Whole body and tissue-specific changes in insulin sensitivity after IL-6 neutralization*

Hyperinsulinemic-euglycemic clamp studies were performed to determine the impact of IL-6 neutralization on insulin sensitivity and glucose metabolism in Lep$^{ob}$ mice. After an overnight fast, but before the clamp studies (3 d after anti-IL-6 and IgG injections), glucose levels were comparable in IL-6-depleted Lep$^{ob}$ mice and the IgG-treated control group (data not shown). During the clamping period, plasma insulin concentrations increased to approximately 10 ng/ml. Using variable glucose infusion, plasma glucose was clamped at approximately 180 mg/dl in both groups. The rate of glucose infusion needed to maintain euglycemia reached a constant state within 90 min in both groups and was continued for a total of 145 min (data not shown). During the final 25 min, [$^3$H]glucose and [$^{14}$C]DG were coinfused to measure glucose turnover and tissue-specific glucose uptake, respectively. The glucose infusion rate that maintained euglycemia was modestly increased by 43% in the IL-6-neutralized group (21.5 ± 2.9 for IL-6-depleted mice *vs.* 15.0 ± 2.2 for controls; Fig. 2A), although this difference did not reach statistical significance ($P = 0.09$). The insulin-dependent suppression of endogenous glucose production was the most affected parameter in response to IL-6 depletion, showing a suppression of 89 ± 23% in IL-6-depleted Lep$^{ob}$ mice *vs.* only 32 ± 11% in Lep$^{ob}$ controls, a 2.8-fold difference (Fig. 2B). Endogenous glucose production essentially reflects hepatic production. Although insulin-dependent suppression of endogenous glucose production was significantly improved after the neutralizing antibody treatment, the whole body glucose turnover rate, which is the ratio of the [$^3$H]glucose infusion rate (disintegrations per minute per kilogram per minute) to arterial plasma glucose specific activity (disintegrations per minute per milligram), was not changed (data not shown). These results, therefore, are suggestive of a selective increase in hepatic insulin sensitivity in genetically obese Lep$^{ob}$ mice after IL-6 neutralization.

Consistent with the observation that the whole body glucose turnover rate was not affected by IL-6 depletion, glucose uptake in skeletal muscle was not significantly altered by this manipulation. There was no improvement in glucose uptake in gastrocnemius and vastus lateralis muscle with IL-6 depletion. Similar results were seen in diaphragm and myocardium. The possible exception to this observation was the soleus muscle, where an increase after IL-6 neutralization (0.049 ± 0.012 *vs.* 0.028 ± 0.003; Fig. 2C) was suggested, but did not reach statistical significance ($P = 0.10$).

### *Hepatic insulin signaling is improved after IL-6 neutralization*

IL-6 depletion partially reversed obesity-associated STAT3 phosphorylation in both liver and adipose tissue, suggesting a decrease in the inflammatory effect of this cytokine. This manipulation also improved hepatic insulin sensitivity, as assessed in the hyperinsulinemic-euglycemic clamp studies. Are an increase in insulin sensitivity and a decrease in STAT3 phosphorylation after IL-6 neutralization associated with improved IR signaling in the affected tissues? To address this question, IR signaling was studied in liver and adipose tissue of Lep$^{ob}$ mice after IL-6 neutralization. Mice fasted overnight were injected ip with insulin. After 10 min, IR signaling was assessed in harvested tissues by immunoprecipitation and Western blot analysis. The first studies addressed two questions related to IR autophosphorylation. 1) Would depletion of IL-6 improve hepatic insulin-dependent IR autophosphor-

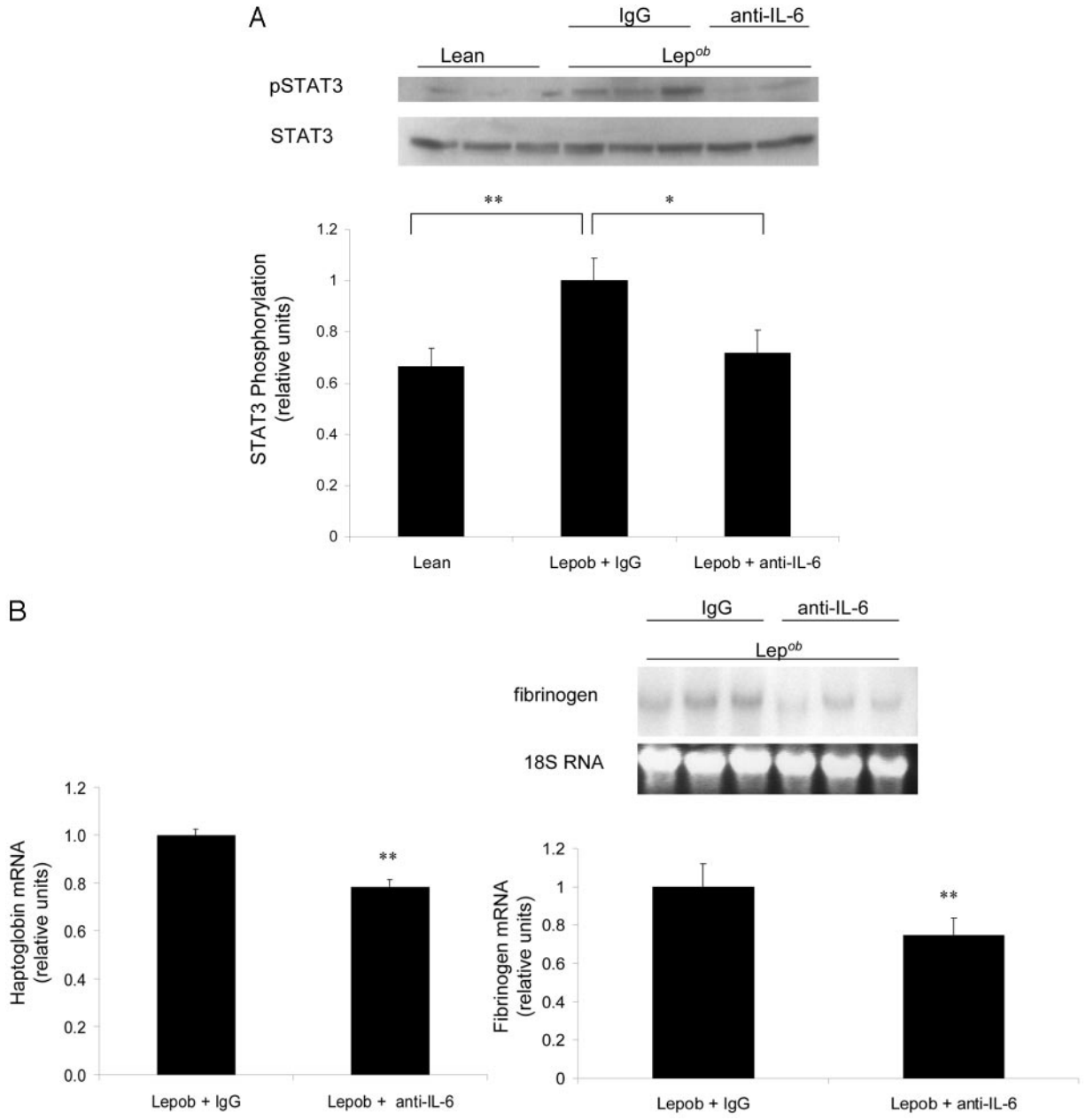


FIG. 1. IL-6-responsive inflammatory markers in Lep$^{ob}$ mice after IL-6 depletion. On d 1, Lep$^{ob}$ mice were injected with IL-6-neutralizing antibody (R&D Systems, Inc.) or IgG control (R&D Systems, Inc.). Lean C57BL/6J wild-type mice received no injection. Mice were fasted overnight before tissue harvesting on d 4. A, Livers from lean and Lep$^{ob}$ mice were homogenized, and samples were analyzed for STAT3 phosphorylation (pY705) and STAT3 mass. A representative Western blot is shown. The *bar graph* represents the mean ± SEM of five experiments, each performed in duplicate or triplicate. B, Relative haptoglobin expression (normalized to β-actin) in liver as a function of IL-6 ablation was measured by real-time PCR. Fibrinogen-γ expression was determined by Northern blot analysis from 25 μg liver RNA. Ethidium bromide staining of 18S ribosomal RNA was a loading control. A representative Northern blot is shown. The *bar graph* represents the mean ± SEM of four experiments, each performed in triplicate. C, Adipose tissue STAT3 phosphorylation (pY705) and STAT3 mass were analyzed in lean and Lep$^{ob}$ mice with or without IL-6 ablation. A representative Western blot is shown. The *bar graph* represents the mean ± SEM of three experiments, each performed in duplicate or triplicate. D, Relative haptoglobin expression (normalized to β-actin) in adipose tissue of Lep$^{ob}$ mice was measured by real-time PCR. The *bar graph* represents the mean ± SEM of three experiments, each performed in triplicate. Data in A–D are normalized to IgG-treated Lep$^{ob}$ mice. *, $P < 0.05$; **, $P < 0.01$.

ylation in Lep$^{ob}$ mice? As shown in Fig. 3A, a direct comparison of responses in Lep$^{ob}$ mice treated with anti-IL-6 *vs.* IgG demonstrated an approximately 50% increase in IR phosphorylation in livers from IL-6-depleted mice (by *t* test, $P < 0.05$). 2) What is the relative contribution of IL-6 to hepatic insulin resistance at the level of IR autophosphorylation in Lep$^{ob}$ mice? In this comparison, which required both obese and lean mice, IL-6 depletion improved IR autophosphorylation in Lep$^{ob}$ mice approximately 35% compared with that in lean controls, but the effect of IL-6 depletion was no longer

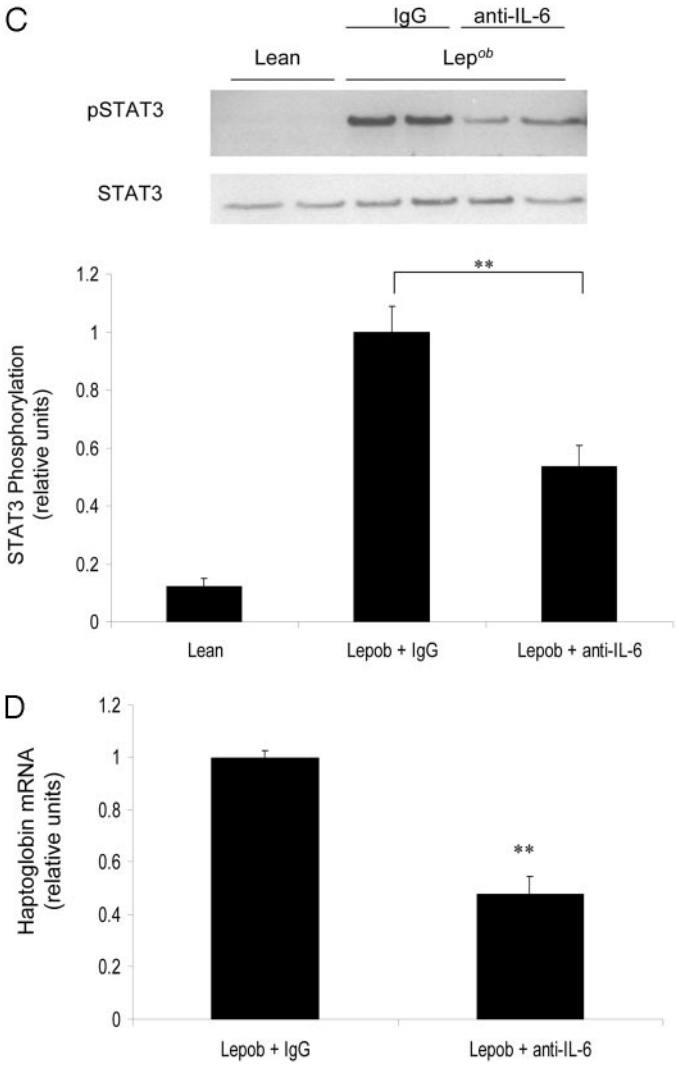


FIG. 1. *Continued*

statistically significant (by ANOVA, $P > 0.05$), due largely to the magnitude of the variance in the lean mouse determinations (Fig. 3A). These data indicate that IL-6 contributes to hepatic insulin resistance at the level of IR autophosphorylation, but other factors are also involved. Basal autophosphorylation in Lep$^{ob}$ mice appeared to be greater than that in lean mice. Although this trend may reflect hyperinsulinemia in Lep$^{ob}$ mice, these differences were not statistically significant and were not influenced by IL-6 depletion.

Insulin-dependent receptor autophosphorylation in adipose tissue of Lep$^{ob}$ mice was markedly suppressed compared with that in lean controls (Fig. 3B). Despite the demonstrated decrease in STAT3 phosphorylation in this tissue with IL-6 depletion, no effect of IL-6 depletion on insulin-dependent IR autophosphorylation in adipose tissue was observed. Basal IR autophosphorylation was very low in all groups, and differences were not statistically significant. These results suggest that additional inhibitory mechanisms predominate in the insulin resistance seen in adipose tissue. The incomplete reversal of insulin resistance in the liver also suggests that factors in addition to IL-6 may contribute to hepatic insulin resistance.

As reported by others, IR substrate-1 (IRS-1) and IRS-2 had very high basal tyrosine phosphorylation in Lep$^{ob}$ mice and showed no response to insulin (26). IL-6 neutralization did not produce a detectable improvement in this defect (data not shown). Akt is a serine kinase that is activated by serine/threonine phosphorylation in response to insulin. It is activated downstream of IRS-1/2 and phosphotidylinositol 3-kinase and plays a critical role in mediating many of the metabolic effects of insulin. Activation of Akt is accompanied by its phosphorylation on serine 473 (pSer$^{473}$Akt). In the livers of Lep$^{ob}$ mice, pSer$^{473}$Akt was similar to that in lean mice in the absence of insulin, indicating that the large pool of tyrosine-phosphorylated IRS-1/2 in the livers of these mice does not represent active signaling molecules. pSer$^{473}$Akt rose markedly in response to insulin and, as expected, was blunted in Lep$^{ob}$ mice compared with lean controls. Interestingly, an insulin-dependent increase in Akt activation was improved approximately 50% in the livers of Lep$^{ob}$ mice after IL-6 neutralization (Fig. 4A). Consistent with IR autophosphorylation data in adipose tissue, no statistically significant effect of IL-6 depletion was observed on pSer$^{473}$Akt in adipose tissue after insulin treatment (Fig. 4B). Improvement in insulin signaling in liver without a significant effect in adipose tissue again indicates that IL-6 plays an important role in the hepatic insulin resistance of obesity and suggests that another factor(s) may be more important to insulin resistance in adipose tissue.

The above data support the hypothesis that abnormal IL-6 production during obesity contributes to insulin resistance. Does IL-6 play a role in insulin action in lean mice? To answer this question, we tested the effect of neutralizing IL-6 antibody on insulin signaling in liver and skeletal muscle of lean mice. Depletion of IL-6 by antibody neutralization in lean mice did not improve insulin-dependent IR autophosphorylation (Fig. 5, A and B) or activation of Akt (Fig. 5, C and D) in either of these insulin-responsive tissues. These results indicate that obesity-associated IL-6 contributes to hepatic insulin resistance, but IL-6 is not an effector of hepatic insulin signaling in lean animals.

### *High-fat diet-induced insulin resistance responds to IL-6 neutralization*

To examine the role of IL-6 in a less severe model of obesity-associated insulin resistance, insulin sensitivity was assessed after IL-6 depletion in C57BL/6J mice fed a high-fat diet for 6 wk. After a 17-h fast, blood glucose levels were determined by tail vein sampling. The DIO mice had fasting blood glucose levels comparable to those in age-matched lean controls (not shown). Additionally, fasting glucose levels were not different between DIO mice receiving ip injections of the IL-6-neutralizing antibody or the IgG control 3 d before these measurements (not shown). Despite comparable fasting glucose levels, a small, but statistically significant, difference was observed between the two obese treatment groups when insulin tolerance tests were performed. IL-6 neutralization significantly improved insulin response in DIO mice (Fig. 6), with a more prolonged response to insulin

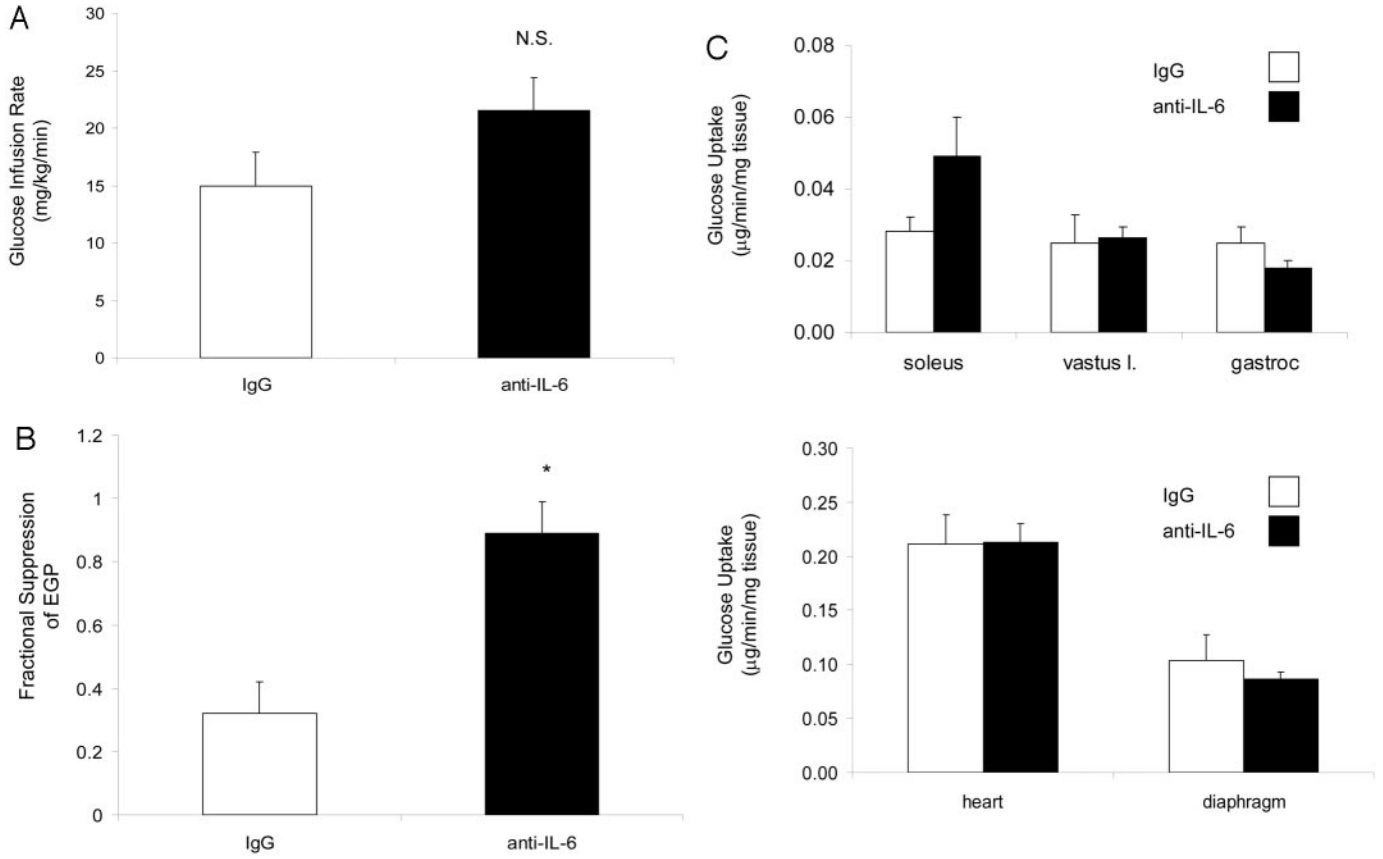


FIG. 2. Effect of IL-6 depletion on whole body and tissue-specific metabolic parameters during a hyperinsulinemic euglycemic clamp in 8-wk-old male Lep$^{ob}$ mice. Mice were injected with either IgG (□) or IL-6-neutralizing antibody (■) 72 h before the hyperinsulinemic-euglycemic clamp. The following parameters were analyzed: A, glucose infusion rate; B, fractional suppression of endogenous glucose production (EGP); and C, glucose uptake in muscle. Vastus l, Vastus lateralis; gastroc, gastrocnemius. Data in A–C are normalized to IgG-treated Lep$^{ob}$ mice. Results are the mean ± SEM. ■, n = 9; □, n = 6. In C, no differences between treatment groups were statistically significant. *, $P < 0.05$; N.S., not statistically significant ($P > 0.05$).

being apparent between 60 and 135 min. These results are consistent with results in Lep$^{ob}$ mice, which indicated that IL-6 depletion improves insulin responsiveness in obese, insulin-resistant mice. Thus, IL-6 appears to be an important contributor to the hepatic insulin resistance of obesity.

## Discussion

IL-6 has been associated with the chronic inflammation and insulin resistance of obesity, but a clear understanding of the role of IL-6 in each of these processes in obesity has not been established. In this report we depleted IL-6 in two obese mouse models by a classic antibody neutralization technique and determined whether IL-6 contributes to the tissue inflammatory responses in these animals and whether IL-6 contributes to the observed insulin resistance. Our results support the model that obesity-associated IL-6 contributes to a chronic inflammatory state and the associated insulin resistance.

In Lep$^{ob}$ mice, STAT3 was chronically activated in both adipose tissue and liver, as assessed by the level of pTyr$^{705}$ STAT3, compared with lean controls. In contrast, levels of activated STAT3 in skeletal muscle were very low under all experimental conditions (data not shown). Importantly, with IL-6 depletion, STAT3 activation in adipose tissue and liver decreased. Similarly, the expression of the IL-6-responsive, acute phase proteins haptoglobin (liver and adipose tissue) and fibrinogen (liver) decreased substantially with IL-6 depletion. These results support the conclusion that IL-6 contributes to an inflammatory response in Lep$^{ob}$ mice, and its targets include liver and adipose tissue.

Based on the above data and reports from our laboratory and others that IL-6 suppresses IR signal transduction in adipocytes and hepatocytes (6, 10, 27), IL-6 depletion was predicted to improve insulin responsiveness *in vivo* in both adipose tissue and liver. Unexpectedly, improved insulin responsiveness was observed in the liver, but not in adipose tissue. The response in the liver to IL-6 depletion was consistent with both *in vitro* data using primary hepatocytes and HepG2 cells (10, 28) as well as our *in vivo* studies (18) and those of others using acute and chronic IL-6 treatments (19). In all cases, IL-6 inhibited hepatic IR signal transduction and insulin action. The role of IL-6 in antagonizing IR signal transduction in adipose tissue, however, has only been investigated in cell lines. Rotter *et al.* (6) demonstrated that IL-6 had no acute effect on insulin action in 3T3L1 adipocytes, but resulted in decreased expression of IRS-1, glucose transporter-4, and peroxisomal proliferator-activated receptor-γ. These changes occurred after 24 h, but did lead to decreased in-

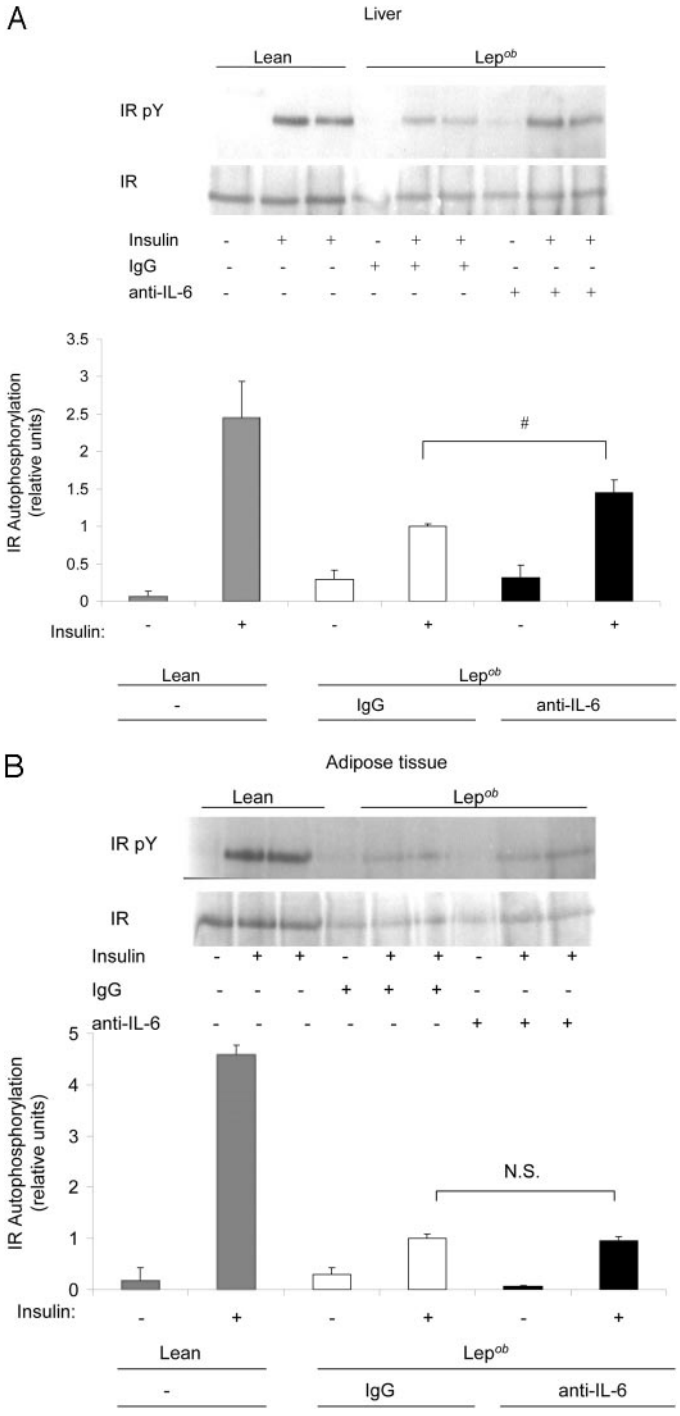


FIG. 3. IL-6 depletion improves hepatic, but not adipose tissue, IR autophosphorylation in Lep$^{ob}$ mice. On d 1, Lep$^{ob}$ mice were injected with IL-6-neutralizing antibody (R&D Systems, Inc.) or IgG. Lean control mice received no injection. Mice were fasted overnight and injected with 2.5 U/kg insulin, and tissue was harvested 10 min later on d 4. IR immunoprecipitates were prepared from liver (A) and epididymal fat pad (B) homogenates. IR autophosphorylation was determined by Western blot analysis with an antiphosphotyrosine antibody. A representative experiment is shown. *Bar graph* data are normalized to insulin-treated, IgG-treated Lep$^{ob}$ mice and represent the mean ± SEM of three experiments, each performed in duplicate. #, Not significant comparing all groups by one-way ANOVA with *post hoc* Fischer's protected least significant difference test, but significant at $P < 0.05$ by *t* test in a single comparison between Lep$^{ob}$ treatment groups; N.S., not statistically significant ($P > 0.05$).

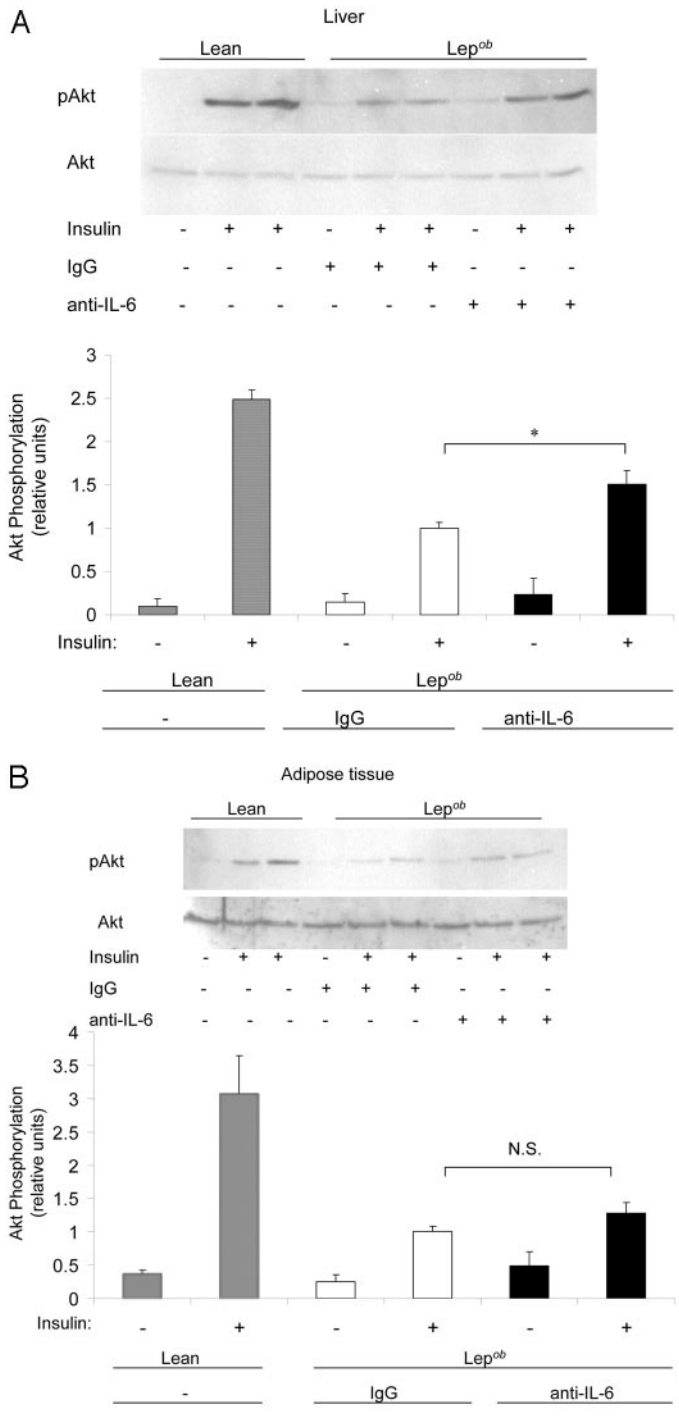


FIG. 4. IL-6 depletion improves hepatic, but not adipose tissue, Akt activation in Lep$^{ob}$ mice. Mice were treated as described in Fig. 3. Akt activity (pSer$^{473}$Akt) and mass were determined by Western blot analysis in liver (A) and adipose tissue (B) homogenates. A representative experiment is shown. Results are normalized to insulin-treated, IgG-treated mice. *Bar graph* data represent the mean ± SEM of three experiments, each performed in duplicate. *, $P < 0.05$; N.S., not statistically significant ($P > 0.05$).

sulin-dependent tyrosine phosphorylation of IRS-1 and decreased insulin-dependent glucose transport. Our inability to observe an effect of IL-6 depletion on adipose tissue may reflect the increased complexity of the *in vivo* approach. It is

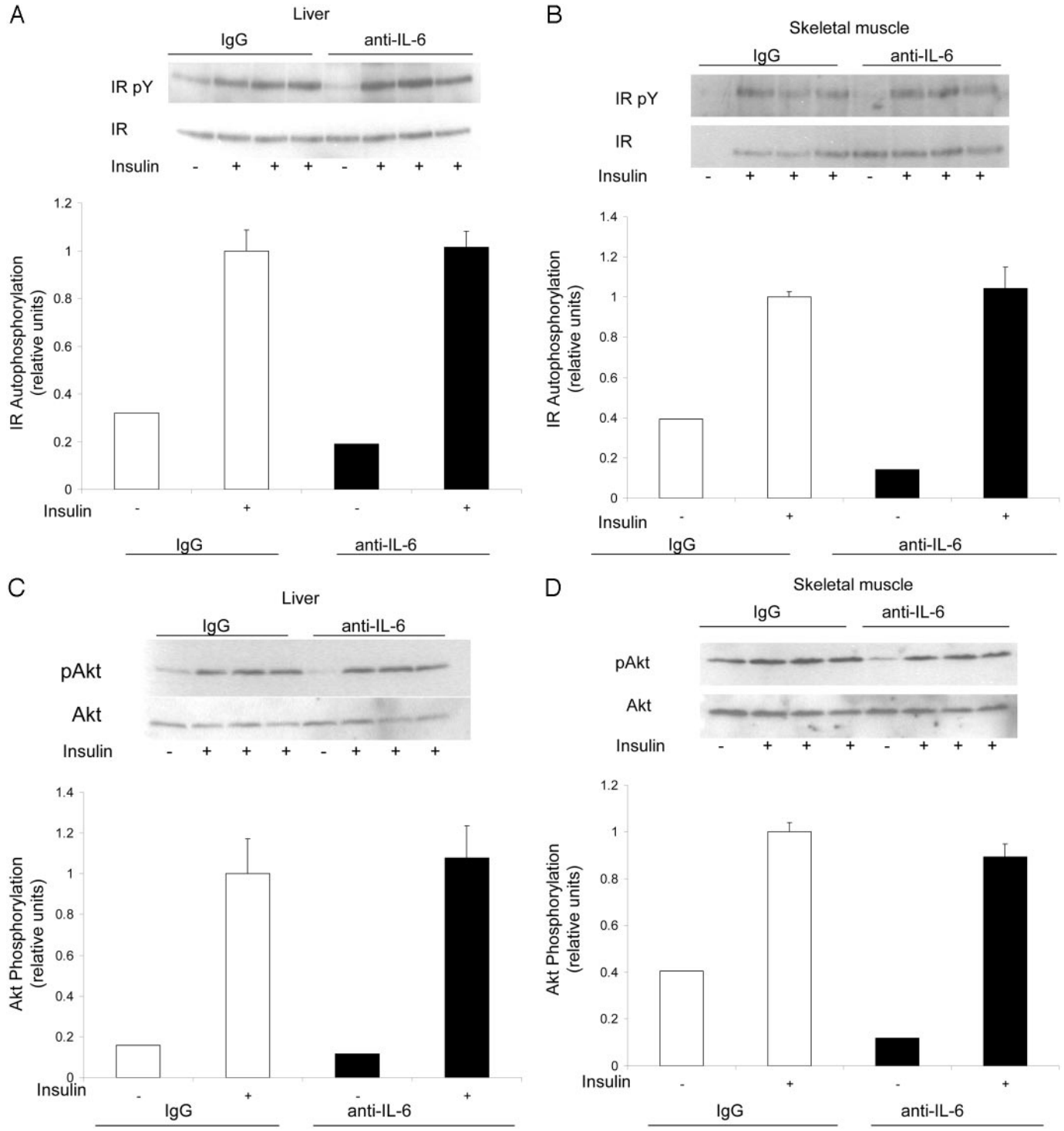


FIG. 5. IL-6 depletion has no effect on insulin-dependent IR signal transduction in lean mice. Lean C57BL/6 mice were injected with IL-6-neutralizing antibody or IgG (control) as described in Fig. 3. After insulin injections on d 4, liver and skeletal muscle homogenates were prepared as described in Fig. 3. Hepatic IR autophosphorylation and mass (A), and Akt activity (pSer$^{473}$Akt) and mass (C) were determined by Western blot analysis. Skeletal muscle IR autophosphorylation and mass (B), and Akt activity (pSer$^{473}$Akt) and mass (D) were similarly determined. A representative experiment is shown. *Bar graph* data are normalized to insulin-treated, IgG-treated mice and represent the mean ± SEM (n = 3).

quite probable, for example, that other important obesity-associated antagonists of insulin action, such as TNF$\alpha$, were continuing to affect insulin action in adipose tissue after IL-6 depletion. It is also possible that paracrine or autocrine production of IL-6 in adipose tissue could not be effectively neutralized by the circulating anti-IL-6 antibody. Alternatively, *in vitro* evidence for an inhibitory effect of IL-6 was generated using IL-6 concentrations in the nanogram per

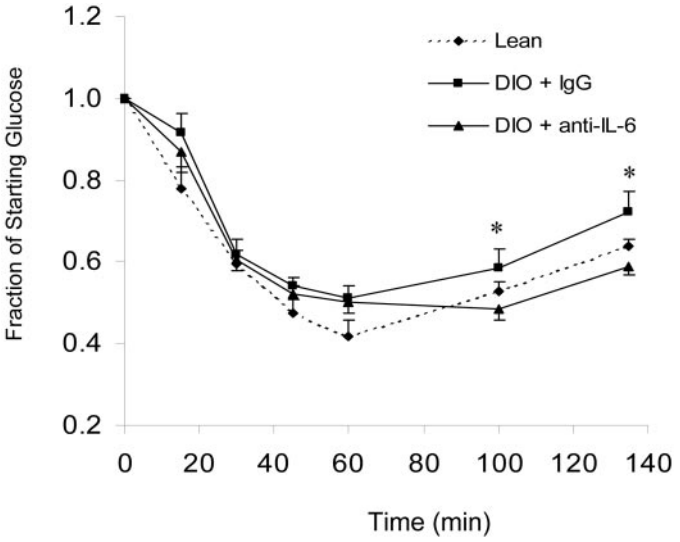


FIG. 6. Effect of IL-6 depletion on glucose homeostasis in DIO mice. Insulin tolerance tests were performed on C57BL/6J DIO mice treated with IL-6-neutralizing antibody or IgG and compared with lean controls. Data are the mean ± SEM of three experiments, each performed in quadruplicate for DIO mice. Lean mouse data are the mean ± SEM of three experiments. *, Statistical analysis (ANOVA with *post hoc* Fisher PLSD test) between the two DIO mouse treatment groups, $P < 0.05$.

milliliter range (6, 10, 27). Although this is a commonly used concentration for *in vitro* studies, circulating levels of IL-6 in mice are in the picogram per milliliter range. It is possible, therefore, that very high levels of IL-6 are necessary in adipose tissue to elicit antagonistic effects on IR signaling.

The current study suggests that skeletal muscle is not an important target for IL-6-dependent insulin resistance of obesity. The whole body glucose turnover rate, which primarily reflects skeletal muscle utilization, was not significantly changed by IL-6 neutralization. Tissue-specific glucose uptake also was unchanged in gastrocnemius and vastus lateralis as well as in diaphragm and heart muscle. The exception was a trend toward increased uptake in soleus muscle. The soleus muscle is predominantly a type 1 fiber type (slow twitch), is more insulin sensitive (29–31), and has greater blood circulation than type 2 (fast twitch) muscle (32). These factors may set the soleus apart, cause this muscle group to be more sensitive to changes in the hormone environment, and may explain its possible improvement in insulin sensitivity resulting from IL-6 depletion. The modest improvement in insulin tolerance test results in DIO mice after IL-6 depletion also is consistent with only a small improvement in glucose uptake and thus, at best, a minor contribution from skeletal muscle. Together our studies indicate a selective hepatic improvement in insulin responsiveness with IL-6 depletion. These conclusions are consistent with our previous study, which demonstrated that insulin signaling in skeletal muscle is unaffected by chronic exposure to physiological IL-6 concentrations in lean mice whereas hepatic IR signaling and insulin action are markedly impaired (18).

Although our results suggest that IL-6 does not antagonize insulin action in skeletal muscle, the work of Kim *et al.* (19) led to a contradictory conclusion. This group demonstrated that acute IL-6 treatment resulted in both skeletal muscle and hepatic insulin resistance, as assessed using hyperinsulinemic-euglycemic clamp studies in mice. IL-6-dependent activation of STAT3 in skeletal muscle was also observed. The apparently contradictory results may reflect differences in the *in vivo* response to physiological *vs.* pharmacological levels of IL-6. Although our investigations (18) involved neutralization of endogenous IL-6 or chronic infusion of IL-6 at 16 ng/h, the infusion rate used in the investigation by Kim *et al.* (19) was 0.5 μg/h. A pharmacologic level of IL-6 may elicit metabolic responses, either direct or indirect, that would not be observed under more physiological conditions.

A few additional reports address the effect of IL-6 on insulin sensitivity in skeletal muscle. In a recent report by Bruce and Dyck (33), IL-6 was found to attenuate insulin's suppressive effect on fatty acid oxidation and its lipogenic effect in isolated rat soleus muscle. Although early IR signal transduction was not examined, the antagonism by IL-6 on insulin action in soleus muscle is not inconsistent with our observation that this muscle may be selectively sensitive to IL-6 depletion. Recently, Weigert *et al.* (34) reported that IL-6 had no effect on IR kinase activity and insulin action in isolated myocytes. Although current data do not rule out a physiological role for IL-6 as a contributor to skeletal muscle insulin resistance, any such effect does not appear to be robust.

There is some controversy about a potential role for IL-6 in insulin resistance. Part of the confusion concerning the role of IL-6 arises from the complex phenotype of the IL-$6^{-/-}$ mouse. A study by Wallenius *et al.* (35) found that these mice have glucose intolerance despite the lack of IL-6. These mice also become obese more rapidly than wild-type mice. An explanation proposed for this finding is that IL-6 acts on the central nervous system and might regulate energy expenditure and appetite (36). Such a complex phenotype makes understanding the mechanism of changes in metabolism and insulin action difficult. In the current study, a temporary depletion of IL-6 over a period of 72 h was used so that changes in body mass were negligible, and any developmental compensations were not relevant. Food intake and energy expenditure were also examined as a function of IL-6 depletion just before the euglycemic-hyperinsulinemic clamp studies. No difference in these two parameters was seen as a result of anti-IL-6 treatment (data not shown).

In humans, circulating IL-6 levels are elevated several-fold in obese, insulin-resistant individuals (37–39). Although IL-6 is produced by monocytes and macrophages, endothelial cells, hepatocytes, and adipocytes among others, the contribution of adipose tissue to IL-6 is of particular interest in obesity. Recently, Weisberg *et al.* (5) and Xu *et al.* (40) demonstrated that macrophages infiltrate adipose tissue and are important contributors to cytokine production by this tissue. Adipose tissue macrophages apparently are responsible for most of the TNFα expression. IL-6 expression is contributed to by both macrophages and adipocytes, with IL-6 expression in adipocytes being induced at least in part by TNFα. The work of Takahashi *et al.* (41) suggests that the localization of macrophages to the adipose tissue may in part be due to elevated expression of monocyte chemoattractant protein-1 (MCP-1) in adipocytes. MCP-1 levels are also increased in plasma in DIO mice. Sartipy *et al.* (42) report that MCP-1 is

an insulin-responsive gene, and that white adipose tissue is a major source of the protein. Taken together, these reports suggest a model in which macrophages are actively recruited to adipose tissue and actively participate in an inflammatory state (43). The increased circulating IL-6 in obesity may reflect this inflammatory mechanism.

Our data indicate that IL-6 is an important contributing factor to the insulin resistance of obesity in mice. This effect appears to be localized to the liver. Although the *in vivo* mechanism by which IL-6 mediates hepatic insulin resistance has not been clarified, our *in vitro* investigations indicate that suppressors of cytokine signaling (SOCS) may be involved. We have shown that SOCS-3 is induced by IL-6 in hepatocytes in a time course that parallels the ability of IL-6 to suppress early IR signaling (10, 11). SOCS-1 and SOCS-3 have recently been demonstrated to cause hepatic insulin resistance when overexpressed by adenovirus infection in mice (44). Using mouse models of suppressed expression, Ueki *et al.* (45) have shown that SOCS-1 and -3 may play a role in the hepatic insulin resistance of obesity. Although induction of inhibitory SOCS proteins may mediate the inhibitory effects of IL-6 on IR signaling in liver, additional studies are required to confirm that this is a direct, physiological mechanism of the hepatic insulin resistance of obesity.

In summary, this study supports the hypothesis that IL-6 contributes to the chronic inflammation of obesity and the associated insulin resistance. Consistent with our previous observations, the impact of IL-6 appears to be predominantly on the liver in experimental mouse models. Additional work is necessary to determine the most critical sources of IL-6 in obesity-associated insulin resistance and the mechanism by which IL-6 exerts its effects.

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